

CEMENTOS ÓSEOS ACRÍLICOS CON CAPACIDAD DE ESTIMULAR LA REGENERACIÓN DE TEJIDO ÓSEO

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TESIS DOCTORAL

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El presente Trabajo de Investigación titulado: ***“Cementos óseos acrílicos con capacidad de estimular la regeneración de tejido óseo”*** fue realizado en el Grupo de Materiales Compuestos de la Escuela de Ingeniería de Materiales de la Universidad del Valle, bajo la dirección de los doctores José Herminio Mina Hernández y Carlos David Grande Tovar, en el marco del proyecto “Investigación y producción de tejidos, órganos y biodispositivos para uso en medicina regenerativa” financiado por el Sistema General de Regalías–FCTel-Colombia (BPIN: 2013000100300) y con el apoyo de la Convocatoria para la formación de alto nivel para la ciencia, la tecnología y la innovación – Capítulo de Doctorados Nacionales de Colciencias (617 de 2013), para optar por el grado de Doctora en Ingeniería, área de énfasis en Ingeniería de Materiales de la Universidad del Valle.

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*¡Adquirir sabiduría es lo más sabio que puedes hacer!
Y en todo lo demás que hagas, desarrolla buen juicio.*

Rey Salomón

Proverbios 4:7

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RESUMEN

Los cementos óseos acrílicos (ABC) son materiales ampliamente usados en ortopedia en la fijación de prótesis articulares, estabilización de fracturas, como material de relleno de defectos óseos, entre otros. Desde el punto de vista de la primera aplicación, las funciones principales de los cementos son la transferencia de las cargas de servicio y del peso corporal de la prótesis al hueso y la inmovilización inmediata de la prótesis. A pesar de su uso extenso, los ABC presentan algunas desventajas relacionadas con la falta de bioactividad debido a su naturaleza inerte y la susceptibilidad a la generación de infecciones por bacterias, todo lo cual causa aflojamiento de las prótesis en el tiempo.

Con el fin de aportar a la solución de algunos de los inconvenientes presentados por los ABC, en este Trabajo de Investigación Doctoral se planteó la incorporación de quitosano en porcentajes entre 0 – 20% en peso con respecto a la fase sólida del cemento y óxido de grafeno en porcentajes entre 0 – 0.5% en peso de la fase líquida. El GO fue sintetizado mediante el método de Hummers modificado y posteriormente caracterizado fisicoquímicamente mediante técnicas Espectroscópicas (FTIR, XPS y Raman), Difracción de rayos-X (DRX), Dispersión de Luz Dinámica (DLS) y Microscopia de Fuerza Atómica (AFM).

El estudio del efecto de la incorporación del CS y el GO en los cementos sobre sus propiedades físicas, químicas, térmicas, mecánicas y biológicas en condiciones *in vitro* e *in vivo*, mostró que el CS le confiere a los cementos propiedades bioactivas además de incrementar la porosidad y la humectabilidad, favoreciendo la biocompatibilidad. Sin embargo, también se presenta la desventaja de que reduce las propiedades mecánicas de compresión y flexión. Por otro lado, la adición de GO incrementó las propiedades mecánicas y le confirió actividad antibacteriana al cemento. Asimismo, tanto el CS como el GO redujeron la temperatura máxima durante la reacción de polimerización e incrementaron el tiempo de curado y la rugosidad y el contenido de monómero residual. Cuando se incorporaron el CS y el GO juntos en la formulación del cemento, el efecto combinado de las cargas mostró una mayor osteointegración después de 3 meses de implantación en hueso parietal de ratas Wistar y las desventajas mecánicas incorporadas por el CS fueron compensadas gracias al refuerzo alcanzado con las nanopartículas de GO.

A partir de los resultados obtenidos en esta investigación se aporta al estado del arte del conocimiento asociado a los ABC cargados con bajo contenido de CS y con GO, consiguiendo principalmente conferir propiedades bioactivas y antibacterianas a los ABC, manteniendo el cumplimiento de las propiedades mecánicas exigidas por la norma ISO 5833-02. Estos resultados evidencian que las formulaciones desarrolladas son susceptibles de ser empleadas en aplicaciones como artroplastias, relleno de defectos óseos, y demás usos donde se requiera un desempeño superior en el material.

Palabras Claves: Cementos óseos, quitosano, óxido de grafeno, bioactividad, propiedades antibacterianas.

ABSTRACT

Acrylic bone cements (ABC) are widely used in orthopedics for joint fixation, antibiotic release, bone defect filling, fractures stabilization, screw augmentation, cranioplasty, vertebroplasty, kyphoplasty, among others. ABCs' primary functions are the transfer of the service loads and the bodyweight of the prosthesis to the bone and the immediate immobilization of the prosthesis. Despite their extensive use, ABCs have some disadvantages related to the lack of bioactivity due to their static nature and susceptibility to bacterial infections, all of which cause loosening of the prosthesis over time.

In order to contribute to the solution of some disadvantages presented by the ABCs, in this doctoral thesis, the incorporation of CS in percentages between 0 - 20 wt.% concerning the solid phase and graphene oxide in percentages between 0 - 0.5 wt.% of the liquid phase was proposed. GO was synthesized by the modified Hummers method and subsequently physicochemical characterized by spectroscopic techniques (FTIR, XPS, and Raman), X-ray diffraction (XRD), Dynamic light scattering (DLS), and atomic force microscopy (AFM).

The study of the effect of CS and GO on the physical, chemical, thermal, mechanical, and biological properties of cements under *in vitro* and *in vivo* conditions showed that CS confers bioactive properties to cements, besides increasing porosity and wettability, favoring their biocompatibility; with the disadvantage that it reduced the mechanical properties to compression and bending. On the other hand, the addition of GO increased the mechanical properties. It conferred antibacterial activity to the cement, and both CS and GO reduced the maximum temperature during the polymerization reaction and increased the setting time and the roughness and residual monomer content. When CS and GO were incorporated together in the cement formulation, the combined effect of the fillers showed the presence of GO reinforced improved biological behavior and the mechanical disadvantages incorporated by CS.

From the results obtained in this research, knowledge is generated about the ABCs loaded with low content of CS and GO. Bioactive and antibacterial properties are conferred to the ABCs, maintaining the ISO's mechanical properties 5833-02 standard. These results show the potential for using the formulations developed in this research in arthroplasties and other orthopedic applications, where their performance is expected to be higher.

Keywords: Acrylic bone cements, chitosan, graphene oxide, bioactivity, antibacterial

Capítulo 1.

INTRODUCCIÓN

1.1 ESTRUCTURA DEL DOCUMENTO

Esta Tesis Doctoral es presentada bajo la modalidad de artículos establecida en el numeral 3 de la resolución 142 del 2015 del Consejo Académico de la Universidad del Valle.

Para mayor comprensión de la información, el documento se encuentra organizado en cinco capítulos. El **capítulo 1** corresponde a la introducción del documento y en él encontramos esta breve descripción de la estructura del documento, los antecedentes y la justificación de abordar esta temática de investigación, junto con los objetivos planteados y las referencias bibliográficas usadas en esta sesión.

El **capítulo 2** contiene el marco referencial y está basado en el artículo de revisión titulado “*The Role of Chitosan and Graphene Oxide in Bioactive and Antibacterial Properties of Acrylic Bone Cements*”, el cual fue sometido para publicación en la revista *Biomolecules* de la editorial mdpi (El anexo A contiene el certificado de sometimiento del artículo).

El **capítulo 3** incluye los artículos que fueron publicados y sometidos para publicación con los resultados obtenidos en esta investigación. A continuación, se mencionan estos artículos junto con su respectiva referencia:

3.1 Ruiz Rojas, L.M.; **Valencia Zapata, M.E.**; Gordillo Suarez, M.; Grande Tovar, C.D.; Mina Hernandez, J.H. *Optimization of mechanical and setting properties in acrylic bone cements added with graphene oxide by using a completely randomized factorial design. RSC Advances*. Artículo sometido para publicación (El anexo B contiene el certificado de sometimiento del artículo).

3.2 **Valencia Zapata, M.E.**; Ruiz Rojas, L.M.; Mina Hernandez, J.H.; Delgado-Ospina, J.; Grande Tovar, C.D. *Acrylic Bone Cements Modified with Graphene Oxide: Mechanical, Physical, and Antibacterial Properties. Polymers (Basel)*. **2020**, *12*, 1773.

3.3 Zamora Lagos, S.I.; Murillo Salas, J.; **Valencia Zapata, M.E.**; Mina Hernandez, J.H.; Grande Tovar, C.D. *Optimization by Central Composite Experimental Design of the Synthesis of Physically Crosslinked Chitosan Spheres. Biomimetics* – 956848. Artículo sometido para publicación (El anexo C contiene el certificado de sometimiento del artículo).

3.4 Zamora Lagos, S.I.; Murillo Salas, J.; **Valencia Zapata, M.E.**; Mina Hernandez, J.H.; Valencia, C.H.; Rojo, L.; Grande Tovar, C.D. *Influence of the chitosan morphology on the properties of acrylic cements and their biocompatibility. RSC Advances*. **2020**, *10*, 31156–31164.

3.5 **Valencia Zapata, M.E.**; Mina Hernandez, J.H.; Grande Tovar, C.D. *Acrylic Bone Cement Incorporated with Low Chitosan Loadings. Polymers (Basel)*. **2020**, *12*, 1617.

3.6 Mina Hernandez, J.M.; Valencia Llano, C.H.; Arango, I.; Castaño, A.; **Valencia Zapata, M.E.**; Hurtado Chavez, N.M.; Lozano Nieva, K.J.; Rodriguez, J. *Cementos Óseos Acrílicos Bioactivos con Aplicación en Ortopedia. Revista Informador Técnico* **2016**, 80, 112–114.

3.7 **Valencia Zapata, M.E.**; Mina Hernandez, J.H.; Grande Tovar, C.D.; Valencia Llano, C.H.; Diaz Escobar, J.A.; Vázquez-Lasa, B.; San Román, J.; Rojo, L. *Novel Bioactive and Antibacterial Acrylic Bone Cement Nanocomposites Modified with Graphene Oxide and Chitosan. International Journal of Molecular Sciences.* **2019**, 20, 2938.

3.8 **Valencia Zapata, M.E.**; Mina Hernández, J.H.; Valencia Llano, C.H.; Grande Tovar, C.D. *Cemento Óseo Acrílico Bioactivo y Antibacterial Modificado con Óxido de Grafeno y Quitosano. Colombia Investiga - ACOFI - Capítulo Nacional de Doctorados en Ingeniería.* **2020**.

3.9 **Valencia Zapata, M.E.**; Mina Hernandez, J.H.; Grande Tovar, C.D.; Valencia Llano, C.H.; Vázquez-Lasa, B.; San Román, J.; Rojo, L. *Osseointegration of Antimicrobial Acrylic Bone Cements Modified with Graphene Oxide and Chitosan. Applied Sciences.* **2020**, 10.

Los artículos referenciados se han ordenado de forma que puedan mostrar la metodología seguida en la investigación para dar cumplimiento a los objetivos planteados, la cual se puede apreciar en la Figura 1.



Figura 1. Esquema de las etapas seguidas en esta investigación

Los artículos de la sección 3.1 y 3.2 presentan los estudios realizados en cementos óseos cargados con óxido de grafeno. En particular el artículo 3.1 abarca los resultados asociados a la síntesis y caracterización del GO, junto con la optimización del tamaño y el contenido del óxido de grafeno y contenido del BPO mediante un diseño factorial completamente al azar, utilizando como variables de respuesta las propiedades mecánicas y de curado exigidas en la norma ISO 5833-02 para los cementos óseos acrílicos. Por otro lado, en el artículo 3.2 se muestran los resultados de la caracterización mecánica, física y antibacteriana de algunas de las condiciones experimentales que se optimizaron en el artículo anterior. Estos artículos aportan al cumplimiento del **objetivo 1** y el **objetivo 3** de esta investigación.

En el artículo 3.3 se optimiza el diámetro de esferas de CS, mediante el empleo de un diseño de superficie de respuesta en el que se estudiaron cuatro variables en el proceso de obtención (concentración de ácido acético, de hidróxido de sodio, de CS y el diámetro de la aguja). Estas microesferas fueron caracterizadas mediante ensayos físicos, químicos y biológicos en

fluido biológico simulado (FBS) y posteriormente incorporadas a las formulaciones de los cementos óseos cuyos resultados se consignan en el artículo 3.4, donde se evaluó el efecto de la morfología del CS (esferas y láminas) sobre las propiedades físicas, químicas, mecánicas y biológicas en FBS y en implantaciones subdérmicas en biomodelos ratas Wistar de los ABC. A partir de los resultados anteriores, se planteó el estudio del efecto del contenido de CS en forma de láminas en las formulaciones de ABC sobre las propiedades físicas, químicas, térmicas, mecánicas, de curado y la bioactividad de los cementos óseos en el artículo 3.5. Finalmente, en el artículo 3.6 se presentan de forma resumida resultados radiográficos obtenidos a partir de la implantación de ABC adicionados con CS en el canal femoral de biomodelos conejos. Los artículos 3.3 – 3.6 dan cuenta del estudio realizado en ABC adicionados con CS y contribuyen al cumplimiento de los **objetivos específicos 1, 2 y 3** de esta investigación.

En los artículos 3.7 y 3.8 se presenta una amplia caracterización física, química, térmica, mecánica, biológica en solución buffer de fosfatos (PBS) y en condiciones *in vitro* con osteoblastos humanos y *Escherichia coli* de ABC que contenían los porcentajes de CS y GO considerados como los más adecuados de acuerdo con las investigaciones anteriores. Para finalizar, en el artículo 3.9 se reportan los resultados de la caracterización de las propiedades de curado y evaluaciones en condiciones *in vivo* en implantaciones subdérmicas y en hueso parietal en ratas Wistar de los cementos cargados con CS y GO. Los artículos 3.7 – 3.9 contribuyen para dar cumplimiento total a los **objetivos específicos 1, 2 y 3** y con esto poder alcanzar el objetivo general planteado en esta investigación.

El análisis relacionado a los 10 artículos presentados en los capítulos 2 y 3 de este documento, se muestra en el **capítulo 4** donde se recoge el panorama general, las conclusiones y recomendaciones encontradas a partir de la investigación y posteriormente se muestran los aportes y las contribuciones de la tesis junto con los logros obtenidos en cuanto a patentes, participación en eventos y pasantías de investigación, principalmente.

Por último, el **capítulo 5** del documento corresponde a los anexos, en este se incluyen los certificados de sometimiento de los tres artículos que se encuentran en estado de sometidos para publicación y adicionalmente se relacionan otros 5 artículos de investigación que fueron publicados en temas afines al presentado en esta Tesis Doctoral.

1.2 ANTECEDENTES

Los cementos óseos acrílicos están basados principalmente en polimetilmetacrilato y por muchos años han tenido un papel importante en la cirugía ortopédica [1–3]. Poco a poco se extiende su aplicación en la remodelación de áreas afectadas por cáncer metastásico u osteoporótico, en la estabilización de fracturas, como relleno de defectos óseos, en la reparación de defectos craneales (craneoplastias) y en la reparación de las fracturas vertebrales (vertebroplastia y la cifoplastia) [1,4–6]. Se conoce como artroplastia a la cirugía de reemplazo de alguna articulación. Durante este procedimiento, la mayoría de las prótesis articulares son fijadas usando un cemento óseo acrílico que actúa como un pegamento entre el implante y el hueso, cuyas funciones principales son la transferencia de las cargas de servicio y del peso corporal de la prótesis al hueso y la inmovilización inmediata de la prótesis. En las artroplastias, la complicación más frecuente a largo plazo es el aflojamiento de la prótesis articular; sin embargo, la causa exacta no está clara, pero en muchos casos se relaciona con problemas en el cemento óseo [7].

La interfaz hueso/cemento es la clave para incrementar la longevidad de las prótesis articulares [8]. Es por eso que en las últimas décadas se ha trabajado fuertemente en superar limitaciones de los cementos tales como su alta temperatura de polimerización (causante de necrosis térmica del tejido próximo) [9–16], su comportamiento inerte que genera la formación de un tejido fibroso a su alrededor después de ser implantado en el cuerpo [17–19] y la liberación de monómero residual que genera necrosis química del tejido alrededor. Es importante el control de todos estos problemas ya que conllevan al aflojamiento aséptico de las prótesis. Igualmente, se están adelantando grandes esfuerzos para superar la susceptibilidad de los implantes a generar infecciones por bacterias, lo cual promueve el aflojamiento séptico en la prótesis.

Los inconvenientes de los cementos que causan el aflojamiento aséptico se han intentado resolver mediante el uso de cargas bioactivas [18,20] y polímeros biodegradables [20–22] que promueven una mayor adherencia entre el hueso y el cemento; sin embargo, estas cargas han generado disminución en las propiedades mecánicas de los cementos. Adicionalmente, se ha intentado modificar la matriz polimérica con el uso de comonómeros [23–26] y la adición de otros polímeros [27] en la formulación, con lo que se ha conseguido mejorar algunas de las propiedades mecánicas.

Debido a que las infecciones crónicas en las prótesis son un problema frecuente después de una artroplastia, se han estudiado estrategias como la incorporación de antibióticos y de agentes antibacterianos en las formulaciones, encontrando que ambas estrategias reducen las propiedades mecánicas, por lo que su empleo se limita solamente al tratamiento de infecciones. Por otro lado, el uso de los antibióticos es ineficiente en el tratamiento contra bacterias resistentes y multirresistentes a los mismos.

Por lo anterior, en este proyecto se planteó el estudio de la influencia de la incorporación de quitosano y óxido de grafeno en las propiedades físicas, térmicas, mecánicas, químicas y biológicas de los cementos óseos acrílicos, con el propósito de desarrollar la formulación de un cemento bioactivo y antibacterial para aplicación en artroplastia.

1.3 JUSTIFICACIÓN

La artroplastia se ha establecido como un procedimiento quirúrgico, encaminado a mejorar la calidad de vida de las personas con osteoartrosis o traumatismos articulares, mediante la mejoría de la función física y el alivio del dolor. En el mundo, el número de operaciones de este tipo ha aumentado a través de los años y se espera un mayor incremento en los próximos años de acuerdo con el aumento en el envejecimiento de la población mundial [28].

En el 2010, la prevalencia del reemplazo total de cadera y de rodilla en la población total de Estados Unidos fue de 0.83% y 1.52%, respectivamente. Se presentó con mayor frecuencia entre las mujeres que en los hombres y aumenta con la edad, alcanzando el 5.26% para el reemplazo total de la cadera (THR) y el 10.38% para el reemplazo total de rodilla (TKR) a los ochenta años. Estas estimaciones corresponden a 2.5 millones de personas (1.4 millones de mujeres y 1.1 millones de hombres) con THR y 4.7 millones de personas (3.0 millones de mujeres y 1.7 millones de hombres) con TKR en 2010 [29]. Actualmente se ha ampliado su uso a pacientes más jóvenes y a afecciones cada vez más complejas, como traumatismos y cáncer, convirtiéndose en una cirugía muy utilizada en el mundo, y se espera que su demanda se duplique en las próximas dos décadas [30,31].

A nivel mundial, aproximadamente el 90% de todas las artroplastias de rodilla y alrededor del 60% de todas las artroplastias de cadera se fijan con cemento óseo acrílico (ABC) [31]. El uso de ABC en las cirugías de reemplazo de articulaciones se ha incrementado a través de los años en países como Japón [32], en Suecia donde más del 95% de las artroplastias de cadera y rodilla utilizan cemento óseo para la fijación de al menos uno de los componentes [33] y en Estados Unidos donde la incidencia de los procedimientos de reemplazo de articulaciones es alta, con más de 1 millón de procedimientos de THR y TKR totales realizadas cada año [29].

En Colombia no se dispone de ningún estudio masivo riguroso que muestre datos epidemiológicos concretos sobre artroplastias, y no existen reportes exactos [30]. No obstante, conviene señalar que Colombia es el segundo país de América Latina que más ha elevado la expectativa de vida de la mujer, con lo que se hace más susceptible de incrementar la incidencia de este tipo de procedimientos [34]. En este sentido, el censo poblacional de 2018, realizado por el Departamento Administrativo Nacional de Estadísticas (DANE) en Colombia, reporta que se ha aumentado el porcentaje de la población mayor de 50 años de 16.9 % en el 2005 a 23.09% en el 2018 y se proyecta que en los siguientes años este valor se incremente, lo que genera mayor riesgo de presentar algún tipo de trastorno en las articulaciones [35,36].

Se encuentran registros de que en Colombia en el 2009 se realizaban 10.000 reemplazos de cadera o rodilla al año; estos reemplazos se presentaron en un 90% de los casos en personas mayores de 60 años, pero en los últimos años se ha visto la necesidad de hacerlas en personas

hasta de 40 años, en jóvenes que han presentado artrosis primaria y en niños que tuvieron una displasia de cadera o traumas en la rodilla [37].

La falla de la fijación entre el implante y el hueso generalmente se atribuye a deficiencias en los ABC y genera la necesidad de revisión, lo que aumenta los costos totales de manera significativa [31]. Debido a la gran importancia de los cementos óseos acrílicos en la artroplastia, a lo largo de las últimas décadas, los esfuerzos se han centrado en la modificación de estos con el fin de incrementar las propiedades mecánicas, mejorar los métodos de mezclado, optimizar sus características de manejo, y promover el desarrollo de nuevas formulaciones y métodos de liberación; además, de la incorporación de aditivos para mejorar la función clínica de los ABC [7].

Debido a las buenas propiedades biológicas mostradas por el quitosano (CS) [17,38–46] y al reforzamiento mecánico [47] y la actividad antibacteriana generado por el óxido de grafeno (GO) en distintas matrices poliméricas [48–52], en esta investigación se planteó como estrategia principal la incorporación de estos componentes en las formulaciones de los cementos, buscando alcanzar una solución frente a algunos de los inconvenientes que presentan los ABC convencionales. En este sentido, el CS fue incorporado con el fin de estimular la regeneración ósea, y el GO para generar actividad antimicrobiana y mejorar el comportamiento mecánico.

1.4 OBJETIVOS

1.4.1 Objetivo General

Desarrollar una formulación de cemento óseo acrílico bioactivo, adicionado con quitosano y óxido de grafeno, para su aplicación en artroplastia.

1.4.2 Objetivos Específicos

- Evaluar la influencia del quitosano y el óxido de grafeno sobre las propiedades físicas, térmicas, mecánicas y químicas de los cementos óseos acrílicos, siguiendo la norma ISO 5833.
- Estudiar las características bioactivas (regeneración ósea) de las formulaciones de cemento óseo desarrolladas, a partir de la ejecución de pruebas en condiciones In Vitro.
- Proponer una formulación de cemento óseo acrílico bioactivo que cumpla con el estándar de la ISO 5833 para cementos óseos acrílicos.

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Capítulo 2. MARCO REFERENCIAL

2.1 **Valencia Zapata, M.E.**; Grande Tovar, C.D.; Mina Hernandez, J.H. *The Role of Chitosan and Graphene Oxide in Bioactive and Antibacterial Properties of Acrylic Bone Cements. Biomolecules*. Artículo sometido para publicación. (El anexo A contiene el certificado de sometimiento del artículo).

Review

The Role of Chitosan and Graphene Oxide in Bioactive and Antibacterial Properties of Acrylic Bone Cements

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Abstract: Acrylic bone cements (ABC) are widely used in orthopedics for joint fixation, antibiotic release, bone defect filling, among others. However, most commercially available ABCs exhibit a lack of bioactivity and are susceptible to infection after implantation. These disadvantages generate long-term loosening of the prosthesis, high morbidity, prolonged and expensive treatments. Due to the great importance of acrylic bone cements in orthopedics, the scientific community has advanced several efforts to develop bioactive ABCs with antibacterial activity through several strategies, including the use of biodegradable materials such as chitosan (CS) and nanostructures such as graphene oxide (GO), with promising results. This paper reviews several studies reporting advantages in bioactivity and antibacterial properties after incorporating CS and GO in bone cements. Detailed information on the possible mechanisms by which these fillers confer bioactive and antibacterial properties to cements, resulting in formulations with great potential for use in orthopedics, are also a focus in the manuscript. To the best of our knowledge, this is the first systematic review that presents the improvement in biological properties with CS and GO addition in cements that we believe will contribute to the biomedical field..

Keywords: acrylic bone cement; antibacterial activity; bioactivity; chitosan; graphene oxide; poly (methyl methacrylate)

1. Introduction

Acrylic bone cements (ABC) are widely used in orthopedics for joint fixation [1,2], antibiotic release [3], bone defect filling, fractures stabilization, screw augmentation, cranioplasty, vertebroplasty, and kyphoplasty [4–6]. One strategy used to provide ABCs with bioactivity is the addition of CS, which, thanks to its excellent biological properties, transfers to the ABC the capacity to generate chemical bonding with the bone in the biological environment, achieving a more stable fixation through the improved interlocking between the bone and cement. On the other hand, GO has demonstrated a high degree of mechanical reinforcement and activity against a wide range of bacteria, which generates antibacterial properties in the cements and reduces the risk of septic loosening of the implants. Recently, a review was written about the biological response of cements added with GO [7]. However, the purpose of that review included a wide range of properties (physical, mechanical, and biological), and there was non an in-depth analysis of the biological properties.

Due to the great importance of acrylic bone cements in orthopedics, many research methods have improved ABCs' bioactivity and antibacterial activity. Figure 1 shows the ascending tendency in the number of publications per year in cements added with GO and the high importance in the last years concerning the study of ABCs added with CS, demonstrating the tremendous present interest in research in these topics.

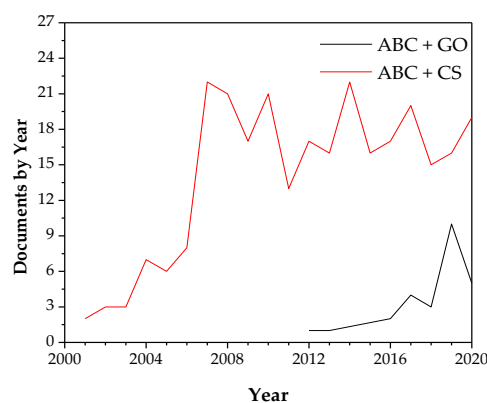


Figure 1. The number of documents published by year (2001 – 2020) about acrylic bone cements loaded with chitosan and graphene oxide. The keywords used were ("Bone cement" AND "Graphene oxide") and ("Bone cement" AND chitosan)—source: Scopus (October 19, 2020).

2. Arthroplasty

Osteoarthritis (also known as osteoarthrosis, osteoarthritis, or degenerative joint disease) is the most common form of arthritis. The knee and hip are the joints most affected by this disease. When a joint develops osteoarthritis, the cartilage is damaged, worn out, or broken, and the exposed bones friction is causing pain, swelling, loss of joint movement, and deformities [8].

Arthroplasty has become consolidated as a surgical procedure to improve people's quality of life with osteoarthrosis or joint trauma by improving physical function and reducing pain. Worldwide, the number of operations of this type has increased over the years, and a more significant increase is expected in the next few years by the increase in the aging of the world's population [9].

In total joint replacement (TJR), also known as arthroplasty, the diseased or damaged parts of the joint are removed (Figure 2a) and replaced by prostheses or implants (Figure 2b). TJR can be performed on various joints, including the hip, knee, ankle, shoulder, elbow, fingers, and wrist. However, hip and knee prostheses are the most common [10].

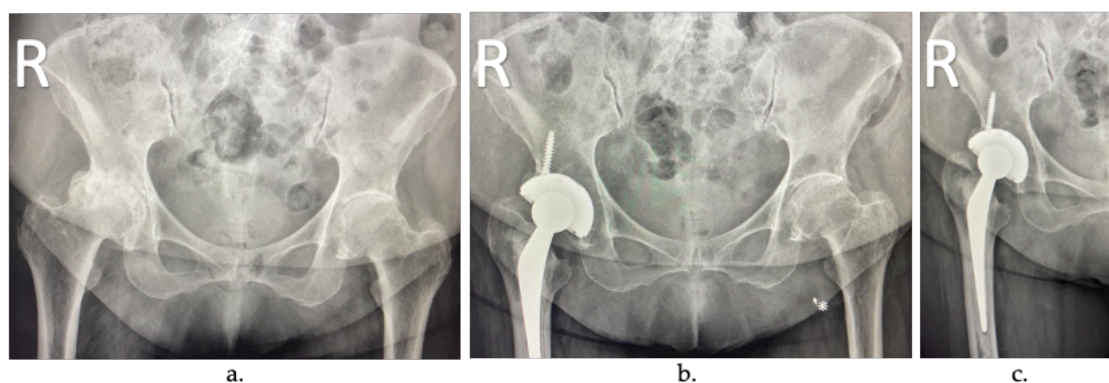


Figure 2. Arthroplasty radiographs a. Patient with severe osteoarthritis in the right hip, b. Joint replacement of the femoral component, and c. Cemented joint replacement.

During TJR, the most common method of fixing the implant is with a load-transfer material's introduction, typically an acrylic bone cement (ABC) (Figure 2c), introduced into the space between the implant and the joint as a flow-able mixture that eventually hardens over time [11]. The main advantage of these cemented joint replacements is the reduction in the recovery time of the surgery.

Once polymerized, the cement can support a load and offer immediate stability, and it has shown excellent long-term results [12]. However, if the cement layer is loosened, the surrounding bone can be reabsorbed, and implant failure can eventually occur. Today, acrylic bone cements use is around 90% of total hip replacement (THR) surgeries in developed countries such as the United Kingdom, the Netherlands, and France.

3. Acrylic Bone Cements (ABC)

Most of the commercial ABC's available today consist of two components, one solid, based mainly on poly (methyl methacrylate) (PMMA) and one liquid based on methyl methacrylate (MMA) [13], which are mixed and through the polymerization reaction of the monomer, transformed into a hardened cement paste [11]. Figure 3 shows the presentation of Surgical Simplex P commercial cement.



Figure 3. Components of commercial ABC Surgical Simplex P. Solid phase in a sterile plastic package and liquid phase in a sterile glass vial.

PMMA cements are the most commonly used to bond and load transfer between the implant and the bone [14,15]. Its use's main advantage is the excellent primary fixation obtained between the implant and the bone and the patient's faster recovery [16]. On the other hand, PMMA is a fragile material with a low resistance to fracture and low fatigue life [11].

Commercial ABCs do not differ drastically between brands. The main differences between them are the addition of PMMA copolymers or antibiotics into the solid phase, comonomers to the liquid phase, solid/liquid ratio variation, radiopaque agent variation, or additives chlorophyll Palacos® [11,17]. The necessary components of the ABCs are shown in Table 1.

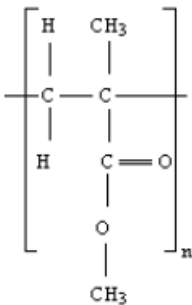
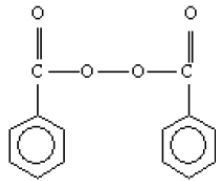
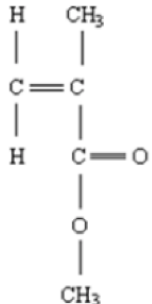
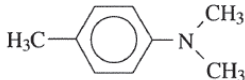
The polymerization process begins with BPO and DMPT mixture with benzoyl radicals [18,19]. The heat produced during polymerization is between 52 and 57 kJ per mole of MMA [3]. The maximum temperature reached during the polymerization reaction is known as T_{max} [20], exceeding 100°C. These high temperatures can cause cellular bone necrosis and contribute to aseptic loosening [10,21–23]. Polymerization temperatures experienced under *in vivo* conditions are much lower (between 40 and 47°C) at the bone interface due to the reduced thickness of the bone cement layer used in TJR, the presence of blood circulation and heat dissipation through the implant and surrounding tissue [19,24–26].

The leading cause of failure of cemented arthroplasties is aseptic loosening of the prosthesis, which usually occurs at the bone-cement-interface and requires a second surgery to replace the whole system [27]. The loose occurs according to:

1. High polymerization temperature of the cements (between 67 and 124°C) [28], which generates thermal necrosis of the bone [29], alteration of the local blood circulation, and predisposition to the formation of a fibrous membrane in the bone-cement interface [16,30].
2. Release of unreacted residual monomer or MMA, which generates chemical necrosis of the bone.
3. Contraction of the cement during polymerization.
4. A significant difference between the cement's stiffness and the adjacent bone generates an inappropriate load transfer [27].

5. Interaction of the cement particles with the surrounding tissues, which produces the inflammatory responses of the periprosthetic tissue and increased bone destruction
6. Lack of osseointegration due to its inert nature [14].

Table 1. Essential components of acrylic bone cement formulations

Component	Composition (~wt.%)	Function	Structural Formula
Solid Phase			
PMMA	87.5 – 89.25	Polymer	
Barium sulfate or zirconium dioxide	10	Radiopaque agent	BaSO ₄ or ZrO ₂
Benzoyl peroxide	0.75 – 2.5	Polymerization reaction initiator	
Liquid Phase			
MMA	97.0 – 97.5	Monomer	
<i>N,N</i> -Dimethyl- <i>p</i> -Toluidine	2.0 – 2.5	Room temperature polymerization reaction accelerator	
Hydroquinone	75 ppm	Inhibitor that prevents premature polymerization of MMA	

All these disadvantages generate a lack of strong interaction cement-bone due to the only adhesive force is the interdigitation of the cement with the bone, without any apparent chemical

reaction. Therefore, fibrous tissue is encapsulated, causing instability and movements in the bone-cement-prosthesis interface, considered the weak bonding zone. These micro-movements can accelerate aseptic loosening, causing implant failure [14,31].

Since a substantial fixation of the ABCs to the bone depends primarily on mechanical anchorage [32], many investigations in two directions are ongoing to overcome loosening in the prosthesis. The first consists of generating bioactivity of the cement, which is a critical factor in achieving long-term stability of the implant [14], since chemical bonding is achieved between the bone and the cement, while the second consists of providing antimicrobial properties to the ABCs since infections are common in arthroplasty and also lead to septic loosening.

4. Chitosan (CS)

CS is a linear, semi-crystalline polysaccharide and the direct derivative of chitin, the second most abundant natural polymer after cellulose [33]. It is a polyelectrolyte with reactive functional groups [34]. Chemically, CS is a copolymer of β -(1-4) D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit) in different proportions, which depend on the degree of deacetylation (DD) [34,35]. As shown in Figure 4, this polycationic polymer has an amino group and two hydroxyl groups in each glycosidic unit. The positive charge through the polymer chain allows many electrostatic interactions with negatively charged molecules [36]. Structurally, it is similar to glycosaminoglycans, the main component of the bone's extracellular matrix [37].

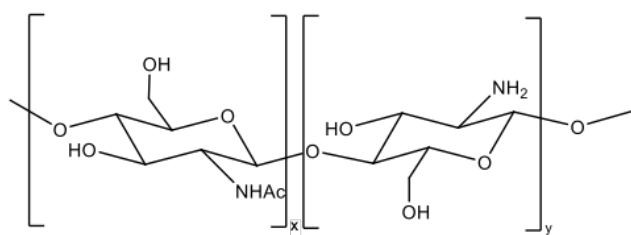


Figure 4. Chemical structure of chitosan

The presence of some functionalities such as -NH_2 and -OH in chitosan molecules provides the basis for interaction with other polymers and biological molecules [38,39]. The amino group's functionality gives rise to chemical reactions such as acetylation, quaternization, alkylation through reactions with aldehydes and ketones, copolymerization, chelation of metals to provide a variety of products with different properties. The two hydroxyl groups in each glycoside unit allow several reactions such as *o*-acetylation, hydrogen bonds with polar atoms, grafting, among others [40].

At low pH, the protonation of the primary amino group present in CS, generating a positive charge that converts CS into a cationic polyelectrolyte, causing electrostatic repulsion between the polymer chains, which facilitates its solubility [41].

The physical properties of CS depend on parameters such as molecular weight (Mw) and DD (in the range of 50 - 95%) [40,41]. It is biodegradable, biocompatible, promotes cell growth and the deposition of a mineral-rich matrix by osteoblasts, easily soluble in diluted acid solutions with pH less than 6, antioxidant, present antimicrobial, antifungal, and antitumor activities, is mucoadhesive and analgesic [34,38–46].

5. Graphene Oxide (GO)

GO is a two-dimensional material that consists of an oxidized form of graphene with hydroxyl, epoxy, carbonyl groups on its basal plans and carboxylic groups at its edges [47–50] (Figure 5). Despite the defects that these groups generate in the graphene sheets, the oxidized sheets keep almost intact their mechanical properties showing Young's modulus values as high as 0.25 TPa. Also, GO is biocompatible [51–53], biodegradable [54–57], sensitive to temperature and pH changes for drug release [58], has excellent antibacterial properties [49,59–62], has a high specific surface area [63], is impermeable to all gases and liquids except water [64], has physiological stability [51], and has easy

biological/chemical functionalization [65]. The functional groups of graphene oxide improve the dispersion of graphene, the solubility in water, and some organic solvents [64,66], possessing photoluminescent in the wavelength range of the near-ultraviolet and the visible blue to the near-infrared [66].

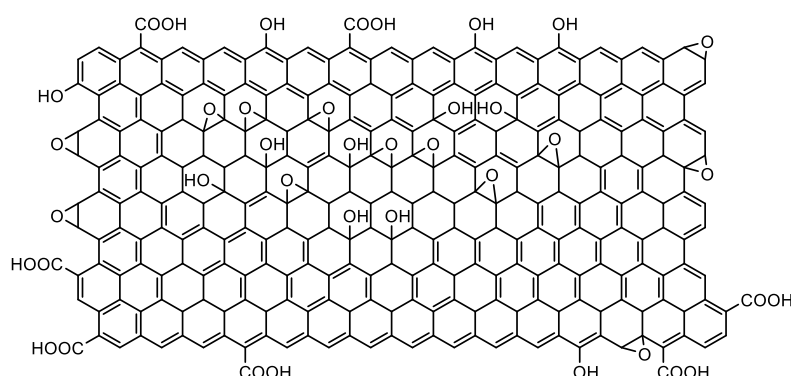


Figure 5. Chemical structure of GO

6. Bioactivity

Bioactive materials interact with bone tissue when implanted inside the bone to stimulate a biological response from the body, such as bonding to tissue and promoting osteogenesis [67,68]. There are three kinds of bioactive materials: osteoconductive, osteoinductive, and osteogenic. Osteoconductive materials support the attachment of new osteoblasts and osteoprogenitor cells, providing an interconnected structure that stimulates bone growth along with the bioactive material [69,70]. Osteoinductive materials induce non-differentiated stem cells or osteoprogenitor cells to differentiate into osteoblasts [69], stimulate the growth of new bone on the material away from the bone/implant interface [70,71]. Osteogenic refers to osteoblasts at the new bone formation producing minerals to calcify the collagen matrix [69].

Bioactive materials have the following functions [69]:

- Stimulate cell differentiation and proliferation
- Stimulate gene and tissue regeneration
- Release bioactive molecules actively and effectively for restoring and repairing the impaired functionality of the organs

Thanks to these desirable bioactive materials, they have become critical in tissue engineering [69].

6.1. Bioactivity in Acrylic Bone Cements

Bone cement is a biologically inert component, so it does not usually promote bone growth [10]. Different strategies look to increase the bone and the cement's interfacial resistance and improve the prosthesis's performance. One of these strategies consists in the incorporation of bioactive fillers in the solid phase of the cement to generate ABC with a bioactive surface, capable of promoting direct bone apposition instead of encapsulation of the implant by fibrous tissue, promoting bone growth and the formation of a strong chemical bond between the cement and the bone [14,32].

Some bioactive fillers used are hydroxyapatite (HA) [72–75], phosphate bioglasses [29,76], silicate and borate bioglasses [30,77], tricalcium phosphates [31,78] and *Sepia Officinalis* cuttlebone [79]. With this type of inorganic filler, it is necessary to control the amount used since they affect the mechanical properties and the cement [32,75]. Besides, most of them tend to make the cement even more brittle, and the matrix must be modified to increase its ductility and decrease its module [27].

Some modifications to the matrix have been achieved with the addition of comonomers in the liquid phase, such as dimethyl aminoethyl methacrylate (DMAEM) [74], diethyl amino ethyl acrylate (DEA) [80], acrylic acid (AA) [73], 4-methacryloxybenzoic acid (MBA), methacrylic acid (MAA), 4-dimethylamino benzyl methacrylate (DEABMA) [81], 5-hydroxy-2-methacrylamidobenzoic acid (5-HMA), and 2-hydroxyethyl methacrylate (HEMA) [82]. The modifications adding other polymers to the solid phase such as hydroxypropyl methacrylate (HPMA) [83] and core-shell nanoparticles with poly butyl acrylate (PBA) core and PMMA shell [84] have also been reported, allowing to modify the stiffness of the matrix and increase properties such as impact resistance.

Bone resorption has been reported after prolonged implantation of some bioactive cements, which compromises the prosthesis's fixation due to the wear of the weak calcium phosphate layer formed on the cement's surface, generating particles that stimulate bone resorption [32,85].

The presence of biodegradable substances in the ABCs generates partially biodegradable cements, which allow the replacement and growth of new bone inside the cement in the pores produced by the hydrolytic and enzymatic degradation in the biological environment, thus improving the mechanical anchorage [32,77,86] or generating systems of controlled drug release [15].

Biodegradable polymers such as polylactic acid (PLA), β -polyhydroxy butyrate (PHB), and thermoplastic starch (TPS) have been added to the solid phase in a range between 49 - 66 wt.% to create partially biodegradable ABCs, finding in all cases a decrease in the mechanical properties of the cement [15,87]. There are studies where percentages from 10 to 30 wt.% of beads composed of PMMA/ ϵ -polycaprolactone were added. The addition of 10 wt.% produced an increase in bending properties while 30 wt.% decreased properties in bending and compression [88]. Other researchers report that a PHB copolymer and hydroxivalerate (PHBV) addition increased the mechanical properties compared to PHB and the cement's biological response [77]. Besides, gelatin microparticles improved the release of antibiotics, leading to decreased mechanical properties [89]. In all cases, these biodegradable polymers' presence increased the cement's water absorption [90]. A recent review of partially degradable acrylic cements published information about cement modifications' advantages and disadvantages [91].

The combination of porosity and bioactive agents in the cement can present a synergistic effect improving material's cell colonization, osteoblasts' activity, osteoinduction processes, and osteoconduction for bone regeneration [92]. The mixture of porosity and bioactive agents is possible, adding chitosan (CS) and a bioglass, but these porous cement's mechanical properties are low to be used in orthopedic applications [32].

6.2. Bioactivity in Acrylic Bone Cements loaded with chitosan

Although chitosan presents similarities with the extracellular matrix and has demonstrated some intrinsic bioactivity, its use as the only material in bone tissue engineering is limited by an inherent polysaccharide's low mechanical with an eventual loss of biological properties over time [93,94].

Several investigations about CS addition's effect reported cement with improved mechanical and thermal properties with chitosan additions from 5 to 10 wt.%, depending on the particle size used [4,16,95]. Also, the presence of CS at 5 wt.% contributed to avoiding the formation of the fibrous tissue interface between the bone and the implanted cement in bone defects of the animal model (rat), improving the bone-cement integration [16], and generating a significant increase in the osteoblast cellular activity compared to the ABC without chitosan [95]. In contrast, other researchers found decreased mechanical properties with CS's addition to the ABCs [96,97].

The inclusion of 10 wt.% chitosan in the solid phase of an ABC with different proportions of HA generated pore spaces for osteoconduction; however, losses in compressive strength of up to 46.3% were presented [92]. Lin et al. [98] report the use of calcium β -phosphate microspheres encapsulated in chitosan gel. Zamora et al. [99] used CS microspheres in the solid phase of bone cements, which generated lower maximum curing temperatures (T_{max}). They increased setting time (t_{set}), also produced a rough surface due to its gradual degradation, which benefited adhesion and cell

proliferation, but reduced mechanical properties. Besides, Zamora et al. [99] reported subdermal implantation studies in Wistar rats, concluding that the cements had excellent compatibility.

The presence of up to 20 wt.% of CS, besides favored the bioactivity of the ABCs by increasing the Ca and P ions deposited on its surface when being immersed in a simulated biological fluid (SBF) for different periods [100], decreased the $T_{\max}$ and increased the t_{set} , and the surface wettability [42,100]. Additionally, the implantation in Wistar rats' parietal bone showed an increase in the cement's osseointegration with CS. This osseointegration was higher when the formulation presented CS + GO, with which a near-total healing bone-cement interface was reached in the bone defect [101]. However, it was evidenced the reduction in the mechanical properties and the increase in the residual monomer content.

Tavakoli et al. [102] found that the incorporation of 25 wt.% of a CS/GO nanocomposite in the solid phase of the cements improved healing properties and increase bioactivity after four weeks of immersion in SBF, increased cell viability, growth, and cell adhesion in MG-63 cell culture. Similar results were obtained by Soleymani et al. [103] when incorporated in the formulation of ABC CS/multiwalled carbon nanotubes composite.

6.3. Bioactivity in Acrylic Bone Cements Loaded with Graphene Oxide

Graphene oxide has attracted attention in bone tissue engineering applications because of their biological, mechanical, and physical interesting properties mentioned before [104,105]. The presence of polar groups on this material's surface improves compatibility with polymer matrices [52,106].

The addition of GO in percentages up to 0.5 wt.% in ABCs has favored the mechanical properties to bending and compression [107–110]. Paz et al. [110] reported increases in fracture toughness and fatigue performance attributed to the GO induced deviations in the crack fronts and hampered crack propagation.

Several authors reported that the increase in GO content in the ABCs decreases the maximum polymerization temperature. It acts as an inhibitory and retardant agent of the MMA polymerization reaction [107,108,110], which decreases the risk of thermal necrosis and improves the cement' bioactivity. On the other hand, this inhibitory effect on the polymerization reaction has generated an increase in the cement's residual monomer content, which increases the possibility of thermal necrosis of the surrounding tissue with percentages of GO higher than 0.3 wt.% [108].

ABCs loaded with GO presented high cell viability, low apoptosis, and extensive spread on disc surfaces of mouse L929 fibroblasts and human Saos-2 osteoblasts [111]. Pahlevanz et al. [112] reported that bone cement bioactivity consisting of PMMA and polycaprolactone (PCL) was improved with fluorapatite and GO, evidenced by an increase in both in apatite formation ability on the polymer surface and in cell viability against MG-63 osteoblast.

Mirza et al. [113] prepared ABCs with GO and evaluated human bone marrow mesenchymal stem cells (hBMSCs). The anabolic and catabolic effects of the incorporation of GO on osteoblast cells at a genetic level, and found early osteogenesis and increased proliferation of hBMSCs on the formulation with higher GO content (0.048 wt.%) compared to the control and the one with lower GO content. On the other hand, Paz et al. [114] evaluated MC3-T3 viability after an incubation period of 72 h when they were exposed to cements with 0.1 wt.% GO and without any cytotoxic effect present.

Valencia et al. [109] reported that GO's presence did not generate any cytotoxic effect in ABCs. The same group [101] implanted GO loaded cement in the parietal bone of Wistar rats. They reported that GO improves the cement's osseointegration since it accelerated the unloaded cement's sealing process.

Contrary to all the results shown above, Sharma et al. [22] compared cements' osseointegration with GO, graphene (G). Amine-functionalized Graphene (AG), showing that the ABCs containing GO and G showed a reduction of cell viability against human osteoblast (MG-63) compared to the cement containing AG, generating cell apoptosis because GO and G penetrated the cytoplasm by endocytosis pathway, disturbing the metabolic activity, gene transcription, and translation processes.

Table 2 shows a summary of the bioactivity in bone cements and CS and GO characteristics. This table appreciates the CS's beneficial contribution to improving the biocompatibility and osseointegration of the ABCs and the significant reduction in mechanical properties. On the other hand, the GO provides mechanical reinforcement and does not present cytotoxicity in most reported investigations. The combination of both components, individually [101,109] or like a nanocomposite [102], has shown synergy in the biological and mechanical properties since there were increases in mechanical properties up to 69% and in viability, proliferation, and cellular adhesion of human osteoblasts and MG-63 cell culture in contact with these cements. Additionally, implants in Wistar rats' parietal bone for three months showed more excellent osseointegration in the cements that contained both loads.

7. Antibacterial Properties

Implanted bone cements carry a high risk of infection when introduced into the body due to the possibility of biofilm adsorption of microorganisms on an inert surface, which usually requires multiple surgeries for treatment [115,116].

Chronic infection of joint prostheses requires surgical removal of the implant to eradicate the infectious process. The procedure can be performed in a single or two steps. In the first place, the intervention consists of removing the infected implant, decontamination of the site, and implanting a new revision prosthesis covered with bone cement with antibiotics. In the second place, the revision prosthesis placement phase is delayed for a pair of months (2-5 months), during which a bone spacer cement impregnated with an antibiotic is placed in the site [117].

In recent years, the increase in resistance of microorganisms to antibiotics has led to severe health problems since most of the bacteria causing the infection are resistant to at least one of the antibiotics that are generally used to eradicate the infection; therefore, worldwide efforts are ongoing to study new antimicrobial agents that can effectively inhibit the growth of bacteria [59].

Table 2. Summary of the effect of CS and GO on the bioactivity of ABCs

Materials	Advantages	Disadvantages	References
ABC	Inert behavior	Insufficient adhesion to the bone	[10,14,32]
ABC + Chitosan	Porous ABCs	Reduction of mechanical properties	[4,16,101–103,42,92,95–100]
	Improvement in osseointegration	Increasing residual monomer content	
	Reduction of T_{max} reached during polymerization		
	Increasing cellular activity of osteoblasts		
ABC + Graphene Oxide	Improvement of mechanical properties	Increasing residual monomer content	[22,101,114,102,107–113]

	Reduction reached during polymerization	T_{max} during	Reduction of cell viability against MG-63 cell culture.
	Good compatibility with bone tissue		
	Accelerates bone formation		
	Non-cytotoxic to human osteoblasts, L929 fibroblasts, MG-63, hBMSCs, and MC3-T3 cells.		
ABC + Chitosan + Graphene Oxide	Increased osseointegration	Increase in residual monomer content	[101,102,109]
	Increased mechanical properties		
	Increased viability and cell adhesion (MG-63 and human osteoblast)		
	Increased antibacterial activity compared to separate loads		

7.1. Formation of the biofilm

All biofilms originate from the same sequence of events, as shown in Figure 6. In clinical uses of biomaterials, the biomaterial surface is first covered with a conditioning film composed of macromolecules adsorbed from the biological environment in which the biomaterial is placed (Figure 6a). In the case of orthopedic biomaterials, such as PMMA, which contact the bone and come into direct contact with the blood, they absorb several proteins from the plasma before the first microorganism appears [118].

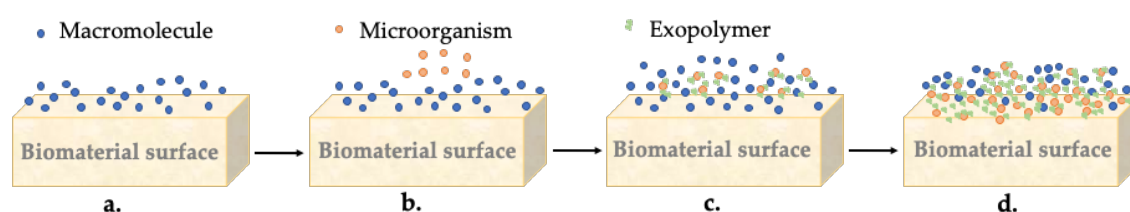


Figure 6. Description of the biofilm formation. a. Formation of the conditioning film, b. Initial adhesion of the first microorganisms, c. Production of exopolymer and d. Glycocalyx formation

Microorganisms arrive at this conditioning surface through various transport mechanisms, such as diffusion, convection, or sedimentation (Figure 6b). Orthopedic implants can be infected by direct contamination during surgery. The initial adhesion of microorganisms is reversible and depends on

the microbial cell surface's general physiochemical characteristics, the biomaterial surface, and the biological fluid. This reversible adhesion of microorganisms can be irreversible by producing an exopolymer (extracellular polymeric secretion produced by bacteria), which generates a firm anchorage of the bacteria (Figure 6c). After that, the exopolymers embedded microorganisms in the biofilm to form a called "glycocalyx" (Figure 6d). This term refers to the accumulation of glycoproteins in the external coating of the biomaterial, which, in addition to serving as an anchorage biofilm, providing a physical and impenetrable barrier for antibiotics and immune cells [116,118].

The growth of the adhering organisms is the primary mechanism of multiplication in a biofilm to a new layer is added, and the bacteria find spaces to multiply, which finally leads to the formation of a denser film. As a final step in forming biofilms, organisms on the biofilm periphery can separate, leading to septic loosening [118].

7.2. Antimicrobial properties in acrylic bone cements

Open surgeries always risk contamination; however, biomaterials' presence increases infection risk due to their susceptibility to bacterial colonization [118]. This event depends on several aspects, such as the physicochemical properties of the surface of the biomaterials, the structure of the cell membrane, and the bacteria receptors, which will define if the bacteria that reach the surface of the biomaterial can easily adhere and proliferate there, causing the septic loosening of the prosthesis [119].

Bacterial infections are a common complication after total joint replacement [120], with infection rates between 1 and 3% [117,118], mainly caused by *Staphylococcus epidermidis*, *Staphylococcus aureus*, and *Staphylococcus capitis* [97,121,122]. Their incidence is expected to increase significantly due to the expanded use of these procedures in older patients and younger people [123]. One way to reduce such infections is by adding antimicrobial agents to the bone cements used for implant fixation [120]. Commonly used antibiotics are tobramycin, gentamicin [122,124], vancomycin, and cephalosporins [125].

In antibiotic-loaded bone cements (ALBC), it is known that most of the antibiotic is retained inside the cement, and only a small amount on the surface is available for diffusion to the desired site [115]. The kinetics of antibiotic elution from bone cements consists of an initial phase of a rapid release of the drug, followed by a much slower release [126].

Some studies have emphasized that antibiotics are mainly released from the surface. The initial fast release is mainly due to the antibiotic dissolution from the surface or near the surface. Subsequently, some antibiotic is released slowly due to the antibiotic diffusion through the cracks, pores, and cement's imperfections [118,127,128]. However, elution of the antibiotic incorporated into the cement is not complete since all in vitro studies indicate that only 5-8% of the total antibiotic incorporated into the ABCs is released after long periods, and in vivo studies also confirm that only 5-18% of the incorporated gentamicin is released [118]. Generally, the release kinetics correlated with the cement's degree of porosity rather than the number of antibiotics [119].

ALBC has been widely used for the treatment of infected arthroplasty, and given the increased incidence of this type of infection, a large number of review papers are found in which aspects such as release mechanisms, clinical efficacy, and safety of the ALBC are discussed [13,118,129–133].

Bioabsorbable charges such as poly (N-vinyl-2-pyrrolidone) [134], lactose, hydroxypropylmethylcellulose [128], gelatin microparticles [89], mesoporous silica nanoparticles [135], and carbon nanotubes [115] have been introduced in their formulations to improve antibiotic's release from ABCs. Their dissolution and porous structure increase the open pores' interconnection with the surface, and the antibiotic can be released into the surrounding material by molecular diffusion. This interconnection of the porosity increases the cement's permeability and leads to an increase in the penetration of fluids, and thus, the antibiotics contained inside the ABCs will be available for elution [126]. These additions generated an increase in the proportion of released antibiotic; however, they produced a loss in the ABCs' mechanical properties. This cement's clinical use is restricted only to treating infections and not to the prostheses fixation [126].

The use of antibiotic-loaded ABCs is a common practice in arthroplasty; however, the development of antibiotic-resistant bacteria due to the prolonged use of antibiotics makes the treatment of infections even more difficult [132,136]. Also, losses in mechanical properties due to the increase in the number of antibiotics that surgeons generally use to reduce the risk of infections rise, generating a premature implant with failures [26,137,138]. Therefore, it is necessary to develop alternatives to antibiotics for the treatment of infections.

The resistant bacterial species list includes multi-drug resistant (MDR) and extremely drug-resistant (XDR) bacteria. These bacterial strains have evolved to exhibit resistance to almost all commercially available antibiotics, affecting millions worldwide [139]. Because of this, there are reports of the addition to ABCs of antimicrobial agents different from antibiotics, such as quaternary amine dimethacrylate iodine comonomer [140], quaternary amine dimethacrylate comonomer [141], gold nanoparticles [142], 2-methacryloyloxyethyl phosphorylcholine, quaternary ammonium dimethylaminohexadecyl methacrylate [143], and Ag₂O [144].

7.3. Antibacterial Mechanism of Chitosan

CS's antimicrobial activity depends strongly on physical properties such as molecular weight (M_w) and the degree of deacetylation (DD). CS with higher DD tends to have higher antimicrobial activity [145,146], while with M_w , it has the opposite effect [147]. Although some authors report that maintaining constant DD, there is an optimal value of M_w in which the highest antimicrobial activity is obtained (90,000 Da) [148]. Others reported that an M_w range between 10000 - 100000 Da is adequate to inhibit *Escherichia coli*, while a molecular weight of 2200 Da accelerated bacteria growth. Concerning CS concentration, it is found that low concentrations (0.2 mg/mL) favored the bonding of polycation to the cell's negative surface, causing its impermeability, while high concentrations could generate a positively charged network by remaining in suspension [149].

The exact mechanism of the antimicrobial activity of CS is not well known. However, so far, the most accepted is based on the electrostatic interactions between positively charged chitosan (NH_3^+) and the negatively charged components of the bacterial cell membrane, such as the anionic cell wall of glycans and proteins or phospholipids in the cytoplasmic membrane. Depending on the type and location of the interaction, various effects are possible. The adhesion of chitosan to the anionic macromolecules of the cell wall forms an impermeable layer around the cell, preventing the transport of nutrients into and out of the cell. In contrast, the interaction with cell membrane constituents alters the permeability, leading to the leakage of intracellular electrolytes, glucose, enzymes, and other low-molecular-weight proteinaceous cytoplasmic materials [149–151].

The chitosan interrupts the physiological activities of the cell but affects *Gram-positive* and *Gram-negative* cells differently since the CS with higher molecular weight forms a polymer shield to prevent the entry and exit of nutrients in *Gram-positive* cells, the low M_w enters the *Gram-negative* cell, and binds to cytoplasmic compounds, causing flocculation with altered physiological processes [151].

Other possible antibacterial mechanisms of CS include chelation of essential nutrients necessary for cell growth or inhibition of messenger RNA and protein synthesis through penetration of chitosan into the cell nucleus of the microorganism and the bonding of CS to DNA. For this penetration to occur, CS must have an M_w below 5000 Da [147], which allows it to pass through the bacterial cell wall, composed of multilayers of cross-linked murein in the plasma membrane [150,151]. The minimum concentration of CS inhibition is between 0.05 and 0.1%, depending on the species of bacteria and the chitosan molecular weight [152].

7.3.1. Antibacterial Effect of Chitosan in Acrylic Bone Cements

The addition of chitosan to ABCs has shown little inhibition of the growth of *S. aureus*, *S. epidermidis*, and *E. coli* bacteria [101,109,120]. Some researchers even report no antimicrobial benefit against the same bacteria, other than *S. Capitis* [96,97].

Researches indicate that using chitosan nanoparticles and quaternary ammonium nanoparticles derived from CS as bactericides in the commercial cements CMW Smartset and CMW Smartset-G against *S. aureus* and *S. epidermidis* have found that these nanoparticles generate inhibition of the

growth of both bacteria. Besides, they do not present toxic effects on 3T3 mouse fibroblasts, and the mechanical properties of both cements are maintained [120]. Wang et al. [46] found that the addition of quaternary chitosan (N-(2-hydroxy)propyl-3-trimethylammonium chitosan chloride)-based hydrogels loaded with nanosized hydroxyapatite gave the cement excellent antibacterial properties against *E. Coli* and *S. aureus* bacteria.

Other studies show the effect of including quaternary chitosan as hydroxypropyltrimethyl ammonium chloride (HACC) in the ABCs, which inhibits the development of rabbit infections tibia caused by antibiotic-resistant bacteria [136]. The effects also include lowering T_{max} , prolonging t_{set} , increasing hydrophilicity, increasing apatite formation when immersed in SBF, and improving adhesion and proliferation of human bone marrow-derived mesenchymal stem cells [153].

In the last years, there has been a great interest in reinforcing polymeric matrices with nanomaterials to generate biocompatible and antibacterial nanocomposites [154]. In the case of CS, it is found that it has an excellent antimicrobial activity, and it is improved against a wide range of *Gram-positive* and *Gram-negative* bacteria with the incorporation of nanoparticles, such as zinc oxide (ZnO) [155,156], Au [4,157], Ag [42,158,159], and reduced GO [94]. Some of these nanocomposites in ABCs demonstrated improvement in antibacterial activity compared to CS alone [4,42]. Future studies could include incorporating CS nanocomposites that lack evaluation in ABCs to studying their effect on cement' antibacterial properties.

7.4. Antibacterial Mechanism of Graphene Oxide

GO is a promising material for developing antimicrobial and antiviral surfaces due to its high surface/volume ratio [160]. The antimicrobial activity of GO is contact-based; thus, it offers a viable alternative to surfaces that release biocides such as antibiotics, which are consumed at the surface over time [161]. It has been proposed that the antimicrobial activity of GO occurs when films come into direct contact with bacterial cells through physical and chemical interactions due to their negative charge and laminar structure [49,62,160,162]. In this process, the cell membrane is the main target of GO cytotoxicity since membrane damage has been evidenced in bacteria exposed to GO through morphological changes in cell structure, leakage of ribonucleic acid (RNA), intracellular electrolytes, absorption of dyes impermeable to the membrane, and changes in transmembrane potential [161].

Membrane damage can be caused by the sharp atomic edges of GO, which can penetrate the cell membrane and physically disrupt its integrity, or it can also be produced through lipid peroxidation induced by the oxidizing nature of GO. Oxidative stress has been considered an essential component of antimicrobial activity for bacterial cells exposed to GO [49,161]. In Table 3, the different mechanisms of antimicrobial activity proposed for GO are highlighted.

Table 3. Primary mechanisms of antimicrobial activity in GO

Proposed mechanism	Definition	References
Extremely sharp edges	It is the primary mechanism of antimicrobial activity in GO. Damage to the membrane when it comes into contact with the edges.	[59,163]
Oxidative stress (through the production of ROS)	It plays a minor role in antimicrobial activity. GO induces reactive oxygen species (ROS), which disrupts the balance in redox processes inside the cell and causes damage to cell components leading to apoptosis in bacteria.	[164]

Oxidative Stress (Independent of ROS production)	GO sheets interrupt a specific microbial process by disturbing or oxidizing a vital cell component or structure without producing ROS.	[50,165]
Formation of GO-cell aggregates	GO sheets envelop the bacteria forming aggregates, locally disturbing the cell membrane and inducing the decrease of the bacterial membrane potential and the leakage of fungal spore electrolytes.	[59,166]

Several researchers suggest that the sharp edges of the GO and the reactive oxygen species (ROS) are not a fundamental part of the antimicrobial mechanism of GO. Instead, the primary mechanism considered is transferring electrons from the bacterial membrane to the GO's surface. When it comes to bacteria, GO acts as an electron receptor that pumps the electron outside the bacteria's membrane, creating oxidative stress independent of the ROS. The antimicrobial activity of GO is strongly related to the number of basal planes of the sheets [165].

The antimicrobial activity of GO and other allotropes of carbon is affected by the nanomaterial's surface's size and chemistry. An increase in the available surface area through particle size reduction improve antibacterial activity due to more significant interaction with bacteria [59]. This behavior was reported by Perreault et al. [161], who worked with GO films on which it was proved that the antimicrobial activity against *E. Coli* bacteria is inversely related to the size of the sheets, reaching an increase in this activity of four times when the area of the sheet varied from 0.65 to 0.01 m². This higher antimicrobial activity of the smaller GO sheets is attributed to the oxidative mechanism associated with the smaller sheets' higher defect density. In contrast, GO suspensions results show that antimicrobial activity increases with sheet size [106,161] against the same bacteria.

The antimicrobial activity of GO has also been questioned by researchers who affirmed not to have found any type of intrinsic inhibition in the growth of *E. Coli* with GO [167], nor *Pseudomonas aeruginosa* [94]. However, they suggest that GO is a cell growth stimulator by increasing the adhesion and proliferation of mammalian cells [167]. These contradictory results encourage further research on the effects of GO on microorganisms. However, because materials in the graphene family have generally demonstrated bacterial toxicity and relatively low cytotoxicity, it has been suggested that these materials can be applied as antimicrobial products [168].

7.4.1 Antibacterial effect of GO on ABCs

When evaluating the antibacterial activity of cements added with GO against *Gram-positive* bacteria *Bacillus cereus*, *S. aureus*, and *Gram-negative Salmonella enterica* and *E. coli*, it was found that the increase in GO content up to 0.5 wt.% increased this property significantly in the cement with both types of bacteria with a level of significantly lower than 0.01 [108]. Likewise, in other investigations, it has been confirmed the antibacterial activity of bone cements against *E. coli*, with GO percentages of 0.3 wt.% [101,109]. In these investigations, it was reported that although the significant contribution to the antibacterial activity of ABCs loaded with GO and CS is generated by GO, CS also has a minor contribution to this property.

On the other hand, according to the results presented by Paz et al. [114], ABCs modified with 0.1 wt.% of GO showed a lack of antimicrobial activity against *S. Aureus*, possibly because the GO powder level was below the threshold required for a positive antimicrobial response. This confirms the results obtained by several authors, who affirmed that GO's antibacterial activity depends on the concentration [108,163,164].

Table 4 shows a summary of the strategies used to confer antimicrobial activity to bone cements and the characteristics offered by CS and GO. From this table, we conclude that the development of bacteria resistant to antibiotics due to their excessive use has generated massive research potential

towards using new agents that generate antimicrobial activity in ABCs. Despite the good antibacterial properties conferred to CS nanometric size cements or modified with some functionalization, GO has conferred to cement outstanding antimicrobial properties. This makes that cements added with GO or GO+CS are promising for preventing and treating infections in arthroplasties.

Table 4. Summary of strategies employed to confer antimicrobial activity in ABCs and the effect of CS and GO on these cement properties

Strategies	Studied bacteria	Advantages	Disadvantages	References
ABC + Antibiotics	<i>S. Aureus</i>	Releases antibiotic	the Most of the antibiotic is trapped inside the cement Antibiotic-resistant bacteria have been developed	[118,124–127]
ABC + Antibiotics + Bioactive fillers	---	A higher percentage of antibiotic released Improves osseointegration	Reduces mechanical properties Antibiotic-resistant bacteria have been developed	[89,115,126,128,134,136]
ABC + Chitosan	<i>S. epidermidis</i> <i>S. Aureus</i> <i>E. coli</i> <i>S. Capitis</i>	Presents good antimicrobial activity when the CS is functionalized or in nano size.	CS without modifications Presents extremely low or no antimicrobial activity in ABCs	[96,97,101,109,120,136,153]
ABC + Graphene Oxide	<i>B. cereus</i> <i>S. Aureus</i> <i>S. enterica</i> <i>E. coli</i>	Shows antimicrobial activity against <i>Gram-positive</i> and <i>Gram-negative</i> bacteria		[101,108,109,114]
ABC + Chitosan + Graphene Oxide	<i>E. coli</i>	Increased antibacterial activity compared to cement with separately added		[101,109]

Paper does not report studies with bacteria; the only report is the antibiotic elution improvement.

8. Future Perspectives

Given that the residual monomer in ABCs can generate chemical necrosis of the surrounding tissue and thus cause the prosthesis's loosening, an in-depth study of the different related factors to decrease residual cement monomer is important with formulations loaded with CS and GO.

The good antibacterial properties shown by cements added with GO and CS against *Gram-positive* and *Gram-negative* bacteria studied so far are promising results. An in-depth study of the

potential of this type of addition in developing bone cements with antibacterial properties is needed, broadening the range of bacteria studied and including those that occur more frequently after a total joint replacement.

9. Conclusions

Despite ABCs being inert materials that do not stimulate any type of chemical adhesion with bone, the incorporation of CS has improved the biological interaction in cements, increasing cell viability, growth, cell adhesion in osteoblasts and increasing the deposition of Ca and P ions on the surface of the cement under *in vitro* conditions. It also prevents fibrous tissue formation around the cement and promotes osseointegration with bone under *in vivo* conditions in animal biomodels. The contribution of CS to the antibacterial properties of cements has been limited and increased when its size is reduced to the nanometric scale or when it has determined functionalities. However, the mechanical properties of these cements are decreased.

ABCs loaded with GO show high potential for use in the prevention and treatment of infections in arthroplasties. They have shown elevated antibacterial activity against some types of *Gram-positive* and *Gram-negative* bacteria and have also been shown to promote osteogenesis.

The addition to the ABCs of CS and GO could be an attractive alternative to generate bioactive and antibacterial ABCs with adequate mechanical properties since GO has contributed to improving the mechanical properties with a reinforcing effect in the cements loaded with CS.

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Capítulo 3.

ARTÍCULOS

PUBLICADOS Y

SOMETIDOS

3.1 Ruiz Rojas, L.M.; **Valencia Zapata, M.E.**; Gordillo Suarez, M.; Grande Tovar, C.D.; Mina Hernandez, J.H. *Optimization of mechanical and setting properties in acrylic bone cements added with graphene oxide by using a completely randomized factorial design. RSC Advances*. Artículo sometido para publicación (El anexo B contiene el certificado de sometimiento del artículo).

ARTICLE

Optimization of mechanical and setting properties in acrylic bone cements added with graphene oxide by using a completely randomized factorial design

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Although the extended use of acrylic bone cements (ABC) in orthopedics presents some disadvantages related to the generation of high temperatures during methyl methacrylate polymerization that produces thermal necrosis of the surrounding tissue and low mechanical properties, both disadvantages cause an increase in costs for the health system and a decrease in the patient's quality of life due to the prosthesis's loosening. Materials such as graphene oxide (GO) have a reinforcing effect on ABC's mechanical and setting properties. Thus, GO addition occurred in the liquid phase. The mechanical and setting properties established for cements in the ISO 5833-02 standard were optimized using a completely randomized experimental design with a factorial structure. The factors studied were the sonication time and the GO percentage in the liquid phase, together with the percentage of benzoyl peroxide (BPO) in the solid phase. Nine formulations selected as the most suitable presented an increase in the properties to compression, flexion, and the time of cure and decreased the maximum temperature reached during the polymerization. From the selected formulations, those containing 0.3 wt.% of GO highlighted because all the possible combinations of the studied factors generate desirable properties for the ABC.

Introduction

Acrylic bone cements (ABC) are biomaterials used since 1960 in orthopedics, and their applications extend from arthroplasty procedures¹ to neurosurgery², vertebroplasty, and kyphoplasty^{3,4,5}. Arthroplasty procedures generally use ABCs with impressive results⁶. The cement mantle's prominent roles are to stabilize the implant and transfer body weight and service loads from the prosthesis to the bone^{1,7,8}. Successful arthroplasty is related to the implants; therefore, the cement's good mechanical properties must be guaranteed⁷.

ABCs are commercialized in two components, a solid phase containing the polymer, which is usually polymethylmethacrylate (PMMA), or a derived copolymer, benzoyl peroxide (BPO) as the polymerization reaction

initiator^{9–12} and a radiopacifier agent as the barium sulfate (BaSO₄) or zirconium dioxide (ZrO₂). The liquid phase is composed of a monomer (generally methyl methacrylate (MMA)), N,N-Dimethyl-*p*-Toluidine (DMPT)) as an activator for the formation of radicals^{7,11} and hydroquinone as a stabilizer to room temperature^{1,5}.

When the BPO and the DMPT are mixed, benzoyl radicals are produced, which start the polymerization process^{7,11}. The polymerization reaction's exothermic character may play a role in thermal necrosis of the periprosthetic tissue^{6,8}, which could induce the early loosening of an implant¹.

Graphene oxide (GO) is a two-dimensional material, a graphene sheet with oxygen groups over its surface¹⁵. It has attracted significant attention in biomedical applications due to its high mechanical performance, biocompatibility, biodegradation, and excellent antibacterial properties^{16,17}. Several research types have demonstrated that GO content in ABCs generates a reinforcement on the mechanical properties flexion^{18,19}, compression, fracture toughness⁸, and fatigue performance³. Reduction in the maximum temperature reached during polymerization ($T_{\max}$) increases the setting time (t_{set})^{3,20–22}, and additionally, improvements in bioactivity^{22–24} and antibacterial properties^{21,25} are also obtained. Despite these improvements, it has also been reported that GO in ABCs can inhibit the polymerization reaction, which can be counteracted by increasing the radical species. However, since the BPO content influences the polymerization exotherm ($T_{\max}$)¹, it is necessary to optimize its content.

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Electronic Supplementary Information (ESI) available: Supporting information file is available free of charge and contains Table S1. Completely random factorial design. Table S2. Analysis of variance of the variables: Maximum temperature ($T_{\max}$) and Setting time (t_{set}). Table S3. Analysis of variance of the variable Compressive strength and Compressive modulus. Table S4. Analysis of variance of the variables Bending strength and Bending modulus. See DOI: 10.1039/x0xx00000x

The effect of GO's sonication time has been studied in materials different from ABCs^{26–28}, and it has been found that this parameter is related to two key characteristics of GO: the lateral size and the thickness of the sheets^{29,30}. Increasing the sonication time decreases the thickness and particle size, as previously reported³¹. However, excessive sonication times can decrease sheets of hydrophilicity and functional groups³² with a simultaneous increase of the defects in the GO structure^{33,34}.

Although there are several types of research where the GO content alone is studied, there are no studies on the interactive effects of the size and content of GO and the BPO content. This study aimed to optimize the parameters of sonication time in the GO, GO/liquid ratio, and BPO/solid ratio. A completely randomized factorial design was used to evaluate these factors' interactive effect on the response variables $T_{\max}$, t_{set} , compressive strength, compressive modulus, bending strength, and bending modulus ISO 5833-02³⁵. Some of the formulations and conditions selected have not been studied and have great potential for arthroplasty.

Materials and methods

Materials

The solid phase was composed of PMMA beads (Veracril® New stetic S.A), BPO, and BaSO₄ (Alpha Aesar). The liquid phase was composed of MMA (MERK), 2-(diethylamino) ethyl acrylate (DEAEA), 2-(diethylamino) ethyl methacrylate (DEAEM) (Sigma Aldrich), DMPT (Merck), and graphene oxide synthesized by our group using a modification of the Hummers method^{21,22}.

Experimental design

A completely randomized experimental design with a factorial structure was applied to analyze the information obtained in this study statistically. Three study factors were considered: The Sonication time (1 and 5 h), BPO/Solid ratio (2 and 2.5 wt.%) and GO/Liquid ratio (0, 0.1, 0.2, 0.3, and 0.5 wt.%), for the generation of a total of 20 treatments with three repetitions. Each of the treatments was performed at random under identical conditions. The responses variables in the statistical analysis were the maximum temperature reached during polymerization ($T_{\max}$), setting time (t_{set}), compressive strength, compressive modulus, bending strength, and bending modulus. The analysis of variance (ANOVA), post-ANOVA tests (Tukey test), and graphs of the data obtained was carried out in the statistical package MINITAB version 19. Simultaneously was evaluated the interaction significance and the factor effects over the response variables.

At the same time, we assessed the importance of the interaction and each factor's primary effects on the response variable. Equation 1 expressing the utterly randomized design model with a factorial structure is given by

$$Y_{ijkl} = \mu + Z_i + \beta_j + \alpha_k + (Z\beta)_{ij} + (Z\alpha)_{ik} + (\beta\alpha)_{jk} + (Z\beta\alpha)_{ijk} + \varepsilon_{ijkl} \quad (1)$$

Y is the response variable, μ is the general mean, Z is the effect due to sonication time, β is the effect due to BPO/solid ratio,

and α is the effect due to GO/liquid ratio. $(Z\beta)$, $(Z\alpha)$, and $(\beta\alpha)$ are the interaction effect, and ε is the experimental error.

Preparation of GO

GO was obtained by the modified Hummers method as reported by Valencia et al.²¹ Briefly, 3 g of graphite were oxidized with H₂SO₄ and KMnO₄ for four days, then purified by dialysis and dried by lyophilization. The characterizations made to GO are reported in a previous study^{21,22}. The lateral size of GO dispersed for one hour measured by dynamic light scattering (DLS) was 400 nm, while the one dispersed during five hours presented a size of 367 nm.

Preparation of ABCs

Cement solid phase (SP) preparation followed specifications shown in Table S1 (supporting information). BaSO₄ was constant (10.0 wt.%) in all formulations. PMMA completed the 100% SP according to the BPO percentage. The SP components were mixed manually.

In the liquid phase (LP), the percentage of DMPT was constant for all formulations at 2.5 wt.%, and the 50:50 mixture of the alkaline comonomers DEAEA and DEAEM was 2.0 wt.%. The MMA content was adjusted to obtain 100% of the LP according to the GO's corresponding percentage in the design. LP was obtained by mixing the components and subsequent sonication in an EF 13057 ultrasonic bath (Branson, Danbury, CT, US) at 25 °C and 40 kHz frequency for one or five hours according to the treatment given in the experimental design.

LP was added to SP in an SP/LP ratio to 2 g/mL, and the dough was manually mixed and deposited in Teflon molds with the form required for each test.

Characterization of ABCs

The cements' characterization was based on the evaluation methods proposed in the ISO 5833-02³⁵ standard for ABCs.

Setting properties. After mixing SL and LP, the dough was put in molds with 68 mm in diameter and 20 mm in height, and the temperature and time were registered. $T_{\max}$ was the higher temperature reached during the polymerization reaction, and the t_{set} was calculated as the time where the T_{set} was reached. T_{set} was determined by equation 2. The test was conducted in duplicated.

$$T_{\text{set}} = \frac{T_{\max} + T_{\text{amb}}}{2} \quad (2)$$

Where:

T_{set} is the setting temperature, and

T_{amb} is the ambient temperature.

Mechanical Properties. Mechanical properties were carried out in a universal testing machine Tinius Olsen-H50KS (Tinius Olsen, Redhill, United Kingdom). Compressive strength and compressive modulus were carried out in cylinders with 6 mm

in diameter and 12 mm in height to crosshead displacement rate of 20 mm/min. Each formulation tested six samples.

A four-point bending test was conducted on rectangular beams of 75 mm × 10 mm × 3 mm with a 5 mm/min crosshead displacement rate. Bending strength and bending modulus were calculated according to the ISO 5833-02³⁵ specifications.

Results and Discussion

Setting Properties

For analysis of the maximum temperature and setting time variables based on the effect of sonication time, BPO/SP ratio, and GO/LP ratio, an utterly randomized design model with a factorial structure was proposed, which meets the assumptions of normality about error of the model, at significance levels greater than p-value = 0.15.

Maximum Temperature. The analysis of variance is shown in table S2 (support information). The T_{max} shows that all the interaction effects were significant at levels below 0.021 (Sonication time*BPO/Solid ratio; Sonication time*GO/Liquid ratio; BPO/Solid ratio*GO/Liquid ratio and Sonication time*BPO/Solid ratio*GO/Liquid ratio). A determination coefficient of 94.64 % was obtained, which is a good measure of the controlled effects' contribution to the total variation.

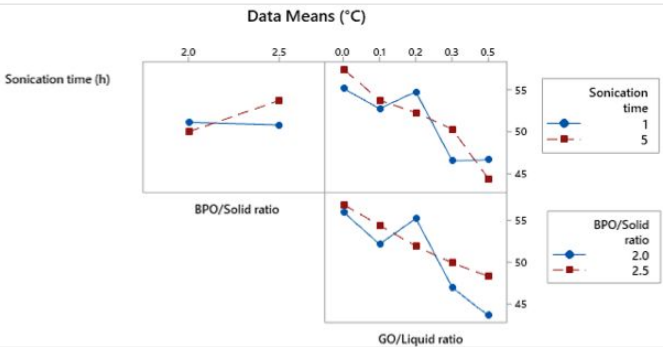


Figure 1. Interaction Plot for the variable T_{max}

Figure 1 exhibits the interaction plot for T_{max} . Increasing the sonication time and the BPO/Solid ratio promoted T_{max} 's higher values, while higher GO percentages decrease the T_{max} reached during polymerization. The T_{max} values of all the formulations studied were found between 41 and 58 °C, below the maximum value allowed by the ISO 5833-02 standard, which is 90 °C. In ABC, a reduction in the T_{max} reached during polymerization is desirable since it reduces the thermal necrosis of the periprosthetic tissue^{6,12,36}, which is related to the loosening of the prosthesis³⁷.

Several researchers have highlighted the influence of the BPO content on the maximum temperature^{5,6,37}, finding that a higher BPO content can increase the concentration of free radicals available during polymerization generating an increase in the amount of energy released during the reaction^{11,38,39}.

The sonication time is related to the degree of dispersion³⁴ and the thickness of the GO particle²⁸. Longer exfoliation times

decrease the size of GO^{29,31}, and possibly its heat dissipation capacity during the polymerization reaction due to the increase of the defects in the GO structure^{33,34,40}.

The decrease of T_{max} with the increase in GO content has been attributed to this component's inhibitory effect during the polymerization reaction because it acts as a sink for free radicals^{18,21,22,41}.

Table 1. Grouping information for T_{max} using the Tukey method and 95% confidence.

Sonication time - BPO /Solid ratio – GO/Liquid ratio	Mean (°C)	Grouping*			
5 - 2.5 - 0.0	58.3	A			
5 - 2.0 - 0.0	56.8	A	B		
1 - 2.0 - 0.2	55.8	A	B	C	
1 - 2.5 - 0.0	55.5	A	B	C	
5 - 2.5 - 0.1	54.8	A	B	C	
5 - 2.0 - 0.2	54.7	A	B	C	
1 - 2.0 - 0.0	54.5	A	B	C	D
5 - 2.5 - 0.3	54.2	A	B	C	
1 - 2.5 - 0.1	54.0	A	B	C	
1 - 2.5 - 0.2	53.8	A	B	C	
5 - 2.0 - 0.1	52.8	A	B	C	D
1 - 2.0 - 0.1	51.5		B	C	D E
5 - 2.5 - 0.2	50.7			C	D E
5 - 2.5 - 0.5	50.5		B	C	D E F
1 - 2.0 - 0.3	49.5			C	D E F
1 - 2.5 - 0.5	47.3			D	E F G
1 - 2.0 - 0.5	46.0				E F G
5 - 2.0 - 0.3	44.5				F G
1 - 2.5 - 0.3	43.5				F G
5 - 2.0 - 0.5	41.3				G

*Means that do not share a letter are significantly different.

Tukey's multiple comparison tests shown in Table 1 indicate that group G formulations are the most suitable from a T_{max} point of view since they had the lowest T_{max} (< 48 °C). This suggests that formulations 1 - 2.5 - 0.5; 1 - 2.0 - 0.5; 5 - 2.0 - 0.3; 1 - 2.5 - 0.3; 5 - 2.0 - 0.5 are statistically equal to a significance level of 5%. All group G samples are characterized by presenting the highest GO contents studied, which indicates that this variable's effect was the most significant on T_{max} . Compared to the other two studies, the lower p-value shown for the GO/liquid ratio confirms the previous statement (Table S2).

Setting time. According to Table S2 (support information), for the setting time variable, only the interactions Sonication time *BPO/Solid ratio and Sonication time* GO/Liquid ratio were significant, at levels below 0.076. A determination coefficient of 97.2 % was obtained, indicating an adequate contribution of the controlled effects to the total variation.

According to Figure 2, higher values in sonication time and BPO/solid ratio reduced t_{set} , while the increase in the GO/liquid ratio increased t_{set} . ISO 5833-02 stipulates that the t_{set} should be between 180 - 900 s (3 - 15 min), so all formulations studied are within the acceptable range.

Setting time is one of the most representative setting parameters of the ABCs^{5,37}, and in orthopedics, it is expected to have a moderate value⁷ because it is directly related to the time the surgeon has to mold and apply the cement during the procedure²¹.

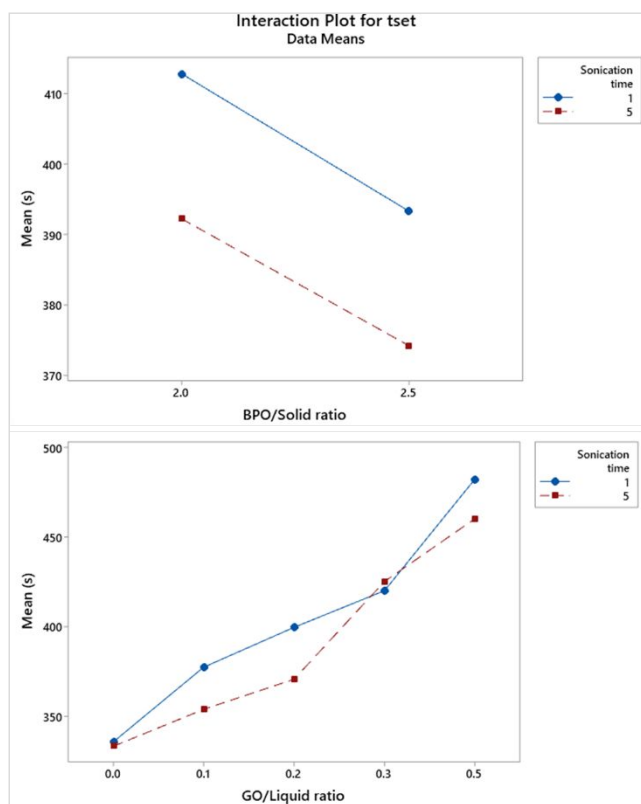


Figure 2. Interaction plot between the effect sonication time vs. BPO/solid ratio and sonication time vs. GO/liquid ratio for the variable t_{set} .

The concentration of BPO plays a vital role in the kinetic parameters of ABC^{1,37,38}, and it is found that the decrease in t_{set} with the increase of the BPO content is due to the higher amount of free radicals available to initiate the polymerization reaction faster^{5,38}.

The longer exfoliation time when generating smaller GO sizes with reduced heat dissipation capacity promotes that the cement hardening reaction occurs in less time.

According to the research of Goncalves et al.¹⁸, the presence of the double bonds C=C in the GO generates extensive electron donor-acceptor capability, so it becomes remarkably reactive toward free radicals, acting as polymerization retardation and polymerization inhibition simultaneously during the polymerization reaction of the MMA in the ABC.

Based on Tukey's post-ANOVA test for the t_{set} variable (Table 2), although all the formulations are within the range allowed by ISO 5833-02, group A provides higher values in both interactions for cement setting time. Formulation with one hour of sonication time, 2.0 wt. % of BPO, and 0.5 wt.% of GO presented the highest setting time

Mechanical properties

Compressive properties. The models proposed for the variables compressive strength and compressive modulus met the assumption of normality on errors with significance levels greater than 10%. The variance analysis presented in Table S3 showed that in both properties, the sum of squares (SS) of the effects studied is greater than the sum of squares of the errors, evidenced by R^2 values of 96.26% and 96.6% for the strength and modulus, respectively. These results indicate that there is a more outstanding contribution from the controlled effects than the random effects.

Compressive strength. Figure 3 shows that the compressive strength decreases with an increasing percentage of BPO and increases when the sonication time and GO content in the formulation. All the formulations studied presented compression strength values higher than the minimum established in ISO 5833-02 of 70 MPa³⁵.

The decrease in the compressive strength with the increase in the BPO content can be related to the fact that the higher amount of free radicals generated by BPO during the polymerization reaction activates more monomer that acts as nucleation sites for the growth of the polymer chain, generating lower molecular weights that directly influence the mechanical behavior of the cement⁵.

Table 2. Grouping information for t_{set} using the Tukey method and 95% confidence

Sonication time *BPO /Solid ratio	Mean (s)	Grouping*	Sonication time *GO/Liquid ratio	Mean (s)	Grouping*
1 - 2.0	413	A	1 - 0.5	482	A
1 - 2.5	393	B	5 - 0.5	460	A
5 - 2.0	292	B	5 - 0.3	425	B
5 - 2.5	386	B	1 - 0.3	420	B
			1 - 0.2	400	B C
			1 - 0.1	377	C D
			5 - 0.2	372	D
			5 - 0.1	355	D E
			1 - 0.0	336	E
			5 - 0.0	333	E

*Means that do not share a letter are significantly different.

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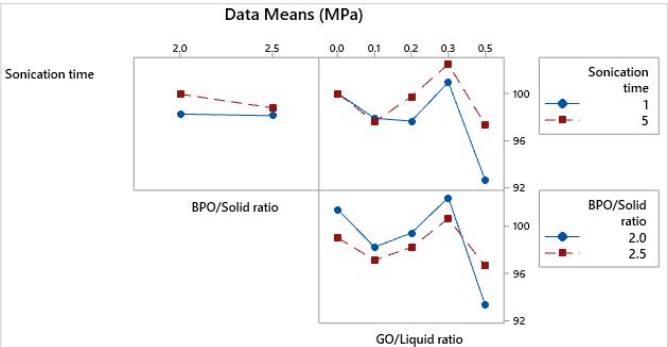


Figure 3. Interaction plot between the effect sonication time, GO/liquid ratio, and BPO/solid ratio for the variable compressive strength.

Some researchers reported an association between the sonication time, the degree of dispersion, and the thickness of the GO particle²⁸. Therefore, the degree of mechanical reinforcement is generated in the matrix⁴². This higher dispersion in the matrix favored the compression strength of the studied ABCs²². The presence of GO favors the chemical interaction between the OH groups of GO and OCH₃ and oxygen bonding groups with double bond oxygen (=O) of PMMA, improving the interfacial adhesion and the strength of the cements^{20,43}. On the other hand, GO contents above 0.3 wt.% can generate agglomerates that diminish the mechanical properties of the cement^{3,21}.

The results obtained to verify the hypothesis tests on the interactions and main effects (Table S3, supporting information) indicate that the third and second-order interactions were significant at levels of significance in the strength and compressive modulus lower than 0.007 (P-value). The analysis of the multiple comparison tests (Tukey's test) is presented in Table 3.

Table 3. Grouping information for Compressive strength using the Tukey method and 95% confidence

Sonication time *	Mean (MPa)	Grouping*			
BPO /Solid ratio					
* GO/Liquid ratio					
5 - 2.0 - 0.3	102.90	A			
1 - 2.0 - 0.3	102.11	A			
5 - 2.5 - 0.3	101.99	A	B		
1 - 2.0 - 0.0	101.43	A	B	C	
5 - 2.0 - 0.0	101.43	A	B	C	
5 - 2.0 - 0.2	100.74	A	B	C	D
1 - 2.5 - 0.3	99.75	B	C	D	E

1 - 2.5 - 0.0	99.04	C	D	E	F	
5 - 2.5 - 0.0	99.04	C	D	E	F	
5 - 2.5 - 0.2	98.70		D	E	F	
1 - 2.0 - 0.1	98.52		D	E	F	
5 - 2.0 - 0.1	98.06			E	F	
1 - 2.5 - 0.2	97.74			E	F	G
5 - 2.5 - 0.5	97.69			E	F	G
1 - 2.0 - 0.2	97.49			E	F	G
1 - 2.5 - 0.1	97.25				F	G
5 - 2.5 - 0.1	97.08				F	G
5 - 2.0 - 0.5	97.00				F	G
1 - 2.5 - 0.5	95.62					G
1 - 2.0 - 0.5	89.71					H

*Means that do not share a letter are significantly different.

Table 3 shows the comparisons between all treatments for compressive strength. Each of the six treatments of group A has the same statistical effect on the compressive strength, and all of them presented values higher than 100 MPa. Within each of the groups B, C, D, E, F, and G, the treatments are statistically equal. It is important to highlight that in group A, formulations with 0.3 wt.% of GO predominated, indicating that this percentage of GO in the formulation could optimize the compressive strength.

Compressive modulus. According to figure 4, shorter sonication time and higher BPO and GO content in the solid and liquid phase, respectively, generate increases in the compression modulus. ISO 5833-02 does not establish a reference value for this property; however, since the cement is responsible for transferring mechanical loads between the implant and the bone¹, mainly of the compressive type in an arthroplasty⁷ is necessary to understand how this property performs.

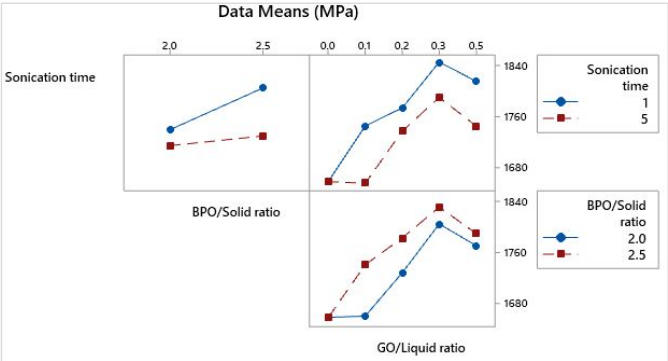


Figure 4. Interaction plot between the effect sonication time, GO/liquid ratio, and BPO/solid ratio for the variable compressive modulus.

Larger particles (shorter sonication time) and the higher quantity of polymer chains (higher BPO content) generated a physical

impediment for the chains' movement, which increased the ABCs' stiffness. As in the compressive strength, the GO content increased the compression modulus of the cement. This mechanical reinforcement of GO over ABCs formulations has been reported by several authors^{20,21,44}.

Based on Tukey's test (Table 4), it is possible to establish that the formulations with one hour of sonication time, 2.5 wt.% of BPO, and 0.3 or 0.5 wt.%, maximize the compression module and present equal effect on this property. The module's lowest values are reached with the lowest GO percentages (Groups H, I).

Table 4. Grouping information for Compressive modulus using the Tukey method and 95% confidence

Sonication time* BPO /Solid ratio* GO/Liquid ratio	Mean (MPa)	Grouping*			
1 - 2.5 - 0.3	1881.50	A			
1 - 2.5 - 0.5	1848.42	A B			
1 - 2.5 - 0.2	1826.75	B	C		
1 - 2.0 - 0.3	1808.89	B	C	D	
5 - 2.0 - 0.3	1799.82	B	C	D	
1 - 2.0 - 0.5	1781.43		C	D	E
5 - 2.5 - 0.3	1779.85		C	D	E F
1 - 2.5 - 0.1	1760.27		D	E	F G
5 - 2.0 - 0.5	1759.19		D	E	F G
5 - 2.5 - 0.2	1738.15			E	F G
5 - 2.0 - 0.2	1735.74			E	F G
5 - 2.5 - 0.5	1730.38				F G
1 - 2.0 - 0.1	1722.33				G
1 - 2.0 - 0.2	1719.82				G
5 - 2.5 - 0.1	1710.46				G H
1 - 2.0 - 0.0	1657.98				H I
5 - 2.0 - 0.0	1657.98				H I
1 - 2.5 - 0.0	1657.62				H I
5 - 2.5 - 0.0	1657.62				H I
5 - 2.0 - 0.1	1618.39				I

* Means that do not share a letter are significantly different.

Bending properties. To model the effect of the sonication time, BPO/solid ratio, and GO/liquid ratio, a completely randomized design model with a factorial structure was proposed, which meets the assumptions of normality about error of the model, at significance levels greater than p -value = 0.15. Through the analysis of variance, the hypotheses about the interaction between effects are tested (Table S4, supporting information), show that effects interaction (Sonication time*GO/Solid ratio; BPO/Solid ratio*GO/Liquid ratio) were significance at levels below 0.001 in the proposed model. For both bending strength and bending modulus, with these models, determination coefficients of 93.47% and 92.13% respectively were obtained, which are a good measure of the controlled effects' contribution to the total variation.

Bending strength. In figure 5, we observe that the increase in sonication time and GO content increase the bending strength of ABCs; in contrast, the increase in BPO content tends to decrease this

property. ISO 5833-02³⁵ establishes a minimum bending strength of 50 MPa, so formulations without GO and the highest GO content studied do not achieve this requirement.

As mentioned earlier for the compressive strength, a higher BPO content has an important influence on the molecular weight and polymerization degree of PMMA¹² and, therefore, lower cement's mechanical performance. The size reduction generated by the longer sonication time generated a higher mechanical reinforcement³³ and a GO content of 0.3 wt. % produced the highest bending strengths, which coincides with the one reported by Paz et al.³ and by our group in other investigations^{21,22}.

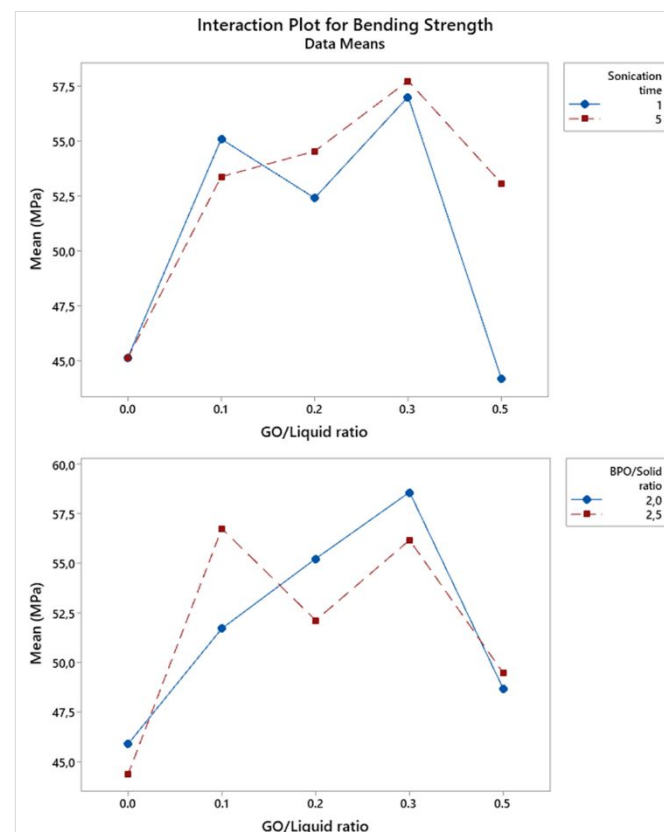


Figure 5. Interaction plot between the effect sonication time vs. GO/liquid ratio and BPO/solid ratio vs. GO/liquid ratio for the variable bending strength.

Tukey's post-ANOVA test for the variable bending strength shown in table 5 indicates that the formulations that maximize bending strength are those with GO contents between 0.1 - 0.3 wt.%. There are no significant differences in the bending strength value of the formulations to which the same letter is assigned so that all formulations grouped as A generate the highest possible values in this property and are above the minimum requirement in ISO 5833-02³⁵.

On the other hand, the addition of 0.5 wt.% of GO does not benefit the ABC from the bending strength point of view. The results of Tukey's test show that the addition of the maximum GO content statistically has the same effect on this property as the basic

formulation without the addition of GO (Group D in both interactions) and that both GO contents are below the minimum strength required in the standard (50 MPa).

Table 5. Grouping information for Bending strength using the Tukey method and 95% confidence

Sonication time * GO/Liquid ratio	Mean	Grouping*	BPO/Solid ratio *GO/Liquid ratio	Mean	Grouping*
5 – 0.3	57.74	A	2.0 – 0.3	58.58	A
1 – 0.3	57.01	A B	2.5 – 0.1	56.75	A
1 – 0.1	55.10	A B C	2.5 – 0.3	56.17	A
5 – 0.2	54.54	A B C	2.0 – 0.2	55.11	A B
5 – 0.1	53.38	A B C	2.5 – 0.2	51.84	B C
5 – 0.5	53.06	B C	2.0 – 0.1	51.72	B C
1 – 0.2	52.41	C	2.0 – 0.5	48.67	C D
1 – 0.0	45.14	D	2.5 – 0.5	48.56	C D
5 – 0.0	45.14	D	2.0 – 0.0	45.91	D
1 – 0.5	44.17	D	2.5 – 0.0	44.37	D

* Means that do not share a letter are significantly different.

Bending modulus. According to figure 6, the bending modulus and the compression modulus increase with higher BPO content, shorter sonication time, and higher GO content up to 0.3 wt.%. The higher the polymer chains during polymerization, the larger particle size, and GO nanoparticles' presence increases the ABCs' stiffness.

been reported the increase in the mechanical properties of ABCs^{3,19,45} and other types of polymeric materials with the addition of GO thanks to the presence of hydroxyl, carboxylic, and epoxy groups on their surface and is consequently more accessible to disperse^{31,46,47}.

Concerning the sonication time, Ormsy et al.⁴⁸ reported an improvement in the cement mechanical properties by mixing multilayer carbon nanotubes in the liquid phase with ultrasound due to the homogenization and dispersion of the nanotubes in the liquid phase.

Based on Tukey's test (Table 6), it is possible to establish that all formulations' bending modulus was between 2.4 - 2.7 GPa, which means that all formulations studied present bending modulus above 1800 MPa, which is the minimum required for ABCs, according to ISO 5833-02³⁵. The formulations with 0.3 wt.% of GO, 1 or 5 h of sonication, and 2.5 wt.% of BPO presented the highest values in this property.

In the ABCs formulations, it is necessary to comply with the mechanical and setting properties described in ISO 5833-02³⁵ to guarantee the long-term stability of the implant¹¹. Considering that most of the formulations studied met all the mechanical and set requirements specified in the standard. A selection criterion based on the bending strength was employed since some formulations did not reach the minimum value required.

In an ABC it is desirable low values of T_{max} , moderate t_{set} to facilitate the application of cement (in applications such as vertebroplasties and kyphoplasties are desirable longer setting times that allow the surgeon to inject the cement), and high mechanical performance that allows the adequate transfer of loads during the patient's daily activities.

In table 7, there is a summary of the studied properties of the formulations with better performance in bending strength. Although all the formulations mentioned in this table accomplish the requirements outlined in ISO 5833-02, those with 0.3 wt.% of GO independent of sonication time and BPO content showed the highest mechanical performance. That allows adequate load transfer during

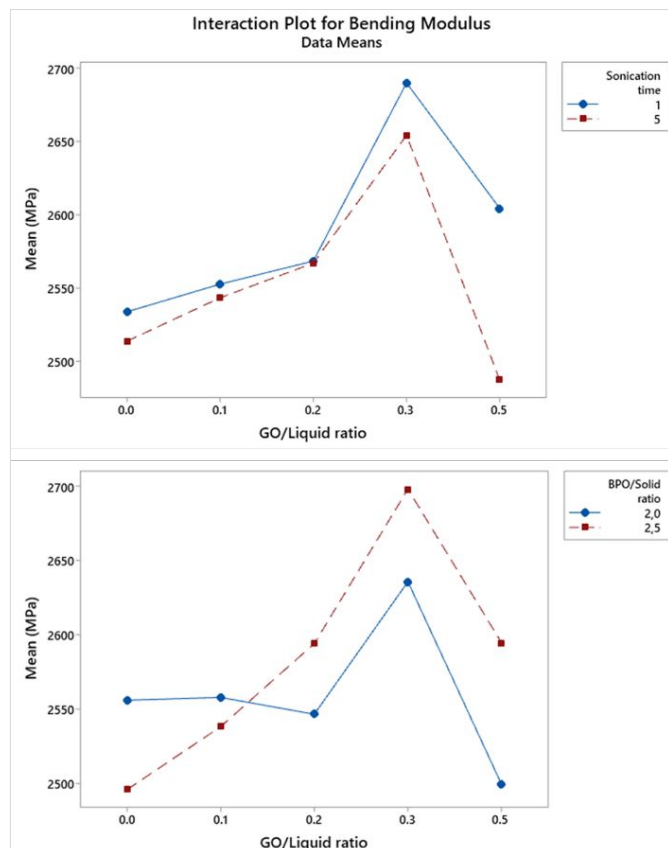


Figure 6. Interaction plot between the effect sonication time vs. GO/liquid ratio and BPO/solid ratio vs. GO/liquid ratio for the Bending variable modulus.

Other researchers found a similar behavior of the modulus concerning the BPO content in ABCs formulations^{1,39}. Also, it has

the patient's daily activities⁷. The longest t_{set} that facilitates cement application and the lowest T_{max} values decrease the risk of thermal necrosis of the surrounding tissue.

Table 6. Grouping information for Bending modulus using the Tukey method and 95% confidence

Sonication time * GO/Liquid ratio	Mean (MPa)	Grouping*		BPO/Solid ratio *GO/Liquid ratio	Mean (MPa)	Grouping*	
1 – 0.3	2690.23	A		2.5 – 0.3	2706.26	A	
5 – 0.3	2651.86	A	B	2.0 – 0.3	2635.83	B	
1 – 0.5	2595.33		B C	2.5 – 0.2	2594.27	B	C
5 – 0.2	2574.34		C D	2.5 – 0.5	2583.08	B	C
1 – 0.2	2568.70		C D	2.0 – 0.1	2558.13		C D
1 – 0.1	2552.97		C D	2.0 – 0.0	2556.24		C D
5 – 0.1	2543.64		C D E	2.0 – 0.2	2548.78		C D
1 – 0.0	2534.07		C D E	2.5 – 0.1	2538.47		C D
5 – 0.0	2521.01		D E	2.0 – 0.5	2499.69		D
5 – 0.5	2487.44		E	2.5 – 0.0	2498.84		D

* Means that do not share a letter are significantly different.

Table 7. Summary of grouping information for T_{max} , t_{set} , compressive strength, compressive modulus, bending strength, and bending modulus for ABCs formulations with higher bending strength. For all properties, letter A represents the highest values, and the letter G the lowest.

Sonication time* BPO /Solid ratio* GO/Liquid ratio	T_{max}	t_{set}	Compressive strength	Compressive modulus	Bending strength	Bending modulus
5 – 2.0 – 0.3	G	C	A	B	A	B
1 – 2.0 – 0.3	F	B	A	B	A	B
5 – 2.5 – 0.3	C	B	A	C	A	A
1 – 2.5 – 0.3	G	D	B	A	A	A
1 – 2.5 – 0.1	C	F	F	D	A	C
5 – 2.5 – 0.1	C	G	F	G	A	E
5 – 2.0 – 0.2	C	F	A	E	A	D
1 – 2.0 – 0.2	C	D	E	G	A	C
1 – 2.0 – 0.1	E	F	D	G	A	C

The characteristics mentioned in the formulations with 0.3 wt.% of GO make them promising to be applied as ABCs; therefore, complementary studies of properties such as residual monomer content, cell viability, antiviral activity, among others, are suggested to evaluate how these properties are affected by the BPO content and the sonication time of GO nanoparticles, in order to limit these factors.

Conclusions

The use of a factorial experiment design entirely randomized for evaluating mechanical and setting properties of ABCs allowed the optimization of BPO/Solid ratio, sonication time, and GO/liquid ratio factors, obtaining a group of formulations that satisfy the requirements proposed in ISO 5833-02 and that they are susceptible to complementary studies to extend the knowledge of these novel formulations and conditions.

The increase in the BPO content favored the cement's stiffness by increasing the compression and bending modulus; however, it increased the T_{max} and decreased the t_{set} , the compression, and bending strength.

The sonication time favored the compressive and bending strength of the ABCs; simultaneously, increased T_{max} when incorporating a more effective content of BPO, reduced the t_{set} and the cement's stiffness by reducing the compressive and bending modulus.

The increase in GO content favored the setting properties, as it decreased T_{max} and increased t_{set} . Moreover, GO's add up to 0.3 wt.% had a beneficial effect on compression and bending's mechanical properties. The addition of 0.5 wt.% of GO generated a significant decrease in the mechanical properties due to possibly agglomerates' formation.

In the formulations that meet the mechanical and setting requirements, the formulations with a GO content of 0.3 wt.% highlight since they presented higher mechanical performance, longer t_{set} , and the lowest T_{max} values.

Conflicts of interest

There are no conflicts to declare.

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Supporting Information

Table S1. Completely random factorial design

StdOrder	Sonication time	GO/liquid ratio	BPO/solid ratio	StdOrder	Sonication time	GO/liquid ratio	BPO/solid ratio
51	5	0	2	3	1	0.1	2
29	1	0.5	2	36	5	0.2	2.5
50	1	0.5	2.5	14	5	0.1	2.5
37	5	0.3	2	23	1	0.1	2
38	5	0.3	2.5	17	5	0.3	2
49	1	0.5	2	39	5	0.5	2
6	1	0.2	2.5	52	5	0	2.5
58	5	0.3	2.5	1	1	0	2
41	1	0	2	2	1	0	2.5
28	1	0.3	2.5	9	1	0.5	2
54	5	0.1	2.5	45	1	0.2	2
43	1	0.1	2	34	5	0.1	2.5
15	5	0.2	2	21	1	0	2
32	5	0	2.5	31	5	0	2
4	1	0.1	2.5	26	1	0.2	2.5
46	1	0.2	2.5	12	5	0	2.5
57	5	0.3	2	53	5	0.1	2
27	1	0.3	2	5	1	0.2	2
11	5	0	2	60	5	0.5	2.5
42	1	0	2.5	59	5	0.5	2
19	5	0.5	2	35	5	0.2	2
7	1	0.3	2	44	1	0.1	2.5
24	1	0.1	2.5	47	1	0.3	2
13	5	0.1	2	10	1	0.5	2.5
25	1	0.2	2	18	5	0.3	2.5
8	1	0.3	2.5	22	1	0	2.5
30	1	0.5	2.5	55	5	0.2	2
56	5	0.2	2.5	20	5	0.5	2.5
48	1	0.3	2.5	33	5	0.1	2
40	5	0.5	2.5	16	5	0.2	2.5

Table S2. Analysis of variance of the variables Maximum temperature ($T_{\max}$), and Setting time (t_{set})

Source	$T_{\max}$		t_{set}	
	Adj SS	P-Value	Adj SS	P-Value
Sonication time	4.572	0.1850	1864.8	0.001
BPO/Solid ratio	21.613	0.0070	1588.7	0.002
GO/Liquid ratio	499.721	0.0000	80028.3	0
Sonication time*BPO/Solid ratio	44.078	0.0000	453.9	0.076
Sonication time*GO/Liquid ratio	35.653	0.0210	1634.1	0.036
BPO/Solid ratio*GO/Liquid ratio	67.579	0.0010	901.5	0.18
Sonication time*BPO/Solid ratio*GO/Liquid ratio	114.734	0.0000	752.4	0.253
Error	48.478		2727.2	

Table S3. Analysis of variance of the variable Compressive strength, and Compressive modulus

Source	Compressive strength		Compressive modulus	
	Adj SS	P-Value	Adj SS	P-Value
Sonication time	30.047	0.000	31083	0.000
BPO/Solid ratio	3.534	0.010	14800	0.000
GO/Liquid ratio	226.126	0.000	168435	0.000
Sonication time*BPO/Solid ratio	3.911	0.007	7773	0.000
Sonication time*GO/Liquid ratio	34.581	0.000	9568	0.000
BPO/Solid ratio*GO/Liquid ratio	41.486	0.000	7252	0.000
Sonication time*BPO/Solid ratio*GO/Liquid ratio	15.219	0.000	13106	0.000
Error	13.432		9464	

Table S4. Analysis of variance of the variables Bending strength, and Bending modulus

Source	Bending strength		Bending modulus	
	Adj SS	P-Value	Adj SS	P-Value
Sonication time	42.32	0.001	11387	0.000
BPO/Solid ratio	2.22	0.415	6404	0.006
GO/Liquid ratio	766.74	0.000	115539	0.000
Sonication time*BPO/Solid ratio	1.55	0.495	1246	0.194
Sonication time*GO/Liquid ratio	142.33	0.000	17546	0.001
BPO/Solid ratio*GO/Liquid ratio	89.19	0.001	31661	0.000
Sonication time*BPO/Solid ratio*GO/Liquid ratio	25.01	0.138	5271	0.146
Error	74.18		16781	

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Acrylic Bone Cements Modified with Graphene Oxide: Mechanical, Physical, and Antibacterial Properties

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Abstract: Bacterial infections are a common complication after total joint replacements (TJRs), the treatment of which is usually based on the application of antibiotic-loaded cements; however, owing to the increase in antibiotic-resistant microorganisms, the possibility of studying new antibacterial agents in acrylic bone cements (ABCs) is open. In this study, the antibacterial effect of formulations of ABCs loaded with graphene oxide (GO) between 0 and 0.5 wt.% was evaluated against *Gram-positive* bacteria: *Bacillus cereus* and *Staphylococcus aureus*, and *Gram-negative* ones: *Salmonella enterica* and *Escherichia coli*. It was found that the effect of GO was dependent on the concentration and type of bacteria: GO loadings ≥ 0.2 wt.% presented total inhibition of *Gram-negative* bacteria, while GO loadings ≥ 0.3 wt.% was necessary to achieve the same effect with *Gram-positives* bacteria. Additionally, the evaluation of some physical and mechanical properties showed that the presence of GO in cement formulations increased wettability by 17%, reduced maximum temperature during polymerization by 19%, increased setting time by 40%, and increased compressive and flexural mechanical properties by up to 17%, all of which are desirable behaviors in ABCs. The formulation of ABC loading with 0.3 wt.% GO showed great potential for use as a bone cement with antibacterial properties.

Keywords: acrylic bone cement; antibacterial activity; graphene oxide; mechanical properties; physical properties

1. Introduction

Acrylic bone cements (ABCs) are employed widely in arthroplasties as a fixation agent between the bone and the implant [1]. Polymethylmethacrylate (PMMA) bone cements consisted of two components: solid and liquid. The solid phase consists mainly of a polymer-based on PMMA, a radio-opaque agent such as barium sulfate, and benzoyl peroxide as the initiator for the polymerization reaction. The liquid phase consists of a methyl methacrylate monomer and a polymerization reaction activator such as *N,N*-Dimethyl-*p*-Toluidine. When the liquid and solid phases are mixed, the polymerization reaction of the MMA begins, which causes the cement to cure [2–4].

Like all implant materials, bone cements have a high risk of infection when they are implanted in the human body owing to the possibility of biofilms' formation of microorganisms on an inert surface, which usually requires multiple surgeries for treatment [5].

Chronic infection of articular prosthesis requires surgical removal of the implant in order to eradicate the infectious process. The procedure can be performed in one- and two-stage methods. In the first case, the intervention consists of the removal of the infected implant, decontamination of the site, and implantation of a new revision prosthesis covered with antibiotic-loaded bone cement. In the second case, the placement phase of the revision prosthesis is delayed for several months (2–5 months), during which a bone spacer cement impregnated with an antibiotic is placed in the site [6].

In recent years, increased resistance of microorganisms to antibiotics has led to severe health problems, owing to most of the bacteria causing the infection being resistant to at least one of the antibiotics generally used to eradicate the disease. Therefore, the requirement of significant efforts worldwide to study new antimicrobial agents that can effectively inhibit the growth of bacteria is evident [7]. Furthermore, it is known that, in cements loaded with antibiotics, most of the antibiotic is trapped inside the cement and only a small amount on the surface is available for diffusion to the desired site [5].

Graphene oxide (GO) is essentially a sheet of graphene with a random distribution of oxygen-rich functionalities on its surface. It is characterized by its high performance mechanical [8], biocompatibility [9], biodegradability [10,11], and excellent antimicrobial properties [7,9,12].

Studies reported that the addition of GO to ABCs resulted in increased mechanical properties in compression [13], bending [14], fracture toughness, fatigue [15], and wear [16]; and increased biocompatibility with MC3-T3 cells [17], human bone marrow mesenchymal stem cells (hBMSCs) [18], mouse L929 fibroblasts, and human Saos-2 [19]. There are also studies about modifications to GO by silanization to improve the mechanical properties of cements [20]. Additionally, Tavakoli et al. [2] evaluated the addition of a chitosan/GO composite to increase the mechanical properties, handling, and injectability of the cement, and to improve the cell viability, growth, and cell adhesion to MG-63 cell culture.

Despite the results shown above, the antimicrobial activity of ABCs with GO for use in orthopedics is a little-explored field. Few studies are evaluating this topic. Paz et al. [17] evaluated the antimicrobial activity of ABCs loadings with 0.1 wt.% against *S. aureus* and found no antibacterial effect, possibly owing to the low concentration used. Additionally, our group has evaluated the antibacterial activity against *E. coli*, reporting promising results with 0.3 wt.% GO [16].

In the present study, the effect of GO incorporation between 0 and 0.5 wt.% on the antibacterial activity of ABCs was evaluated against *Gram-positive* (*B. cereus* and *S. aureus*) and *Gram-negative* (*S. enterica* and *E. coli*). Properties such as roughness, handling, residual monomer content, water contact angle, strength, modulus of compression, and bending were studied to evaluate the possible application of these cements with antibacterial properties in orthopedics.

2. Materials and Methods

2.1. Materials

The solid phase was composed of PMMA beads (New Stetic SA, Medellin, Colombia), barium sulfate (BaSO_4) (Alfa Aesar, Tewksbury, MA, USA), and benzoyl peroxide (BPO) (Sigma-Aldrich, Palo Alto, CA, USA). The liquid phase was composed of methyl methacrylate (MMA), 2-(diethylamino) ethyl acrylate (DEAEA), 2-(diethylamino) ethyl methacrylate (DEAEM) (Sigma-Aldrich, Palo Alto, CA, USA), *N,N*-dimethyl *p*-toluidine (DMPT) (Merck, Burlington, MA, USA), and GO synthesized by a Hummers method modified.

2.2. Synthesis and Characterization of GO

GO was synthesized by the Hummers method modified as reported by Valencia et al. [16]. Briefly, 3.0 g of graphite was oxidized with KMnO_4 for three days and then washed with ethanol and Milli Q water in a centrifuge. The sediment was purified by dialysis tube (Thermo Scientific, Suwanee, GA, US) and then lyophilized at $-51\text{ }^\circ\text{C}$ and 0.12 mBar (Labconco, Kansas City, MO, US).

GO was characterized by Fourier transform infrared in attenuated total internal reflectance mode (ATR-FTIR) (Perkin-Elmer, Waltham, MA, US); X-ray photoelectron spectra (XPS) (Specs, Berlin, Germany) with a PHOIBOS 150 1D-DLD analyzer (PHOIBOS, Kowloon, Hong Kong, China), using a monochromatic source of Al-K α (1486.7 eV, 13 kV, 100 W), C1s spectrum from GO was analyzed, and dynamic light scattering (DLS) in a Zetasizer Nano ZS DLS (Malvern Panalytical, JarmanWay, Royston, UK).

2.3. Preparation of ABCs

ABCs consisted of two phases (liquid and solid). The solid phase (SP) was composed of 88 wt.% of PMMA, 10.0 wt.% of BaSO₄, and 2.0 wt.% of BPO. Constituents of the liquid phase (LP) are shown in Table 1. For each formulation, the SP/LP ratio was 2.0. LP after stay 1 h in ultrasound to disperse the GO (Figure 1) was added to the SP and manually mixed. Then, the dough was placed in Teflon molds with the dimensions specified for each test.

Table 1. Composition (wt.%) of the liquid phase of the acrylic bone cements' (ABCs) formulations. GO, graphene oxide; MMA, methyl methacrylate; DEAEA, 2-(diethylamino) ethyl acrylate; DEAEM, 2-(diethylamino) ethyl methacrylate; DMPT, *N,N*-dimethyl *p*-toluidine.

Formulation	MMA	DEAEA	DEAEM	DMPT	GO
ABC 0% GO	88	1	1	2.5	0
ABC 0.1% GO	83	1	1	2.5	0.1
ABC 0.2% GO	78	1	1	2.5	0.2
ABC 0.3% GO	73	1	1	2.5	0.3
ABC 0.5% GO	68	1	1	2.5	0.5



Figure 1. Photographs of the liquid phase of the acrylic bone cements' (ABCs) formulations. From left to right, the percentage of graphene oxide (GO) increases from 0 to 0.5 wt.%.

2.4. Characterization of ABCs

2.4.1. Fourier Transform Infrared (FTIR)

The chemical composition of ABCs specimens was studied by Fourier transform infrared in attenuated total internal reflectance mode (ATR-FTIR) (Perkin-Elmer, Waltham, MA, US).

2.4.2. Thermogravimetric Analysis (TGA)

TGA of ABCs specimens was performed in a TGA Q500 analyzer (TA Instruments, New Castle, DE, US), between 50 and 500 °C, at a heating rate of 10 °C/min.

2.4.3. Scanning Electron Microscope (SEM)

The surface morphology of the specimens fractured during the compression test was coated with gold and analyzed by an environmental scanning electron microscope (ESEM) Philips XL30 (Philips, Eindhoven, The Netherlands).

2.4.4. Water Contact Angle (WCA)

The water contact angle (WCA) was measured at 25 °C using a KSV CAM200 tensiometer (KSV Instruments, Helsinki, Finland). At least ten separate measurements were taken on each sample.

2.4.5. Handling Properties

The maximum temperature reached during polymerization ($T_{\max}$) and setting time (t_{set}) was determined by the procedure specified in ISO 5833-02 [21]. The cement dough was deposited in Teflon molds of 68 mm diameter and 20 mm height, the temperature and time were recorded, and $T_{\max}$ and t_{set} were calculated from the temperature versus time plot. The test was performed in duplicate.

2.4.6. Mechanical Properties

Four-point bending and compression tests were conducted following the specifications of the ISO 5833-02 [21] in a universal testing machine Tinius Olsen-H50KS (Tinius Olsen, Redhill, United Kingdom). Specimens for bending and compression consisted of 75 mm × 10 mm × 3 mm beams and 6 mm diameter and 12 mm high cylinders, respectively. The bending test was conducted at a crosshead displacement rate of 5 mm/min, while the compression test was conducted at 20 mm/min. For each of these tests, a minimum of six specimens was tested for each of the ABCs' formulations.

2.4.7. Dynamic Mechanical Analysis (DMA)

The dynamic mechanical analysis was carried out in a DMA Q800 (TA Instruments, New Castle, DE, US) in Dual Cantilever mode to 1 Hz of frequency in a range from −100 to 200 °C at a heating rate of 3 °C/min. Specimens consisted of 46 mm × 6 mm × 4 mm beams. T_g was defined as the temperature where the maximum of $\tan \delta$ occurs. At least three specimens were tested per formulation.

2.4.8. Residual Monomer Content (RMC)

Proton nuclear magnetic resonance spectra ($^1\text{H-NMR}$) were conducted in a Bruker Avance III HD-400 spectrometer (Bruker, Billerica, MA, US) at 25 °C. Specimens were dissolved in deuterated chloroform (CDCl_3) one week after being prepared. Residual monomer content (RMC) in a sample was calculated by integrating the signals of the methoxy protons of the MMA ($\delta = 3.7$ ppm) and the PMMA ($\delta = 3.5$ ppm) using Equation (1).

$$\text{RMC}(\%) = \frac{A_{\text{MMA}}}{A_{\text{MMA}} + A_{\text{PMMA}}} \times 100 \quad (1)$$

where A_{MMA} is the signal area of methoxy protons of MMA and A_{PMMA} is the signal area of methoxy protons of PMMA.

2.4.9. Antimicrobial Activity

The antimicrobial activity of ABC was determined against *Gram-positive* bacteria *Bacillus cereus* (ATCC 13061) and *Staphylococcus aureus* (ATCC 55804), and *Gram-negative* bacteria *Salmonella enterica* (ATCC 13311) and *Escherichia coli* (ATCC 11775). The inoculums were obtained from cultures in liquid medium of 24 h of growth and adjusted to a concentration of 10^6 colony-forming units (CFU) mL^{-1} in a spectrophotometer Eppendorf BioSpectrometer® kinetic at 600 nm (Eppendorf, Hamburg, Germany). The dilutions were made utilizing cell washing, centrifuging the strains at 4000 rpm, and resuspending the pellet obtained in nutrient broth diluted 1 in 500.

The sterile ABCs were deposited in microtubes together with 1000 μL of each of the inoculum and placed in agitation at 250 rpm on an IKA KS 3000i orbital shaker (IKA, Staufen, Germany) for 24 h at 37 °C. The control was performed without the ABCs.

Inhibition of cell activity was determined by adding 20 μL of 0.2% of 2,3,5-triphenyltetrazolium chloride (TTC) to an aliquot of 100 μL of the strain exposed to the treatments in a microplate well and

incubated for 2 h at 37 °C. Turning the coloring of the well contents to red indicated the metabolic activity of the pathogen. Inhibition of the activity was tested by direct seeding of 100 µL of each of the treatments by making serial dilutions on Müller Hinton agar and incubating at 37 °C for 24 h. The tests were conducted in triplicate.

2.5. Statistical Analysis

Quantitative properties are shown as the mean $\pm$ standard deviation. For each property, the Student *t*-test compared the means for significant differences. The difference was considered statistically significant when $p < 0.05$. Statistical analysis was performed comparing ABCs loaded of GO with respect to ABCs without GO.

3. Results and Discussion

3.1. GO Characterization

FTIR characterization of synthesized graphene oxide by the modified Hummers method [16] showed the characteristic bands of this material reported by other authors (Figure 2a). At 3369 and 1046 cm^{-1} , the stretching vibrations of the hydroxyl functional group; at 1729 cm^{-1} , the vibration of the carbonyl group (C=O); at 1620 cm^{-1} , the vibration of the double bond C=C of the aromatic structure of GO; at 1374 cm^{-1} , the vibration of the carboxyl group; and at 1224 cm^{-1} , the characteristic peak of the epoxy group [20,22–25]. DLS results (Figure 2b) show an average particle size of 400 nm for GO sheets.

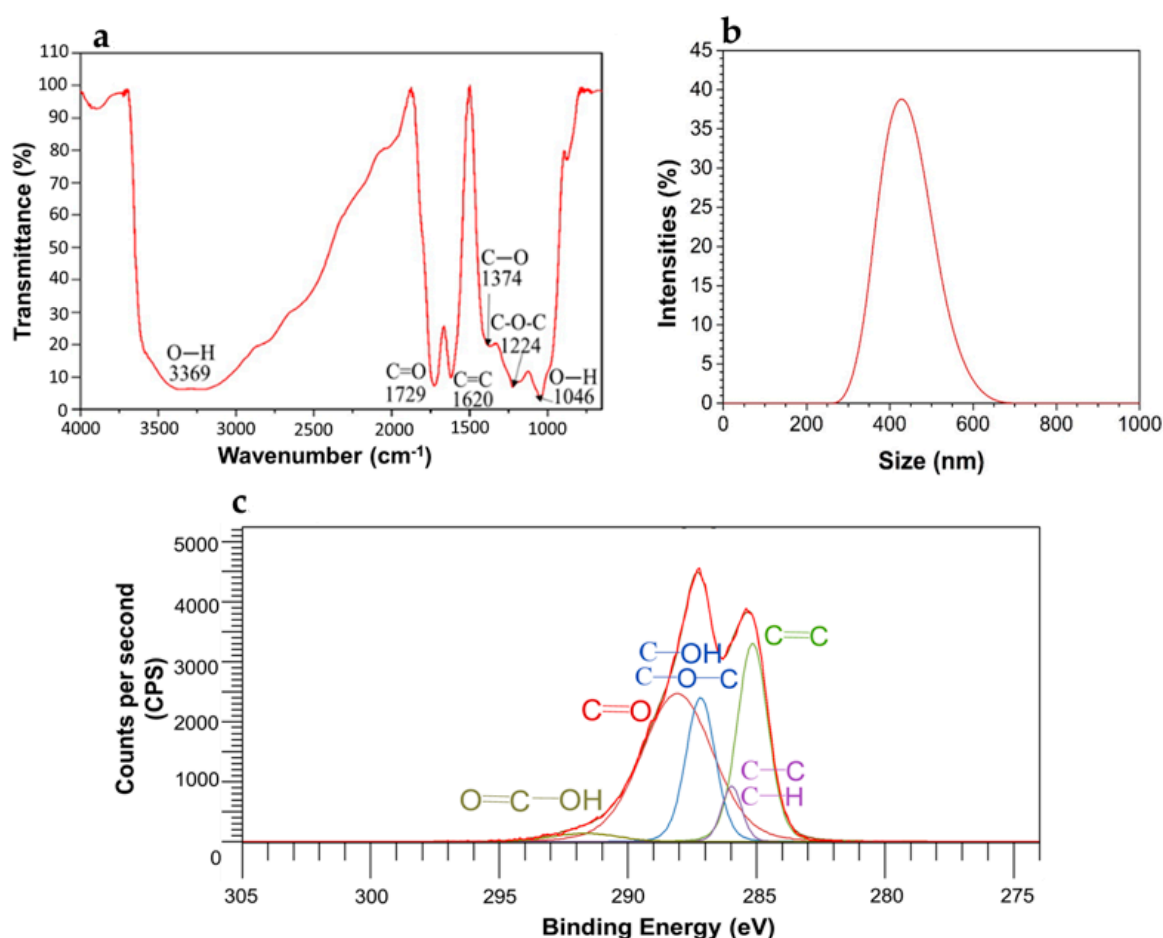


Figure 2. Results characterization of GO. (a) Fourier transform infrared (FTIR) spectrum, (b) Dynamic light scattering (DLS), and (c) Peak deconvolution of C(1s) in the X-ray photoelectron spectra (XPS) of the graphene oxide.

The deconvolution of the C(1s) spectrum of GO in Figure 2c confirms the presence of the functional groups shown in the FTIR spectrum. The results of the binding energies shown in Table 2 allowed quantification of the atomic percentage of these groups and determined the level of oxidation of GO at 67.84% [26]. This degree of oxidation and the O1s/C1s ratio from 1.06 indicates that the graphite oxidation process was successful, producing functional groups such as $-\text{COOH}$, $-\text{C}=\text{O}$, and $\text{C}-\text{OH}$ on the surface of the graphitic structure. Additionally, the presence of the carboxyl group (2.46 at%) could favor the chemical interactions with PMMA and the antimicrobial activity of the cements added with this nanomaterial, because part of the antimicrobial activity of GO is based on the presence of these carboxylic groups at its edges [27–29].

Table 2. Analysis peak deconvolution of C(1s) of the graphene oxide [30].

	Binding Energy (eV)	Atomic Concentration (at.%)
C=C	285.2	27.21
C–C and C–H	286.0	4.95
C–O–C and C–OH	287.2	18.77
C=O	288.1	46.61
O=C–OH	291.7	2.46

3.2. Characterization of ABCs

3.2.1. FTIR

Figure 3a shows the infrared spectrum of bone cements. The characteristic peaks of PMMA at 2995, 1723, 1435, and 1142 cm^{-1} corresponding to C–H stretching, C=O ester carbonyl stretching, C–H bending, and C–O stretching vibrations are shown [31–33]. The presence of GO in the formulations generated weak absorption peaks at 3752 cm^{-1} in the formulation with 0.5 wt.% of GO (Figure 3b), corresponding to the vibration of the O–H groups present in the GO. The presence of this new peak in the spectrum without changes in the characteristic peaks of PMMA confirmed physical interaction with the GO and the ABC matrix, rather than confirming the secondary hydrogen bridge links between GO and the ABC [34].

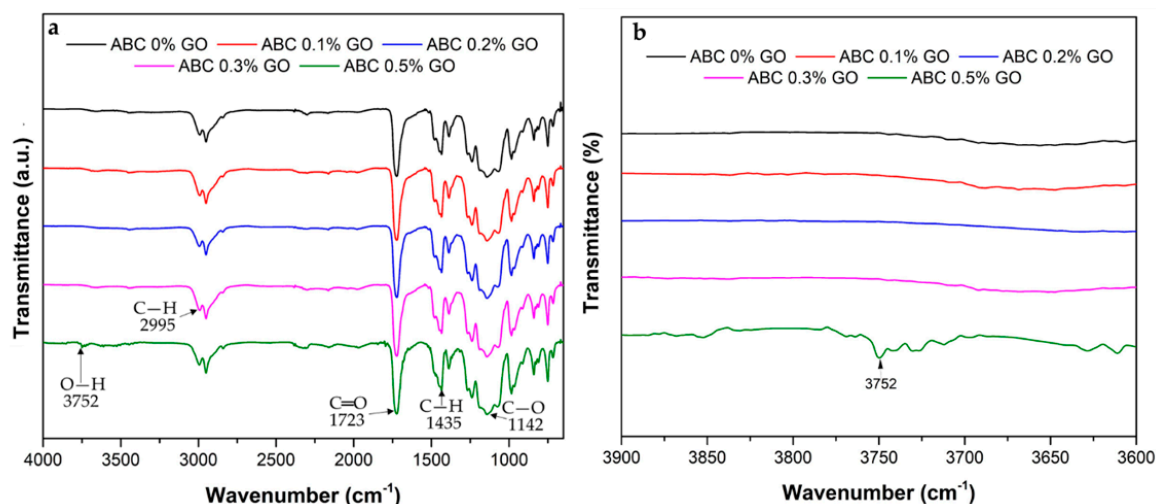


Figure 3. FTIR spectra of ABCs loading with different percentages of GO. (a) Spectrum from 700 to 4000 cm^{-1} and (b) spectrum from 3600 to 3900 cm^{-1} .

3.2.2. TGA

The thermogravimetric analysis of the ABC formulations studied is evident in Figure 4. The graph indicates three stages in the thermal degradation of ABCs that coincide with those reported by other

authors for PMMA [33,35]. These stages are related to the degradation of each type of radical structures formed during the polymerization of PMMA [35]. The first one with a 5% mass loss from 145 to 264 °C corresponds to water evaporation and chain fragmentation of the chains formed by the union of two identical radical-chain halves in growth that are known as a head-to-head attachment. The second phase (10% mass loss) occurs between 264 and 320 °C with double bond cleavages of allyl vinylidene in the final position. The third phase between 320 and 440 °C, with a 78% mass loss, corresponds to thermal depolymerization owing to the cleavage of the head–tail bonds [14].

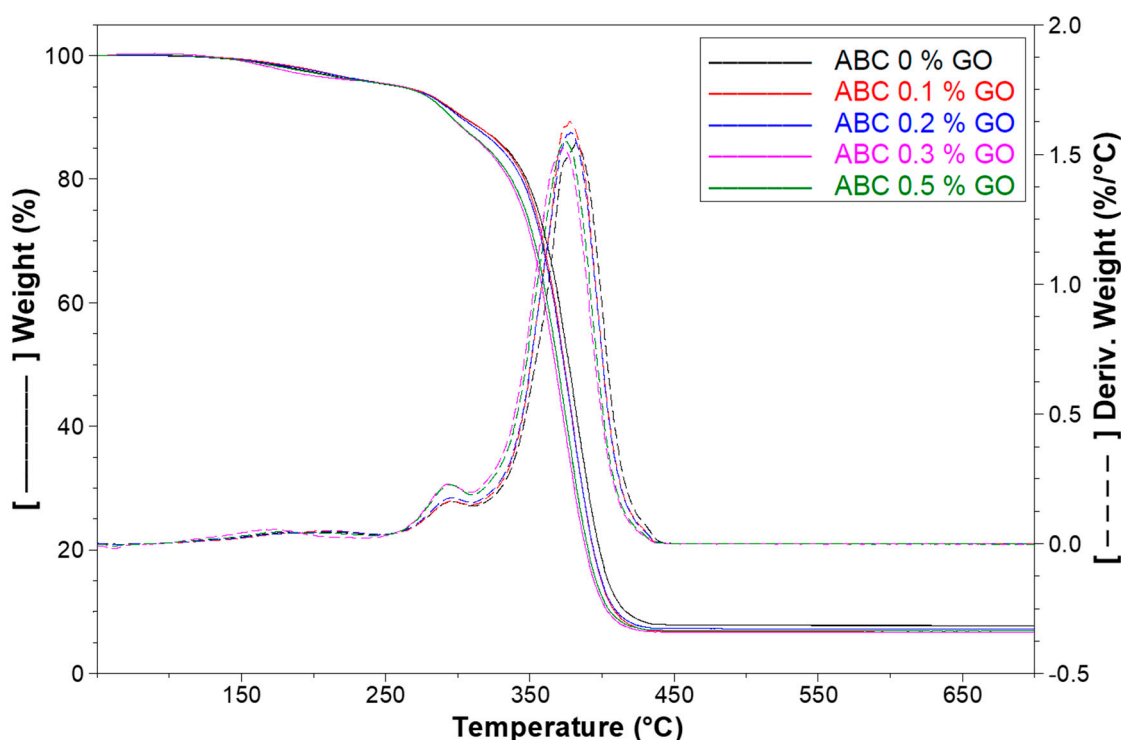


Figure 4. Thermogravimetric analysis (TGA) and Derivate-TGA (DTGA) thermograms of ABCs loading with different percentages of GO.

The thermal stability of the cements is reduced with the addition of GO, because, from the second mass loss phase (264 °C), it is observed that the formulation without GO showed a lower weight loss compared with those containing GO. Furthermore, according to the derivate-TGA curve (DTGA), the temperature with the highest mass loss rate decreases from 382 °C for the formulation without GO to 375 °C for the formulation with 0.5% GO. This decrease in the thermal stability of ABCs may be related to the ability of GO to capture free radicals during polymerization, thus generating slightly shorter chains with fewer secondary links, which are partly responsible for the thermal stability [26,36].

3.2.3. SEM

According to Figure 5, which shows the fracture zone of compression specimens of GO cement formulations, the increase in GO content generates an increase in ABC roughness that is more evident in the formulations with 0.3 and 0.5 wt.%, which can be beneficial in facilitating cell anchorage [16,18]. Also, some authors suggest that the surface roughness of biomaterials, such as Ti and PMMA, show a direct relationship with cell adhesion, proliferation, and differentiation [37,38].

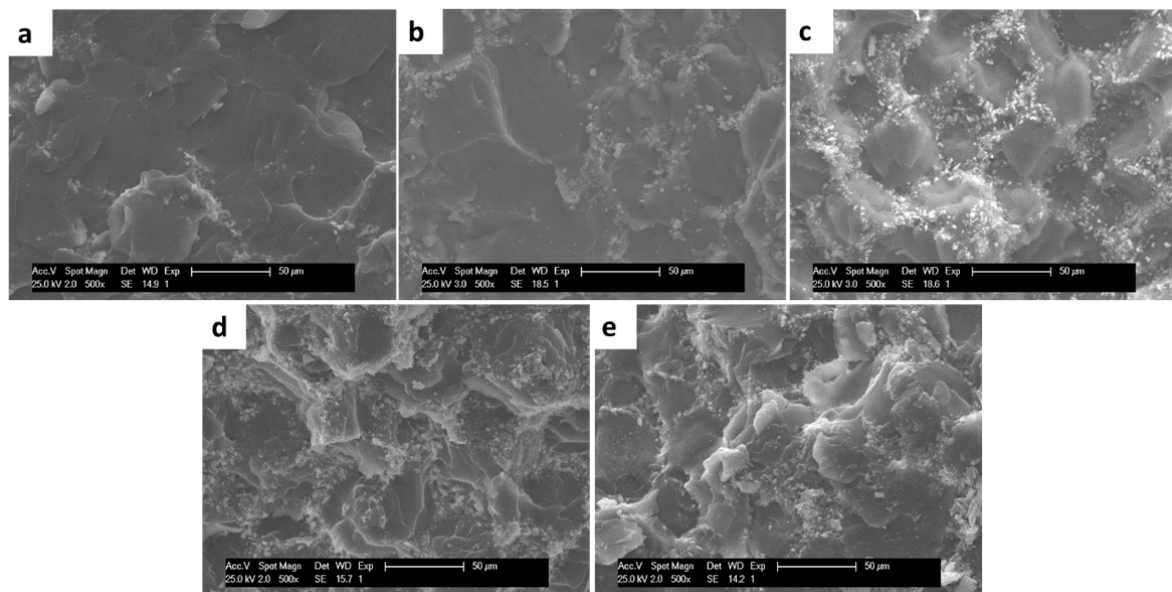


Figure 5. SEM images of the fractured surface of samples to the compression test of ABC modified with GO. (500 $\times$). (a) ABC 0% GO, (b) ABC 0.1% GO, (c) ABC 0.2% GO, (d) ABC 0.3% GO, and (e) ABC 0.5% GO.

Figure 6a shows that GO films are extended in the form of sheets in the PMMA matrix and presented excellent adhesion to the polymer matrix (Figure 6b). The good dispersion and adhesion of GO in the PMMA matrix is beneficial for improving the mechanical properties of ABCs.

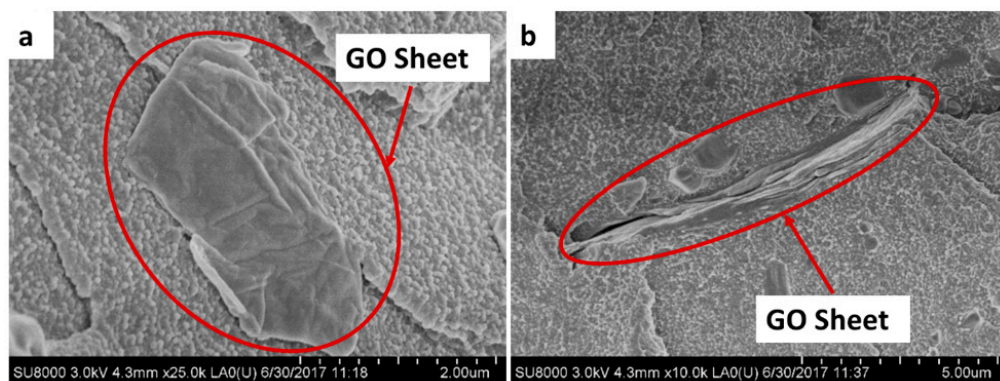


Figure 6. Scanning electron microscope (SEM) images of ABC with 0.5 wt.% GO, where the GO sheets are shown in the cement at different magnifications. (a) 25,000 $\times$ and (b) 10,000 $\times$.

3.2.4. Water Contact Angle (WCA)

Higher contact angles indicate lower surface wettability. Figure 7 shows that wettability in ABCs has a direct relationship with GO content, with an increase of 16.6% in the formulation at 0.5 wt.% GO. This characteristic that confers GO to the surface of the ABC is because of its hydrophilic nature, generated by the presence of hydroxyl, epoxy, carbonyl, and carboxyl functional groups [39].

The hydrophilicity of the materials has been related to increased adhesion of osteoblasts to the surface, which promotes osteogenesis [40,41]. Besides, some studies suggest that the presence of more hydrophilic surfaces in PMMA favors antimicrobial activity owing to the formation of a hydration layer that can bind to the surrounding water and generate a physical and energetic barrier that prevents microbial adhesion [42,43].

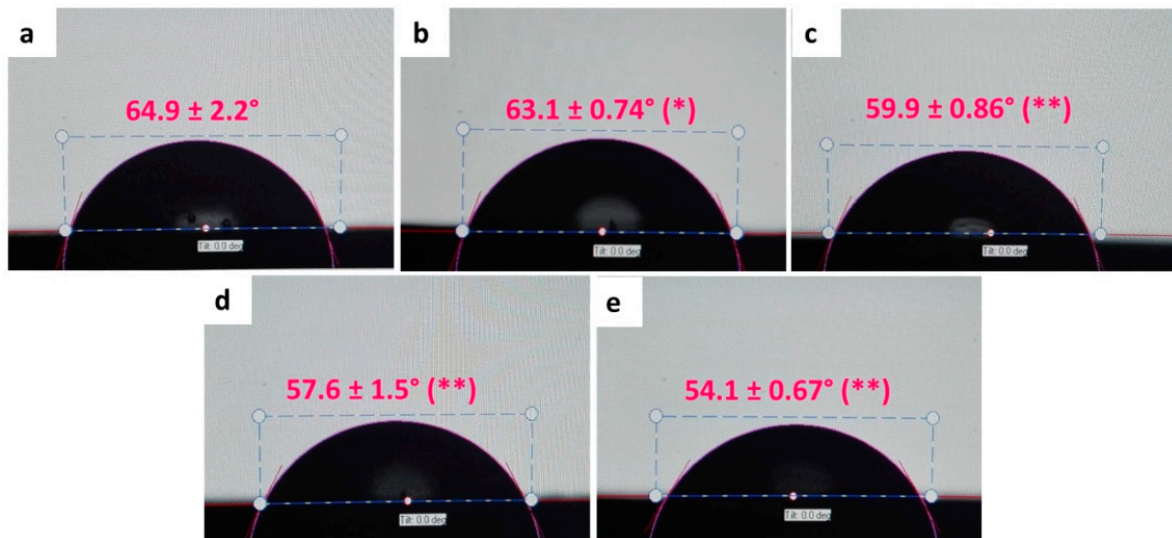


Figure 7. Photographs of water contact angle (WCA) on the surfaces of ABC with different percentages of GO. (a) ABC 0% GO, (b) ABC 0.1% GO, (c) ABC 0.2% GO, (d) ABC 0.3% GO, and (e) ABC 0.5% GO. Asterisks denote significant differences of ABC loading samples respect to ABC 0% GO at a significance levels of * $p < 0.05$ and ** $p < 0.01$.

3.2.5. Handling Properties

According to Figure 8, all the ABCs' formulations studied reached, during polymerization, $T_{\max}$ lower than 90 °C and t_{set} between 3 and 15 min (180 and 900 s), which are values allowed by ISO 5833-02 [21]. In general, $T_{\max}$ decreased and t_{set} increased with increasing GO content, mainly owing to the inhibitory and retarding effect of GO on the polymerization reaction as it acts as a sink for free radicals [15,26,44].

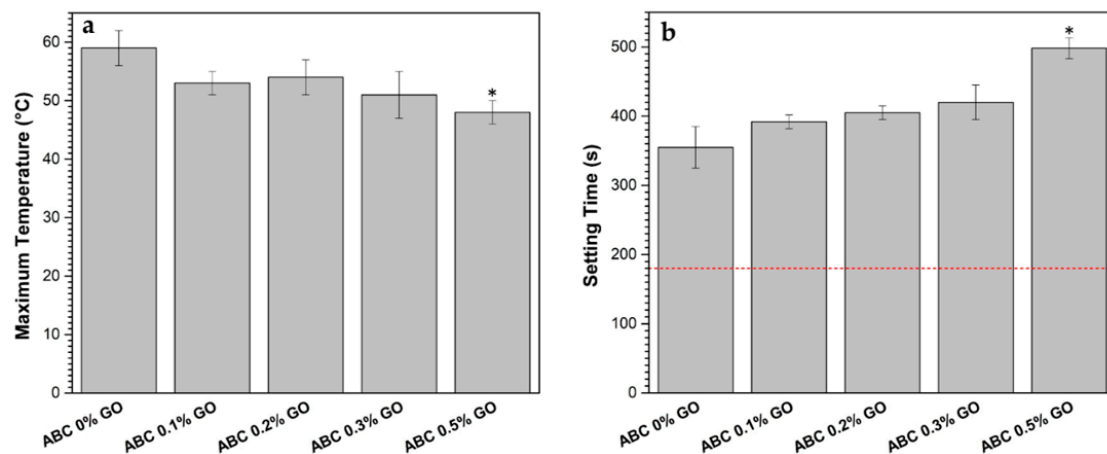


Figure 8. Handling parameters of liquid-powder mixture in ABC loadings with GO. (a) The maximum temperature reaches during the polymerization and (b) setting time of each formulation. The red dash line indicates the minimum value of the property set in ISO 5833-02 [21]. Asterisks denote significant differences of ABC loading samples with respect to ABC 0% GO at a significant level of * $p < 0.05$.

It has been widely reported that high polymerization temperatures generate thermal necrosis of the tissue surrounding the cement, which leads to loosening of the prosthesis [3,15,45–47], and that higher t_{set} values within the limits of the standard give the surgeon more time to conform the cement during the operation [48]. The t_{set} is an essential clinical requirement in applications such as vertebroplasty

and kyphoplasty, where the cement is injected through the vertebra. On the other hand, prolonged t_{set} facilitates the manageability of the ABC during the procedure [49–51].

3.2.6. Mechanical Properties

The results of the mechanical characterization of the ABCs are presented in Figure 9. ISO 5833-02 sets the minimum values for compressive strength, flexural strength, and modulus of bending, as 70 MPa, 50 MPa, and 1800 MPa, respectively. These minimum values are highlighted in Figure 9 with a red dashed line. All formulations loaded with GO complied with the mechanical requirements, except the formulation with 0.5% GO, which did not reach the bending strength value.

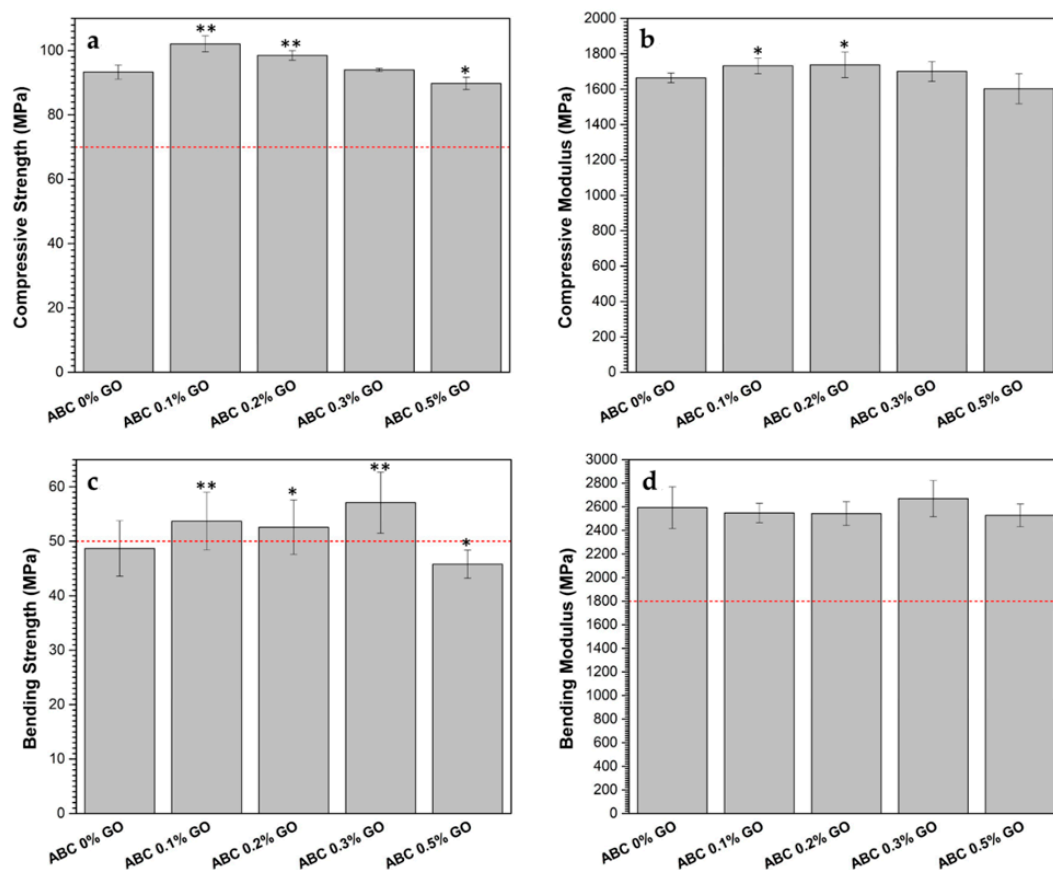


Figure 9. Mechanical properties compression and bending in ABC modified with different percentages of GO. (a) Compressive strength, (b) compressive modulus, (c) bending strength, and (d) bending modulus. The red dash line indicates the minimum value of the property set in ISO 5833-02 [21]. Asterisks denote significant differences of modified ABC samples respect to ABC 0% GO at significance levels of * $p < 0.05$ and ** $p < 0.01$.

Compressive strength and compression modulus were significantly improved ($p < 0.01$ and $p < 0.05$, respectively) with GO contents up to 0.2 wt.%, while flexural strength showed a reinforcement up to GO contents of 0.3 wt.%. The GO content does not seem to have any significant effect on the flexural modulus.

Mechanical reinforcement of GO is probably owing to a homogeneous dispersion of the nanoparticles in the matrix [16]. Furthermore, the increased chemical interaction at the PMMA/GO interface owing to the high surface area of the GO sheets (Figure 6a) improves interfacial adhesion by forming hydrogen bridges between the hydroxyl (OH) groups of GO and the methoxyl (OCH₃) groups of PMMA [2,26,52]. The use of GO loads > 0.3 wt.% resulted in detrimental mechanical properties, possibly owing to the formation of GO agglomerates that act as stress concentrators [15].

3.2.7. DMA and RMC

Figure 10 shows the results of the storage modulus (E') and $\tan \delta$ of the ABCs loaded with GO as a function of temperature. E' increased with intermediate GO percentages (0.2 and 0.3%) and decreased with the lowest and highest GO percentages (0.1 and 0.5 wt.%). The previous result indicates that, possibly, the best dispersion of GO is achieved with the average rates of GO, which is corroborated with the mechanical properties to bending.

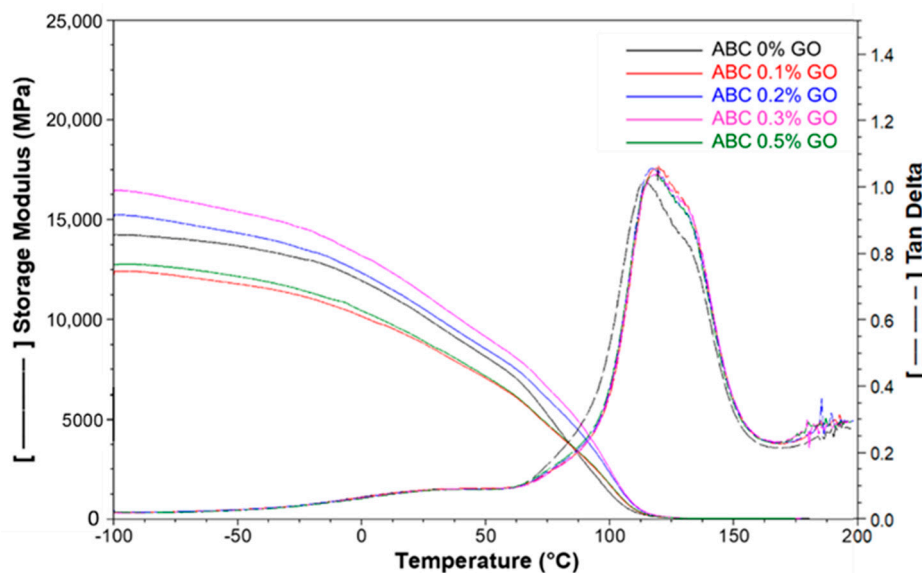


Figure 10. Storage modulus and tangent delta de ABC modified with GO obtained by dynamic mechanical analysis (DMA).

The T_g of the ABCs, obtained from the $\tan \delta$ graph (Table 3), shows a slight increase for cements with GO (approximately 4%) compared with the sample without GO. This is possibly owing to the mechanical reinforcement generated by the nanoparticles and the chemical interactions with the matrix [16].

Table 3. Glass transition temperature (T_g) taken as the maximum tangent of delta in dynamic mechanical analysis (DMA) graphic and residual monomer content (RMC) calculated by Proton nuclear magnetic resonance ($^1\text{H-NMR}$) of ABC modified with different percentages of GO.

Formulation	T_g (°C)	RMC (%)
ABC 0% GO	115	1.06
ABC 0.1% GO	118	1.06
ABC 0.2% GO	120	1.06
ABC 0.3% GO	120	1.32
ABC 0.5% GO	119	1.32

The residual monomer content shows a slight increase in the two formulations with higher GO content (Table 3). These RMC results, together with the decrease in $T_{\max}$ and the rise in t_{set} during curing, confirm the retarding and inhibiting effect that GO generates during the free radical polymerization reaction [15].

3.2.8. Antibacterial Activity

According to the results shown in Figure 11, ABCs without GO showed weak antimicrobial activity against *gram-positive* (25%) and *gram-negative* (21%) bacteria, such inhibition may be related to the release or interaction at the surface with unpolymerized MMA (RMC 1.06%), which may generate an

inhibition by the susceptibility of the bacteria to traces of residual monomer. Moreover, the percentages of GO > 0.1 wt.% in the ABC formulation have a significant effect ($p < 0.01$) in decreasing the number of colony-forming units in both *Gram-positive* and *Gram-negative* bacteria. It was found that, by increasing the concentration of GO in the study range, the concentration of CFU for *Gram-positive* ($r^2 > 0.96$) and *Gram-negative* ($r^2 > 0.82$) bacteria was proportionally decreased linearly, achieving a reduction of 63 and 76% for *Gram-positive* and *Gram-negative* CFU, respectively.

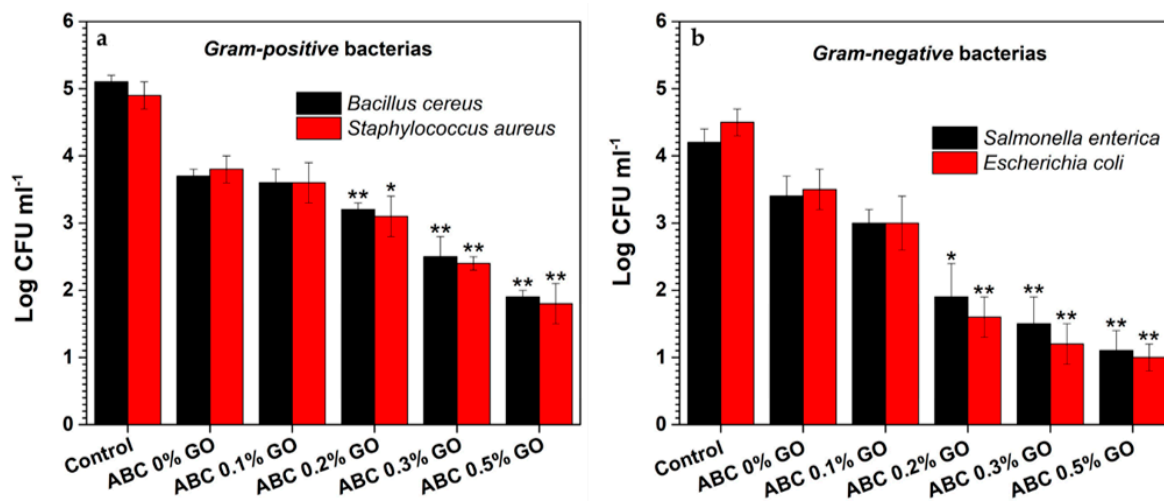


Figure 11. Colony-forming units (CFU) of *Gram-positive* (a) and *Gram-negative* strains (b) after 24 h incubation with GO-modified ABCs. Asterisks denote significant differences of ABC loading samples with respect to ABC 0% GO at significance levels of * $p < 0.05$ and ** $p < 0.01$.

These results differ from those reported in CS/PVA/GO films [53], where *Gram-negative* bacteria had higher resistance to inhibition, suggesting inhibition against *Gram-negative* or *Gram-positive* bacteria, showing GO is dependent on the matrix in which it is included. It is found that the inhibitory effect against *S. aureus* bacteria is slightly higher compared with *B. cereus* (Figure 11a) and that the impact on *E. coli* bacteria is also more significant when compared with *S. enterica* (Figure 11b).

In Table 4, it is observed that the inhibition of the cellular activity of GO is higher on *Gram-negative* bacteria because a total inhibition of the pathogen is achieved with a lower percentage of GO in the cement (GO = 0.2 wt.%) compared with the *Gram-positive* bacteria studied, where ABCs' loadings with 0.3 wt.% GO are necessary to achieve total inhibition. This could be attributed to different cell wall structures, which further cause different GO penetration probability. Once inside, the sharp ends will puncture the membrane and produce irreversible damage to the cells [54]. Another possibility is the generation of reactive oxygen species, which causes oxidative stress and cell membrane destruction after direct contact [55].

The low antibacterial activity of cements with 0.1 wt.% GO is consistent with the results reported by Paz et al. They found no antibacterial activity against *S. aureus* in ABCs loaded with the same amount of GO. That was probably because of the low concentration used [17], as the antibacterial activity of GO has been widely reported as dose-dependent [27,29].

Several mechanisms have been suggested for bacterial inactivation. The first involves direct contact of the bacteria with the extremely sharp edges of the GO, which causes physical damage to the membrane. The second mechanism consists of the generation of oxidative stress on the membrane by the production of reactive oxygen species (ROS), which leads to cell death [9,27,56,57]. Further studies are required to elucidate the possible antibacterial mechanisms presented in ABCs loading with GO to predict how it will behave against other types of bacteria.

Table 4. Inhibition of the cellular activity of cement added with GO against four bacterial strains.

Strain	Control	ABC 0% GO	ABC 0.1% GO	ABC 0.2% GO	ABC 0.3% GO	ABC 0.5% GO
<i>Bacillus cereus</i> ATCC 13061	+++	+	+	+	-	-
<i>Staphylococcus aureus</i> ATCC 55804	+++	+	+	+	-	-
<i>Salmonella enterica</i> ATCC 13311	+++	+	+	-	-	-
<i>Escherichia coli</i> ATCC 11775	+++	+	+	-	-	-

(+++ Strong activity of the pathogen, represented by a turning red of the TTC; (+) Weak activity of the pathogen, represented by a turning pink of the TTC; (-) Without the activity of the pathogen, represented by the absence of turn of the TTC; TTC: 2,3,5-Triphenyltetrazolium chloride, an indicator of dehydrogenase enzyme activity.

Owing to the antibacterial mechanisms reported for GO, it is evident that the high degree of oxidation reached by the graphite during synthesis (71.2%) and the correct exfoliation that led to the obtaining of GO of a few nanometers of thickness (9 nm) favored the antibacterial activity of the studied ABCs.

4. Conclusions

GO was obtained with an oxidation degree of 71.2% and an average lateral size of 400 nm. These characteristics of the nanomaterial allowed to get excellent dispersibility in the liquid phase and an increase in mechanical, physical, and antibacterial properties.

The presence of GO presented the following desirable properties in the ABCs: an increase in roughness, in wettability (by 17%), in t_{set} (by 40%), in the mechanical properties to compression and flexion up to 17%, and in the antibacterial activity against *Gram-positive* and *Gram-negative* bacteria studied, in addition to a decrease in T_{max} up to 19%. However, it also generated a slight reduction in thermal stability and a negligible increase in residual monomer content.

Because the formulation of ABC with 0.3 wt.% GO presented the minimum concentration of GO with which it is possible to inactivate both *Gram-positive* and *Gram-negative* bacteria studied, in addition to complying with the mechanical and handling requirements established in ISO 5833-02, this formulation is suggested to develop complementary studies that confirm its high potential for use as a replacement for bone cements loaded with antibiotics as a solution in the treatment of antibiotic-resistant bacteria in orthopedics.

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3.3 Zamora Lagos, S.I.; Murillo Salas, J.; **Valencia Zapata, M.E.**; Mina Hernandez, J.H.; Grande Tovar, C.D. *Optimization by Central Composite Experimental Design of the Synthesis of Physically Crosslinked Chitosan Spheres. Biomimetics* – 956848. Artículo sometido para publicación (El anexo C contiene el certificado de sometimiento del artículo).

Article

Optimization by Central Composite Experimental Design of the Synthesis of physically crosslinked chitosan spheres

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Abstract: Chitosan (CS) has special properties such as biocompatibility, biodegradability, antibacterial, and biological activity. Whereby this material is currently studied and used in various applications, including tissue engineering. There are different methods to modify the morphology of CS. Most use chemical crosslinking agents; however, those methods have disadvantages such as low polymer degradability and unwanted side effects. For this reason, the physical modification of commercial CS flakes was performed to obtain CS spheres through physical crosslinking without using chemical crosslinking agents, specifically, using the simple coacervation method, dissolving CS in acetic acid (AAC) solution, and dripping it into a sodium hydroxide (NaOH) solution. A central composite experimental design was used to optimize the synthesis of the CS spheres. The drip needle diameter, the composition of the solutions, and the relative humidity of drying were varied, with the spheres' final diameter as the response variable. The response surface methodology was possible to obtain CS spheres with the smallest diameter and the most regular morphology. With the optimal formulation (CS solution 1.8 % (w/v), AAC solution 1% (w/v), NaOH solution 13% (w/v), relative humidity of (10%) and needle diameter of 0.6 mm), a final sphere diameter of 1 mm was obtained. By studying the CS spheres using the SEM technique, it was possible to detail the spheres' morphology and surface.

Moreover, by elemental analysis, the deacetylation degree for CS flakes and spheres was obtained: 88.43% and 58.94%, respectively. The thermal analyzes identified that CS spheres are less thermally stable than CS flakes, which is causally related to the deacetylation degree. By hydrolytic degradation assay in simulated body fluid (SBF), it was concluded that it was possible to transform CS flakes' morphology into a regular, spherical morphology, without considerably modifying CS desirable properties. From these research results, it is possible to say that the synthesized CS spheres have a

high potential to form part of structures where biodegradation is a vital property for application in tissue engineering.

Keywords: chitosan; spheres; morphology; physical, crosslinking, biomedical; application.

1. Introduction

Chitosan (CS) is a partially deacetylated derivative of chitin with biodegradation, biocompatibility, and bioactivity, attractive for biomedical applications (i.e., as a bioactive agent, scaffold manufacturing, hydrogels) and various dental applications [1].

CS can be prepared with different morphologies such as sponges, fibers, films, and other complex structures such as microspheres [2]. CS spheres are widely applied in many fields because of their special sizes and different forms and composition. CS spheres can be functionalized or crosslinked with chemical agents, either to modify their chemical properties such as the addition of active components like antibiotics, curcumin, zinc oxide, or simply to maintain their initial morphology such as the addition of crosslinking agents like glutaraldehyde or sodium tripolyphosphate [3–7].

Crosslinking mainly improves mechanical properties and increases polymer stability by interconnecting molecules. However, it reduces the number of available functional groups, decreases polymer degradability, changes rheology properties [8]. Some crosslinking agents, like glutaraldehyde, are considered highly toxic. Due to its low molecular weight, glutaraldehyde can enter a living organism's cells through several pathways [9,10]. Therefore, the safety of the use of chemical crosslinking is a fact that limits its application in the biomedical field [11].

CS spheres are generally used as a drug delivery system, quick hemostat system, tissue engineering as a microstructural unit in scaffolds, or active bone cement [7,12–14].

Studies report significant advantages when using CS with sphere morphology in different tissue engineering applications. For example, Abdel-Fattah et al. reported that it was possible to develop matrices with various pore size and controllable pore volume by using CS spheres with excellent mechanical properties [15]. Zamora et al. conclude that the use of CS spheres as fillers in acrylic bone types of cement means a significant improvement in mechanical properties compared to the use of CS flakes [16]. In their study, Meng et al. reported that when adding CS spheres in a formulation of bone types of cement, they obtained a material with good bioactivity and biodegradation properties and a mechanical strength comparable to the reported strength of cancellous bone [14]. Mainly, in tissue engineering, the objective of CS spheres uses is related to the biodegradation and biocompatibility properties of the material. Besides, CS spheres use is connected to control the surface area in contact with the fluid and increase or preserve the matrix's mechanical properties.

Therefore, once the CS is in contact with the biological fluid, it is desired that it provides all the physical activity described before, maintaining its biocompatibility properties. The use of chemical crosslinking agents could be a barrier for CS to play its role. It can decrease CS degradability, and the crosslinking agent can cause unwanted side effects such as negative inside the body. Some authors had used physical crosslinking to avoid the undesirable changes and possible side effects of chemical crosslinking [17]. Physical crosslinking generally occurs in response to specific conditions, like temperature or pH, and can be affected by polymer concentration and other components. CS physical

crosslinking is problematic due to the nature of the molecule. Many interactions are involved at the same time, affecting the sol-gel transition.[8]

Although there are different investigations related to CS spheres preparation using chemical crosslinking, in this study, CS spheres we focus on the physical crosslinking by the simple coacervation method but optimizing the formulation for CS spheres preparation. The optimization was performed using a central composite experimental design. In this way, it was possible to maximize their size through the experimental design. The relation between the CS spheres formation solutions' concentration, the drip needle's size, and relative humidity for the CS spheres drying was studied. Finally, the CS spheres with the most regular morphology and smallest size possible demonstrated by scanning electron microscopy (SEM) were characterized, and it was observed that by the proposed method of synthesis of CS spheres, CS presented a chemical modification, specifically in its degree of deacetylation, studied by elemental analysis (EA). The thermal analysis made it possible to verify that the CS spheres are less thermally stable. Finally, by the hydrolytic degradation test by immersion in simulated body fluid (SBF), it was studied the variations in pH of the medium, and it was proven that despite the physical and chemical modification of CS when synthesizing the spheres, they still present interesting degradation properties, characteristic of CS. Therefore, the CS spheres obtained show a regular morphology and suitable size and properties for tissue engineering applications.

2. Materials and Methods

The CS flakes employed in this study were from shrimp shells ($M_w = 190.000\text{--}310.000$ Da, viscosity of 328 CPS, and a deacetylation degree of 83%) according to the technical data from Sigma-Aldrich (St. Louis, MO, USA). For CS spheres synthesis, the last CS flakes were used, acetic acid (AAC) ($M_w = 60.05$ Da), and sodium hydroxide flakes (NaOH) ($M_w = 40.00$ Da) all from Sigma-Aldrich (St. Louis, MO, USA).

2.1 Response surface design

The experimental formulations used in this study were designed based on a central composite experimental design (Table S1). In this case, the effect of the components, needle diameter, and the drying relative humidity (R.H) in CS spheres' final diameter was studied. Three independent central composite experimental designs were included for each R.H studied (10%, 52%, room R.H). The following components were used for experimental design: AAC, CS, NaOH.

Intervals for the experimental region of each model are given in Table 1. The diameter is the response variable for each experimental design (Table 1).

Table 1. Independent variables of the experimental design for the CS spheres synthesis.

Independent variables	Intervals		
	Minimum (% g/mL)	Centered (% g/mL)	Maximum (% g/mL)
x_1 Acid acetic concentration (AAC)	1	3	5
x_2 CS concentration (CS)	1.2	1.7	2
x_3 Sodium hydroxide concentration (NaOH)	10.4	1.4	20
x_4 Needle diameter	0.6 (mm)	-----	0.8 (mm)

The response surface methodology (RSM) was used to optimize the CS spheres' diameter as the response variable using the prediction equation (Eq. 1).

$$Y = B_0 + B_1X_1 + B_2X_2 + \dots + B_3X_1^2 + B_4X_2^2 + \dots + B_5X_1X_2 + \dots + e \text{ (Eq. 1)}$$

Where Y is the response variable (diameter), B_i represent the model coefficients for predictive variables ($i = 0, 1, 2, 3, 4, 5, \dots, 10$), $X_{(s)}$ are the independent variables ($s = 1, \dots, 4$), $X_{(s)}^2$ are the quadratic effects of each of the predictive variables, $X_{(s)}X_{(s)}$ are the interaction effect between variables, and e represents the experimental error.

2.2 CS spheres synthesis

Synthesis of CS spheres was performed using the coacervation methodology, which consisted in the addition of a low concentration (1 w/v%) solution of CS flakes dissolved in diluted AAC (1.8 w/v%) to a NaOH solution (13 w/v%) dropwise, under gentle stirring.

2.2.1 Dripping stage

The assembly shown in Figure 1 was performed, which consisted of connecting a needle to a programmable syringe pump through which the CS solution passed and was deposited by dripping at a rate of 0.3 mL/min in the NaOH solution stirred at 360 rpm with the help of a magnetic stir plate. Subsequently, the spheres were collected by filtration. At the same time, washes with 500 mL of distilled water at 360 rpm were carried out, until a nearly neutral pH was obtained (6.8-7.5) using a pH meter Fisher Scientific Accumet AB150. Finally, the spheres were collected by filtration.

2.2.2 Drying stage

The CS spheres produced were dried for 24 hours in desiccators (R.H. $10.0 \pm 0.1\%$). Once the CS spheres were dried, they were collected to perform the granulometric separation with a 0.75 mm opening screen. Finally, they were collected in polyethylene bags and kept inside the desiccator at R.H. $10.0 \pm 0.1\%$.

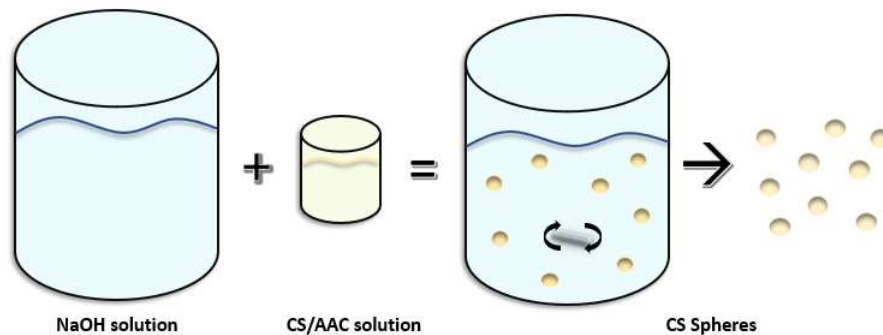


Figure 1. Simple coacervation method for CS spheres synthesis.

2.3 CS spheres characterization

2.3.1 Particle morphology and surface microstructure

Morphology of CS flakes and spheres were studied by scanning electron microscopy (SEM, JEOL JSM-6490LA, Musashino, Tokyo, Japan). Samples were gold-sputtered before the examination. The working conditions were 20 kV as acceleration voltage and mode of secondary backscattered electrons.

2.3.2 Fourier-Transform Infrared Spectroscopy (FTIR)

The chemical identification of the CS flakes and spheres was studied using FTIR in the attenuated total reflectance (ATR) mode (Shimadzu, Kyoto, Japan).

2.3.3 Elemental analysis

The degree of deacetylation (DDA) of CS was also determined by elemental analysis. A Thermo Electron Flash EA 1112 elemental analyzer was used. The test was performed at 40 psi, with a constant flow of helium and oxygen. The DDA was calculated using equation 2 [18].

$$DDA(\%) = 100 - \left(\frac{\frac{C}{N} - 5.2}{6.9 - 5.2} \right) * 100 \quad Eq. 2 .$$

2.3.4 Proton nuclear magnetic resonance (¹H-NMR)

For the ¹H-NMR technique, a BRUKER 400 AVANCE II equipment was used. The degree of deacetylation of CS was determined. For this test, D₂O was used with two drops of trifluoroacetic acid (reactive grade) as solvent and 3- (trimethylsilyl) propionic acid-d₄, (reference salt), 32 runs were taken with an acquisition time of 5.24s and a relaxation time of 1s. Equation 3 was used to find the DDA [19].

$$DDA(\%) = \left(1 - \left(\frac{1}{3} HAc / \frac{1}{6} H26 \right) \right) \times 100 \quad Eq. 3.$$

Where:

HAc: represents the three protons of the methyl group of N-acetyl glucosamine

H26: represents the protons from 2 to 6 ppm

2.3.5 Thermal characterization

Differential Scanning Calorimetry (DSC)

The DSC technique was used to study the thermal behavior. The method was performed with a TA Instruments DSC25 equipment. Each sample was placed on an aluminum tray, and one was sealed with an empty tray of the same type (reference). A nitrogen atmosphere was used. The heating rate was 20 °C/min, and the temperature scans were taken from -100 °C to 550 °C. The second scanning was studied.

Thermogravimetric analysis (TGA)

The thermal degradation of the CS spheres and its maximum decomposition temperature was studied using the TGA and the derivative of the thermogravimetric analysis (DTGA) technique. The technique was performed with a TA Instruments TGAQ50 equipment. A nitrogen atmosphere was used. The heating rate used was 20 °C/min, and the temperature ranges were brought from room temperature to 550 °C.

2.3.6 Hydrolytic degradation assay

CS spheres immersion for 30 days in simulated body fluid (SBF) prepared according to Kokubo and Takadama method was used to evaluate the hydrolytic degradation [20]. SBF was stored in plastic bottles at a temperature between 5 and 10°C and used within less than one month. During immersion, the pH of the sample solution was analyzed.

Each sample (0.12 g) was immersed in SBF and kept in an incubator at 37 °C for 30 days in which pH measurements were made daily.

2.4 Statistical analysis and optimization

The design experiment optimization included analyzing each optimization graph and contour plot thrown by the MINITAB 18 program in the central composite experiment design for each R.H. For

the optimization, the desirability function (D: global; d: individual) was used, which combines the geometric media and optimizing the general metric media [21]

3. Results

Through the development of the experimental design, it was observed that the CS /AAC solution should have a viscosity that would allow it to pass through the drip needle without effort, to produce a spherical drop. If the dissolution had an excessive viscosity, it could not flow through the drip needle. In contrast, if the solution viscosity is too low, it would not produce spherical drops of CS, and instead, it will create a continuous stream of solution. It was observed that NaOH concentration must be enough to able CS/AAC solution to form regular spheres upon contact with NaOH solution. Furthermore, with a controlled drying R.H, spheres with a more regular morphology were obtained.

The response surface methodology (RSM) is a technique that optimizes one or more response variables within an experimentation area. RSM was used to optimize the response variable and obtain the minimum CS spheres diameter depending on the process variables.

The contour graphs allowed us to observe the different CS spheres diameters according to the conditions of the independent variables in each R.H. (Figure S1-S5). According to the contour graphs shown in figure 2, the smallest diameters of the spheres (<1mm) were obtained with the presence of the highest percentages of CS, low concentration of AAC, and low concentrations of NaOH. Further, the optimization charts (Figure S6-S11) offer the possibility to modify the values of the factors conveniently. This design optimization allowed finding the most appropriate conditions to minimize the diameter and obtain a homogeneous morphology in the spheres; these conditions are shown in Table 2.

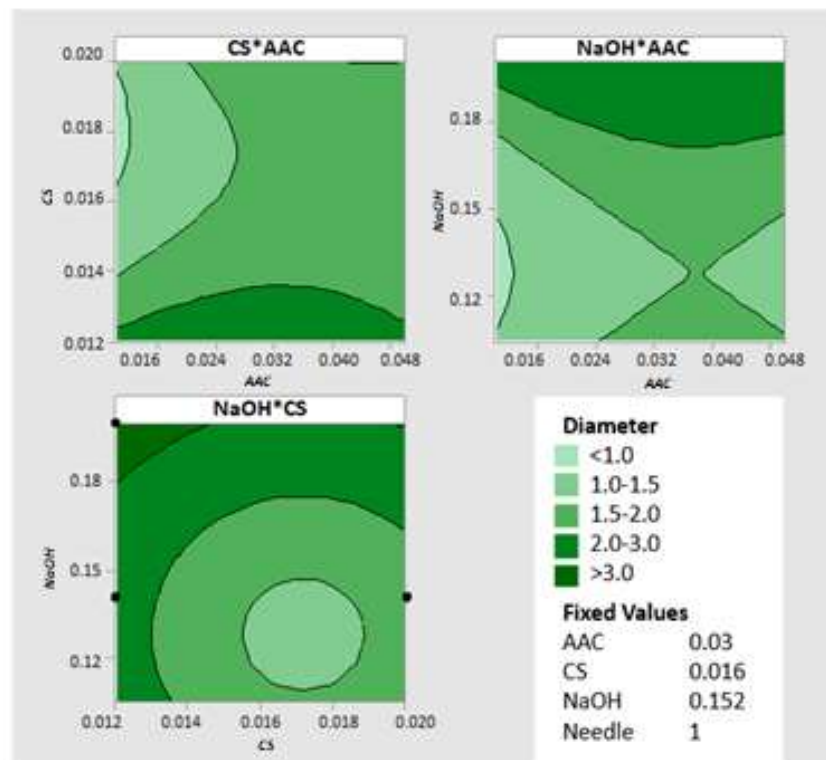


Figure 2. Diameter contour plot for CS spheres. Conditions: Needle 1, HR 10%±0.1%.1.

Table 2. The optimal formulation for CS spheres synthesis.

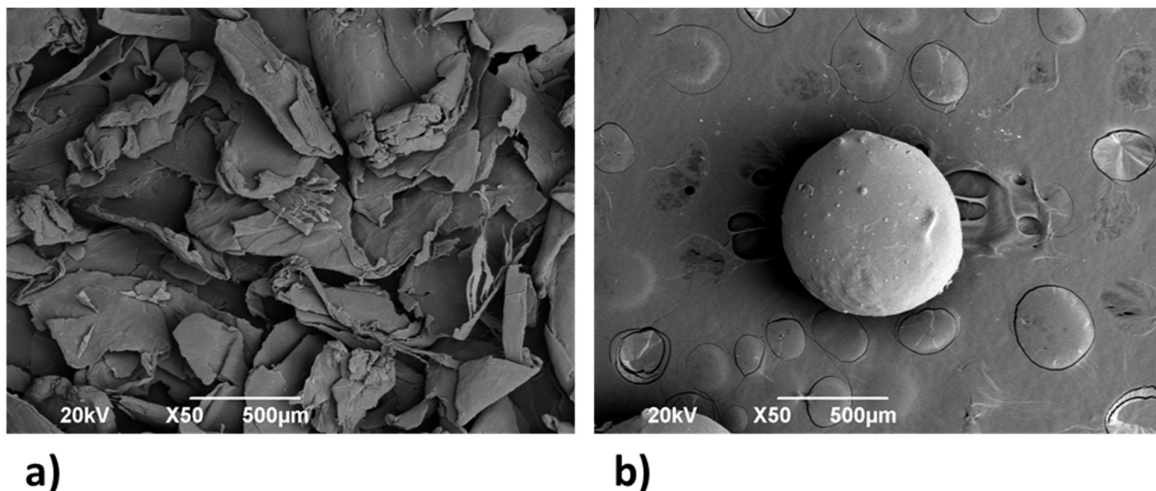
Component	Concentration (% w/v)
AAC	1
CS	1,8
NaOH	13

3.1 Particle morphology and surface microstructure

CS spheres obtained were analyzed using a stereoscope at magnification 80×, using a graph paper as a reference frame to study the variations in the morphology of CS spheres. Sphere morphology was obtained with a diameter of 1 mm or lower, as shown in figure 3.

**Figure 3.** The photograph at 80 × of CS spheres obtained from the optimal formulation, (H.R. 10%).

Furthermore, SEM was performed on the commercial CS flakes and CS spheres. As can be seen in Figure 4, commercial CS has an irregular laminar morphology and angular edges. On the other hand, the coacervation mechanism's CS particles have an almost spherical morphology with a mainly smooth surface, but with some defects introduced after the manual separation process.

**Figure 4.** SEM micrographs at 50× of: a) CS flakes b) CS spheres [16].

3.2 Fourier-Transform Infrared Spectroscopy (FTIR)

Chemical analysis was carried out using FTIR. Figure 5 shows the FTIR spectra of CS flakes and spheres. Both samples showed prominent characteristic bands. At 3286 cm^{-1} , overlapped bands

correspond to the stretching vibration of O-H and NH₂ groups. The bands present at 2915 cm⁻¹ and 2875 cm⁻¹ are associated with NH₂ and CH₂ groups. The band presents at 1643 cm⁻¹ corresponds to the NH₂ (Amide I) group's bending, while the band at 1566 cm⁻¹ corresponds to NH₂ (Amide II). The band at 1367 cm⁻¹ and 1306 cm⁻¹ corresponds to CH bending and C-CH₃ deformation, respectively. Finally, at 1020 cm⁻¹, a band is associated with the vibration of the glucopyranose ring [22–24]. The physical crosslinking performed to synthesize the chitosan spheres did not significantly affect the chemical structure of the polymer. Both spectra show the characteristic bands of CS with a slight variation in intensity. Studies report that variations in the intensity of the peaks are associated with the higher presence of specific functional groups, related to the variation of DDA [25,26].

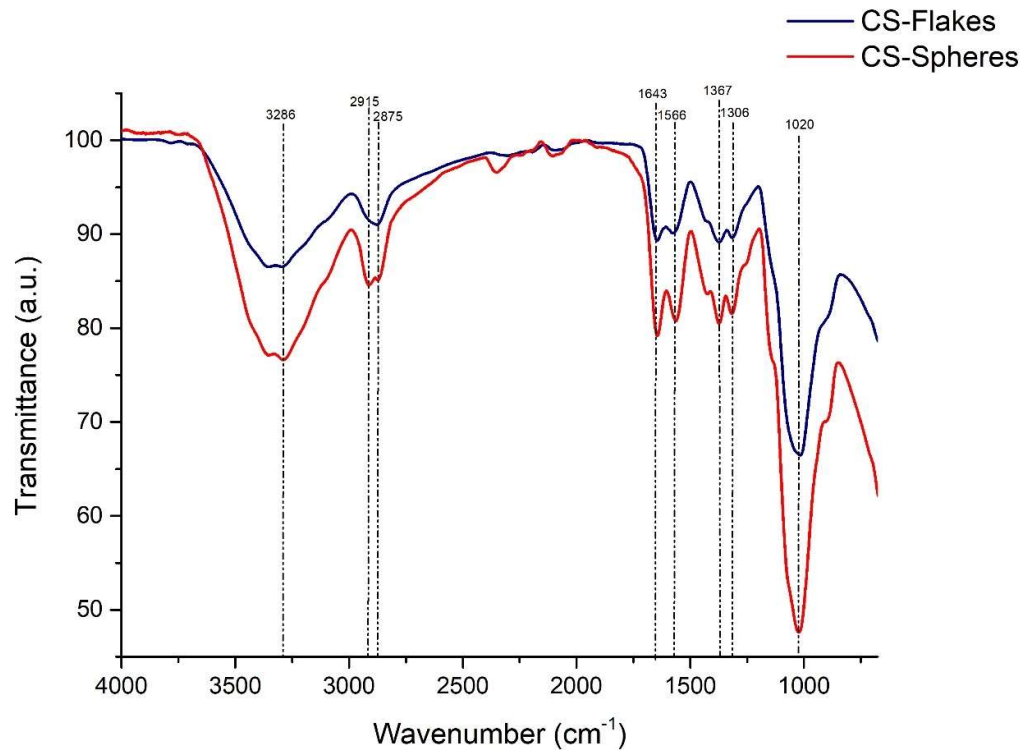


Figure 5. FTIR spectra of CS flakes and CS spheres.

3.3 Elemental analysis

According to the elemental analysis (Table 3), the percentage of the elements are similar in flakes and CS spheres, as expected. However, nitrogen content presents some differences, probably due to the amine group interaction with sodium ions from sodium hydroxide interaction. A similar process occurs in alkaline deacetylation with the acetamide groups of chitin [27]. Therefore, the DDA values (Equation 2) by EA were 88.43% and 58.94% for CS flakes and spheres, respectively, and by ¹H-NMR (Equation 3), the DDA values obtained were of 75.01% and 60.78 % (Figures S13–S14).

Table 3. Elemental analysis for CS flakes and CS spheres.

	CS Flakes	CS Spheres
Element	(%)	(%)
Nitrogen	7.28	6.39
Carbon	38.93	37.38
Hydrogen	6.98	7.04

Sulfur	0	0
Total	53.20	50.82
DDA	88.43	58.94

According to different studies, a higher DDA (%) increases the number of reactive positions for degradation by hydrolysis and provides a lower crystallinity of the polymer, which makes the degradation rate of the polymer higher [28–30]. Furthermore, CS's degradation capacity influences the solubility and mechanical properties due to the structural changes that the material undergoes [31]. Consequently, this property will control facile CS degradation as required in biomedical applications.

3.4 Thermal characterization

Differential Scanning Calorimetry (DSC)

Figure 6 shows the DSC curves of CS flakes and CS spheres. The first endothermic event between room temperature and 150 °C corresponds to the loss of absorbed and constituent water molecules, volatile components, and hydrophilic groups present in CS. Around 156 °C, an additional endothermic peak is observed, associated with the polymer's glass transition temperature. They coincided with Dong et al., who reported a glass transition temperature for CS between 140 °C and 150 °C. Also, Dong et al. reported a thermal transition at 200 °C associated with the liquid-liquid change, which is also present in both analyzes for flakes and spheres at approximately 220 °C [32]. The exothermic events at 320 °C and 290 °C for CS flakes and spheres are associated with the decomposition of amine units, cracking, and CS [24,33].

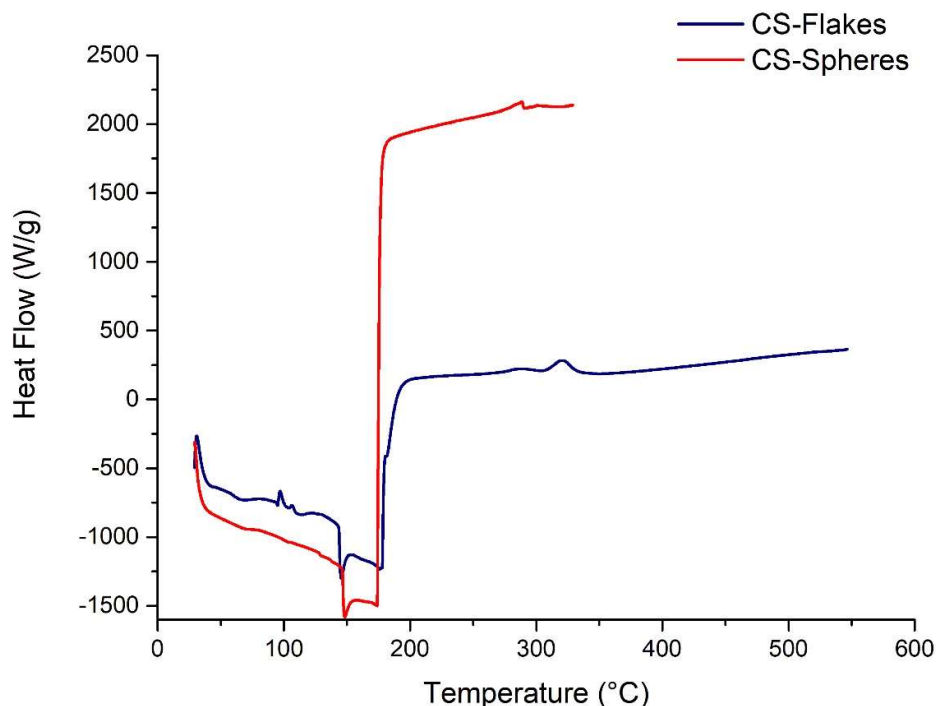


Figure 6. DSC analysis for CS flakes and spheres.

Thermogravimetric analysis (TGA)

Figure 7a shows the TGA curves of CS flakes and spheres. The first stage of degradation occurs between 35 °C to 200 °C, with a weight loss of approximately 12%, related to the evaporation of water molecules or small molecules [34]. According to the curve, these elements' weight loss occurs more pronounced in the CS flakes than the CS spheres.

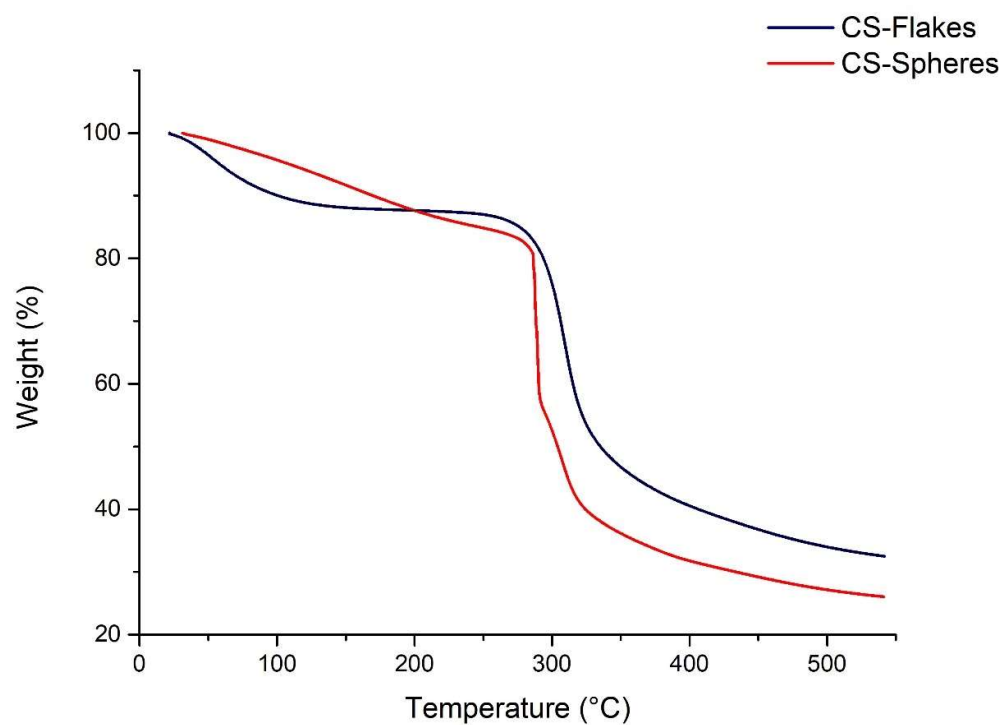
This can be explained by the physical crosslinking when preparing the CS spheres, which reduces the number of available functional groups, limiting the interaction with water molecules present in the environment [35].

The second stage of degradation occurs between 200 °C to 550 °C, with a weight loss of 68% and 74%, corresponding to CS's flakes and spheres, respectively. This stage is related to a complex process that involves the dehydration of the saccharide rings and the decomposition of the acetylated and deacetylated units of CS [34]. Furthermore, in figure 7 b), in the DTGA curves, it can be seen that CS spheres present a first peak representing the maximum rate of degradation, which occurs at 290 °C (decomposition temperature). Then another small peak is observed at 307 °C, while the decomposition temperature of CS flakes is 309 °C. Gámiz-González et al. observed similar behavior when analyzing the TGA and DTGA of chitosan and its acetylated derivatives with different DDA. In the DTGA, they reported the appearance of a second peak as a shoulder on the high-temperature side of the principal peak, concluding that the intensity of this second peak increased as the DDA decreased [36]. According to Young Sik et al., the first decomposition stage corresponds to D-glucosamine chitosan units' depolymerization. In contrast, the second decomposition stage can be attributed to N-acetyl-D-glucosamine [37].

In this study, the peak corresponding to the degradation of N-acetyl-D-glucosamine is present with greater intensity. This is due to the high proportion of these units within the CS spheres' polymer chain compared with CS flakes.

Therefore, the thermal analyzes can be concluded that the CS spheres are less stable thermally than the CS flakes. Some studies indicate that thermal stability of CS is linked to the degree of deacetylation, as a consequence of the decrease in the content of acetyl groups, which provides less crystallinity to CS, and reduces thermal stability because less energy is required for its decomposition [24,34,38]. Takara et al. reported that treating CS with aqueous NaOH solutions causes deprotonation, the formation of new hydrogen bonds, and greater deacetylation of CS [39].

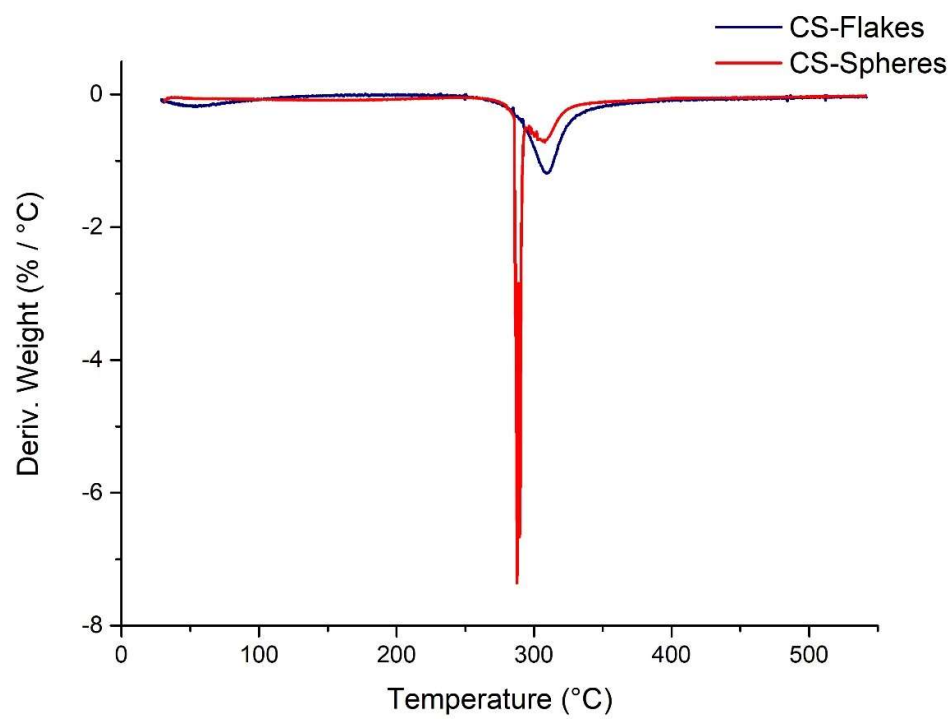
a)



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303 b)



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Figure 7. Thermal analysis of CS flakes and spheres: a) TGA curves b) DTGA curves.

3.4 Hydrolytic degradation assay

Figure 8 shows the medium's pH variation during the immersion of the CS flakes and CS spheres inside the SBF. The medium's pH tends to decrease gradually with days, which might be due to the CS degradation products that are mainly composed of bound 1,4-D-glucosamine and partially -1,4 N-acetyl-D-glucosamine. Studies report that these products have significant potential in medical fields due to their wide bioactivity, antibacterial, antifungal, antitumor activity, radical scavenging, antimicrobial activity, immunity, modulating effect, and wound healing [40].

The pH values of SBF with CS flakes were higher than those with spheres until day 26th. On day 26th, the value of 6.8 for flakes and 5.9 with spheres was observed. The test runs until day 30th. There was no variation in pH, with a final value of 5.6 and 5.7 for flakes and spheres, respectively. The values were too acid for biomedical applications according to the traditional standards [41].

For biomedical applications, the CS compounds should maintain the biodegradation and biocompatibility properties. Therefore, when observing that the physical crosslinking performed for the synthesis of the CS spheres did not have a significant effect on the performance of the spheres within the hydrolytic degradation assay is positive for the research since this means that it was possible to transform the morphology of CS flakes into a regular, spherical morphology without considerably modifying the chemical and biological properties of CS in flakes.

In a previous study carried out, the improvement in CS spheres' mechanical performance was confirmed when compared with CS flakes within an acrylic bone cement matrix [16]. This is a desirable performance in other applications such as scaffolds or films for tissue engineering

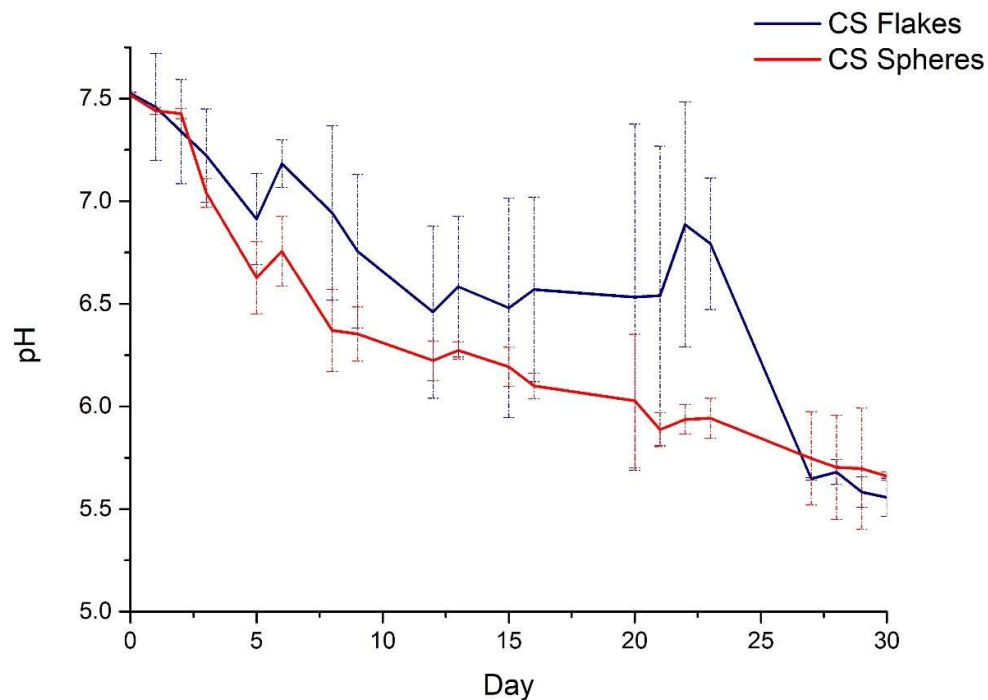


Figure 8. pH changes in CS flakes and CS spheres after 30 days of immersion in SBF.

In figure 9, CS spheres' surface morphology presented modifications with the immersion time in the SBF. Initially, spheres offer a regular edge and a smooth surface, but from day 5th, the surface increases the roughness and porosity, which is very convenient for tissue engineering applications. The formation of porous structures is acceptable behavior because porosity structures mimic the natural extracellular matrix better than a two-dimensional system like CS flakes, supporting adhesion and cell growth [42].

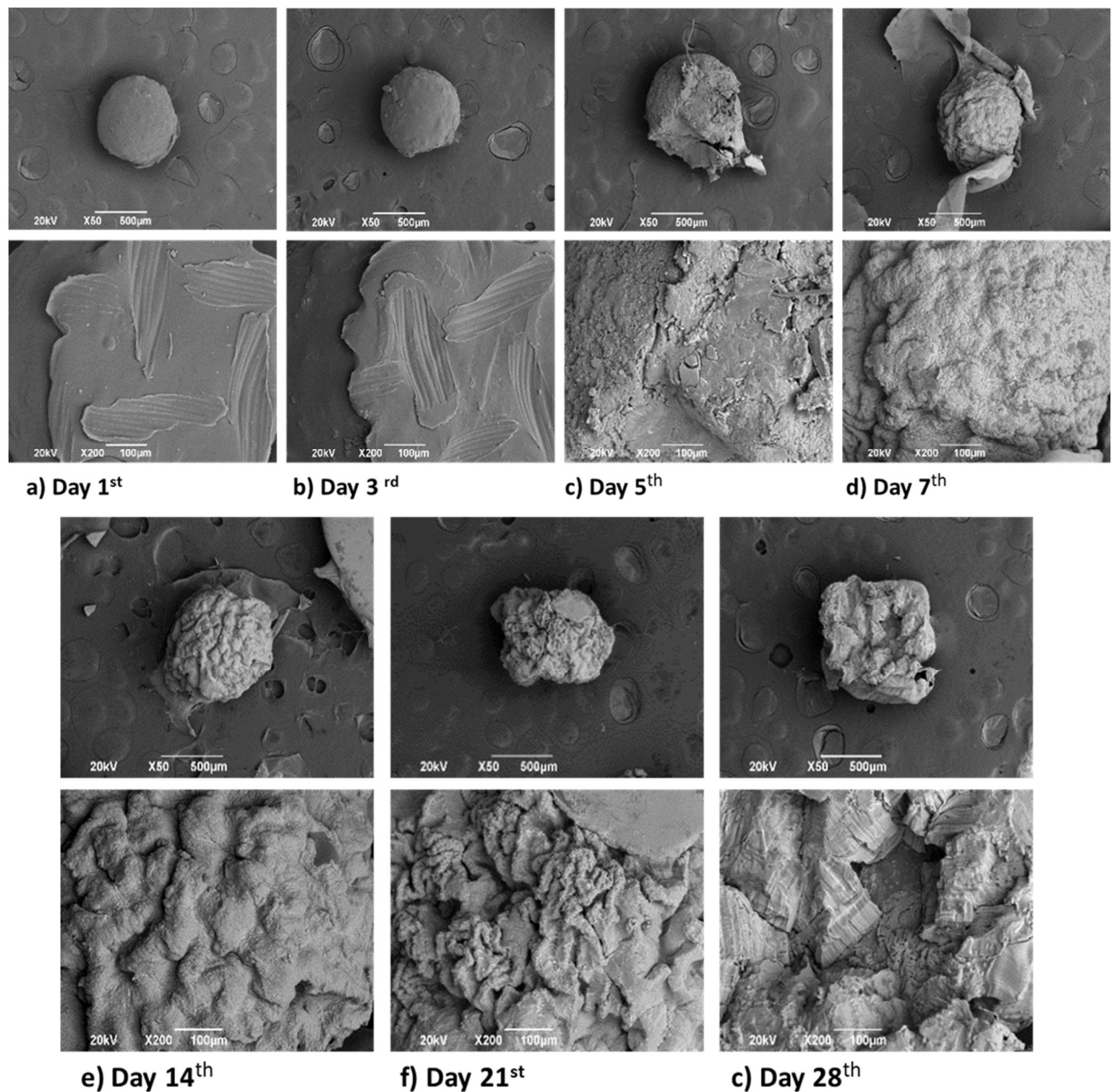


Figure 9. SEM micrographs at 50× and 200× of CS spheres from day 1st until day 28th of SBF immersion.

4. Conclusions

The coacervation methodology successfully produced CS spheres of a diameter of 1.0 ± 0.1 mm with a consistent and homogeneous appearance, varying the solutions' concentrations, the drip needle's diameter, and the relative humidity during drying, demonstrating the reproducibility of the method.

CS sphere deacetylation degree characterization confirmed the physical crosslinking by decreasing the value compared to the CS flakes. The lower hydrogen bonding capacity of the CS spheres also produced a decrease in the maximum rate of degradation, as evidenced by TGA and DSC analysis (309° and 290° for CS flakes and spheres, respectively), demonstrating that physical crosslinking decreases CS crystallinity by reducing available hydrogen bonds. When CS spheres were evaluated within the SBF, they presented a similar behavior regarding the final pH reported during the test, 6.8

for CS flakes, and 5.9 for CS spheres, reporting values allowed by the standard. The synthesis method generated a physical crosslinking that did not modify CS's biological properties; therefore, CS spheres will have remarkably similar properties to CS flakes in terms of degradation and biocompatibility. Furthermore, after the hydrolytic assay, SEM analysis demonstrated that immersion produced a porous structure, which means that the CS spheres will have more significant degradation properties because this structure will help the tissue promoter cells enter the system. And it will also facilitate the transfer of oxygen, nutrients, and metabolic waste due it mimics the extracellular matrix.

Author Contributions:

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Conflicts of Interest: The authors declare no conflict of interest.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1. Table S1. Central composite experimental design for the CS spheres synthesis. Figure S1. Diameter contour plot for CS spheres. Conditions: Needle 2, HR 10%±0.1%. Figure S2. Diameter contour plot for CS spheres, Conditions: Needle 1, R.H. 52%±0.1. Figure S3. Diameter contour plot for CS spheres, Conditions: Needle 2, R.H. 52%±0.1. Figure S4. Diameter contour plot for CS spheres, Conditions: Needle 1, Room R.H. Figure S5 Diameter contour plot for CS spheres, Conditions: Needle 2, Room R.H. Figure S6. Optimization chart, Conditions: Needle 1, R.H. 10%±0.1%. Figure S7. Optimization plot, Conditions: Needle 2, R.H. 10%±0.1%. Figure S8. Optimization plot, Conditions: Needle 1, R.H. 52%±0.1%. Figure S9. Optimization plot, Conditions: Needle 2, R.H. 52%±0.1%. Figure S10. Optimization plot, Conditions: Needle 1, Room R.H. Figure S11. Optimization plot, Conditions: Needle 2, Room R.H. Figure S12. Chemical chitosan structure. Figure S13. ¹H-NMR spectrum for CS flakes. Figure S14. ¹H-NMR spectrum for CS spheres.

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Supplementary Materials

Optimization by Central Composite experimental Design of the Synthesis of physically crosslinked chitosan spheres

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Table S1. Central composite experimental design for the CS spheres synthesis.

Figure S1. Diameter contour plot for CS spheres. Conditions: Needle 2, HR 10%±0.1%.1.

Figure S2. Diameter contour plot for CS spheres, Conditions: Needle 1, R.H. 52%±0.1.

Figure S3. Diameter contour plot for CS spheres, Conditions: Needle 2, R.H. 52%±0.1.

Figure S4. Diameter contour plot for CS spheres, Conditions: Needle 1, Room R.H.

Figure S5 Diameter contour plot for CS spheres, Conditions: Needle 2, Room R.H.

Figure S6. Optimization chart, Conditions: Needle 1, R.H. 10%±0.1%.

Figure S7. Optimization plot, Conditions: Needle 2, R.H. 10%±0.1%.

Figure S8. Optimization plot, Conditions: Needle 1, R.H. 52%±0.1%.

Figure S9. Optimization plot, Conditions: Needle 2, R.H. 52%±0.1%.

Figure S10. Optimization plot, Conditions: Needle 1, Room R.H.

Figure S11. Optimization plot, Conditions: Needle 2, Room R.H.

Figure S12. Chemical chitosan structure.

Figure S13. ¹H-NMR spectrum for CS sheets.

Figure S14. ¹H-NMR spectrum for CS spheres.

Table S1. Central composite experimental design for the CS spheres synthesis.

StdOrder	RunOrder	AAC (Fraction-)	CS (Fraction)	NaOH (Fraction)	Needle
5	1	0.018	0.007	0.176	1
36	2	0.03	0.012	0.14	2
12	3	0.03	0.02	0.14	1
37	4	0.03	0.012	0.14	2
8	5	0.042	0.017	0.176	1
30	6	0.05	0.012	0.14	2
39	7	0.03	0.012	0.14	2
27	8	0.018	0.017	0.176	2
1	9	0.018	0.007	0.104	1
17	10	0.03	0.012	0.14	1
6	11	0.042	0.007	0.176	1
13	12	0.03	0.012	0.08	1
32	13	0.03	0.02	0.14	2
34	14	0.03	0.012	0.2	2
9	15	0.01	0.012	0.14	1
24	16	0.0419	0.0168	0.1043	2
31	17	0.03	0.004	0.14	2
15	18	0.03	0.012	0.14	1
38	19	0.03	0.012	0.14	2
19	20	0.03	0.012	0.14	1
21	21	0.0181	0.0072	0.1043	2
3	22	0.0181	0.0168	0.1043	1
29	23	0.01	0.012	0.14	2
40	24	0.03	0.012	0.14	2
18	25	0.03	0.012	0.14	1
33	26	0.03	0.012	0.08	2
11	27	0.03	0.004	0.14	1
20	28	0.03	0.012	0.14	1
16	29	0.03	0.012	0.14	1
25	30	0.018	0.007	0.176	2
14	31	0.03	0.012	0.2	1
4	32	0.0419	0.0168	0.1043	1
2	33	0.0419	0.0072	0.1043	1
23	34	0.0181	0.0168	0.1043	2
28	35	0.0419	0.0168	0.1757	2
26	36	0.0419	0.0072	0.1757	2
22	37	0.0419	0.0072	0.1043	2

10	38	0.05	0.012	0.14	1
7	39	0.018	0.017	0.176	1
35	40	0.03	0.012	0.14	2

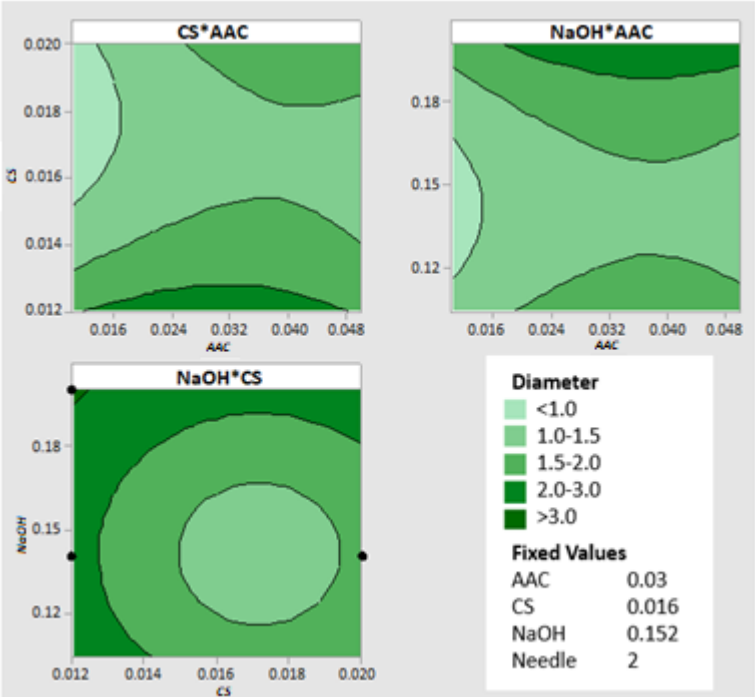


Figure S1. Diameter contour plot for CS spheres. Conditions: Needle 2, R.H. 10%±0.1.

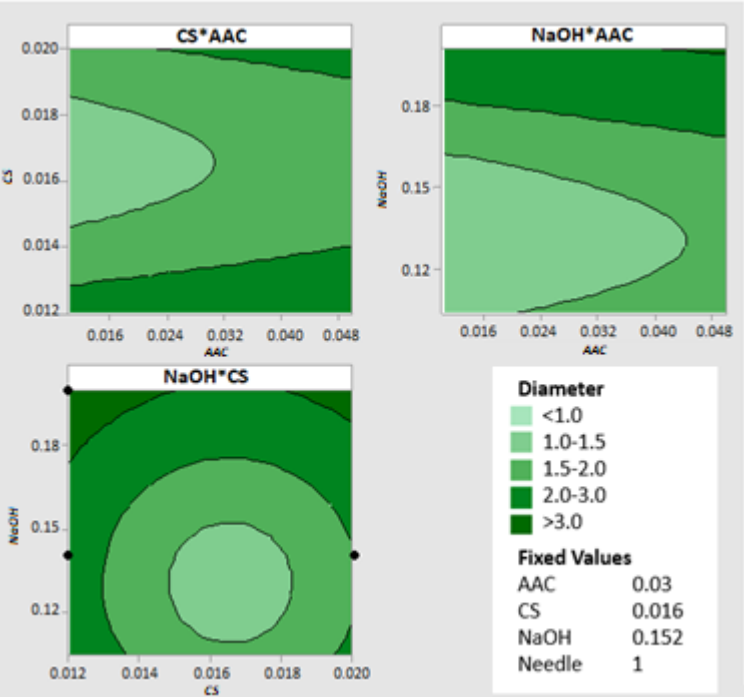


Figure S2. Diameter contour plot for CS spheres, Conditions: Needle 1, R.H. 52%±0.1.

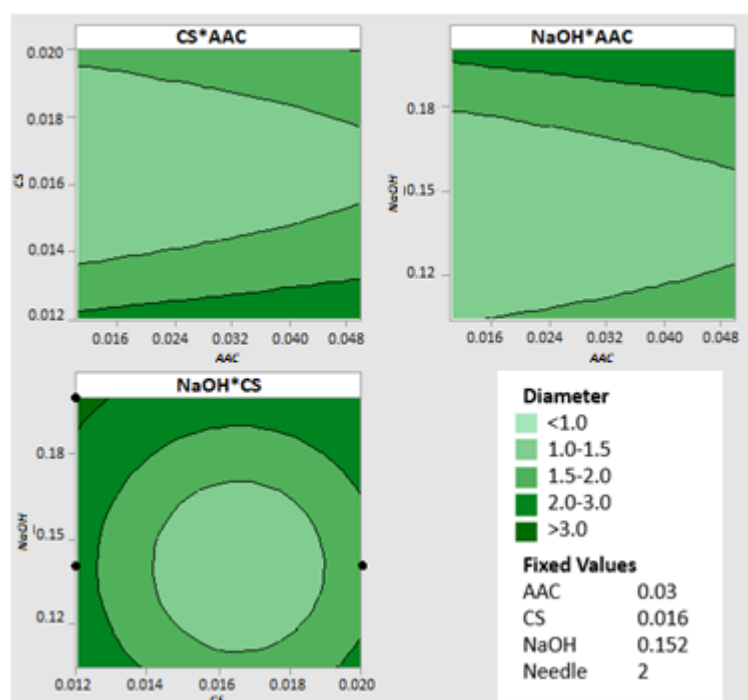


Figure S3. Diameter contour plot for CS spheres, Conditions: Needle 2, R.H. 52%±0.1.

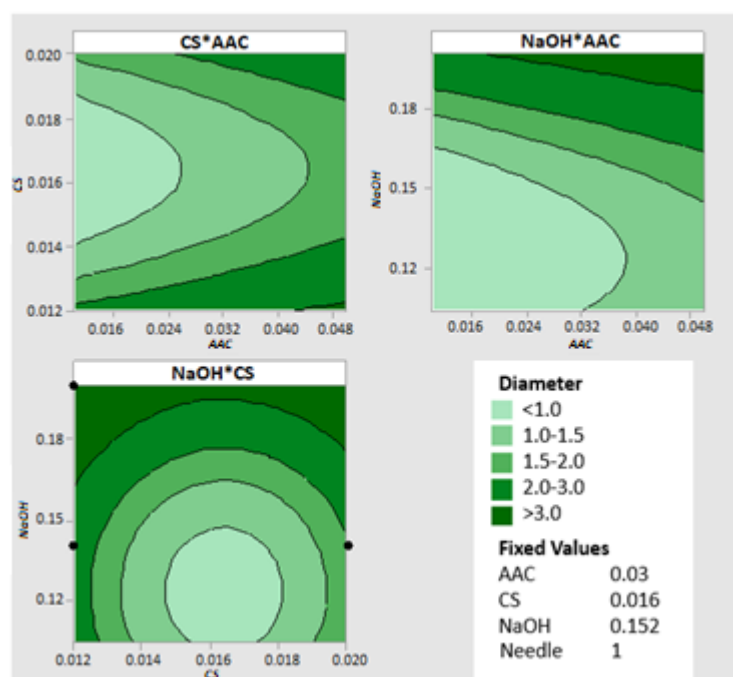


Figure 4. Diameter contour plot for CS spheres, Conditions: Needle 1, Room R.H.

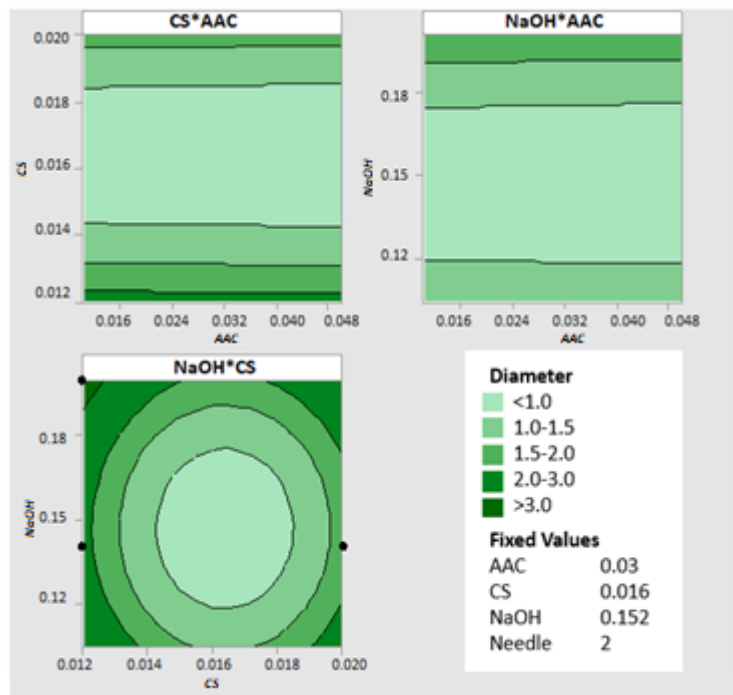


Figure S5. Diameter contour plot for CS spheres, Conditions: Needle 2, Room R.H.

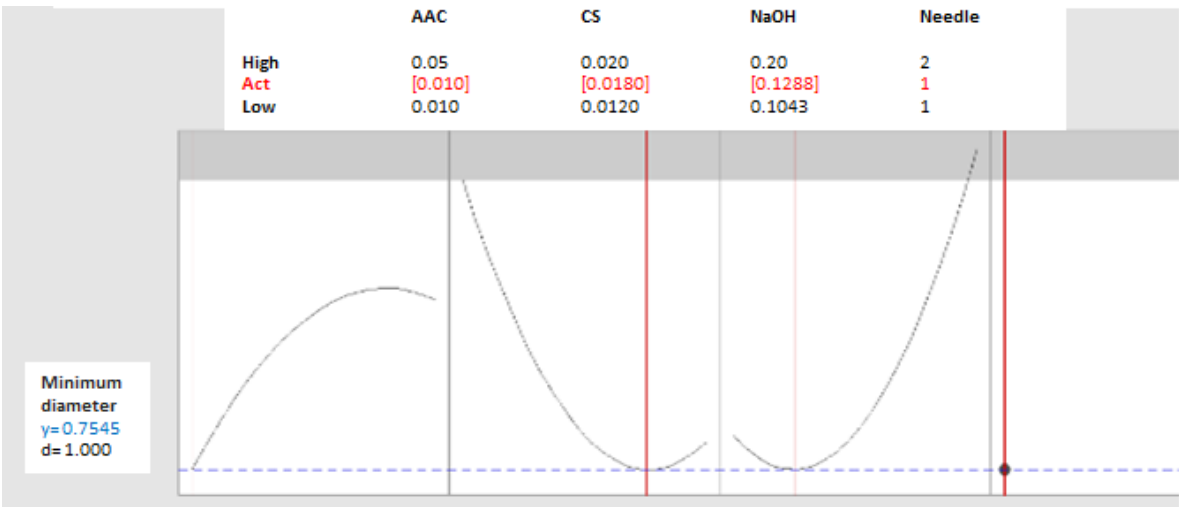


Figure S6. Optimization chart, Conditions: Needle 1, R.H. 10%±0.1%.

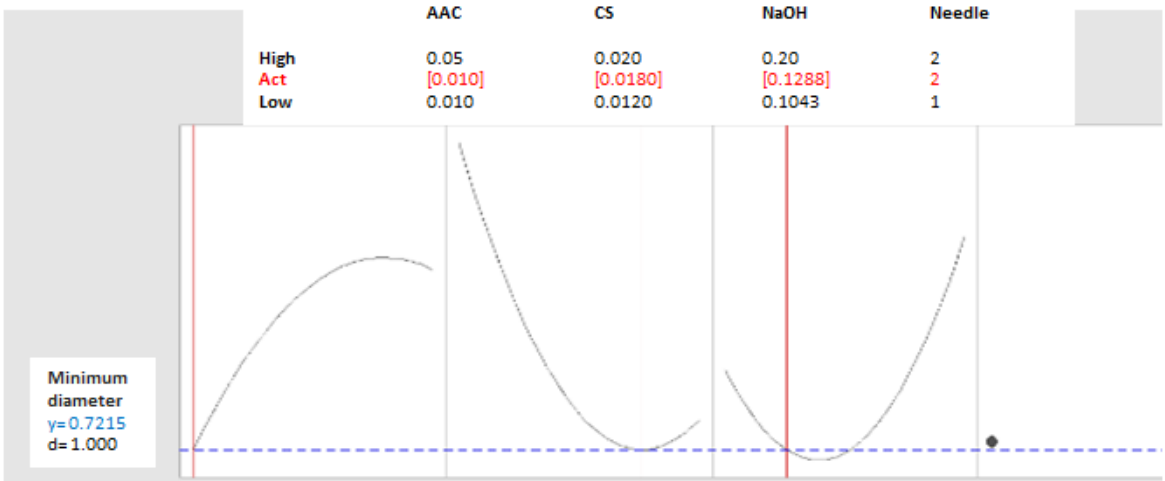


Figure S7. Optimization plot, Conditions: Needle 2, R.H. 10%±0.1%.

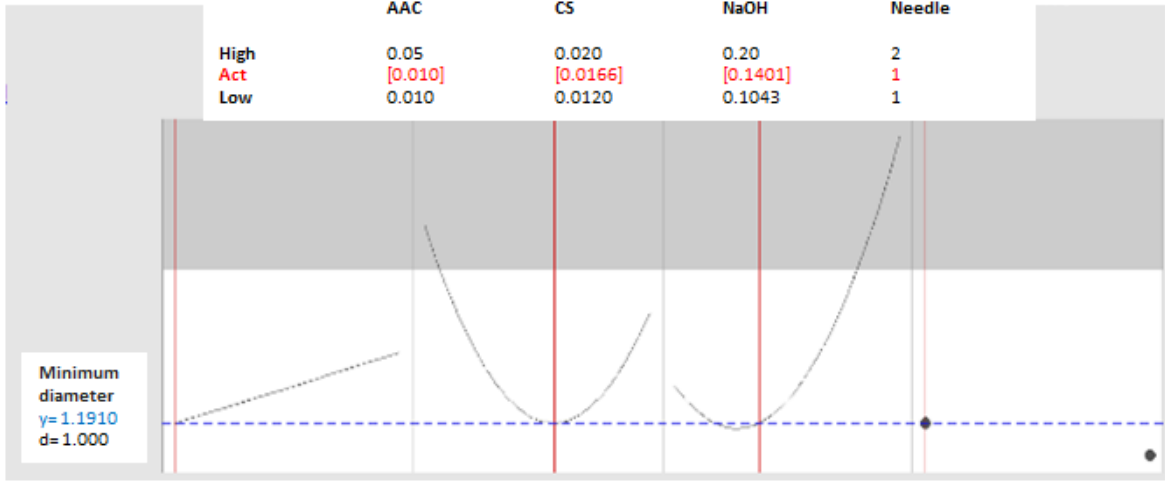


Figure S8. Optimization plot, Conditions: Needle 1, R.H. 52%±0.1%.

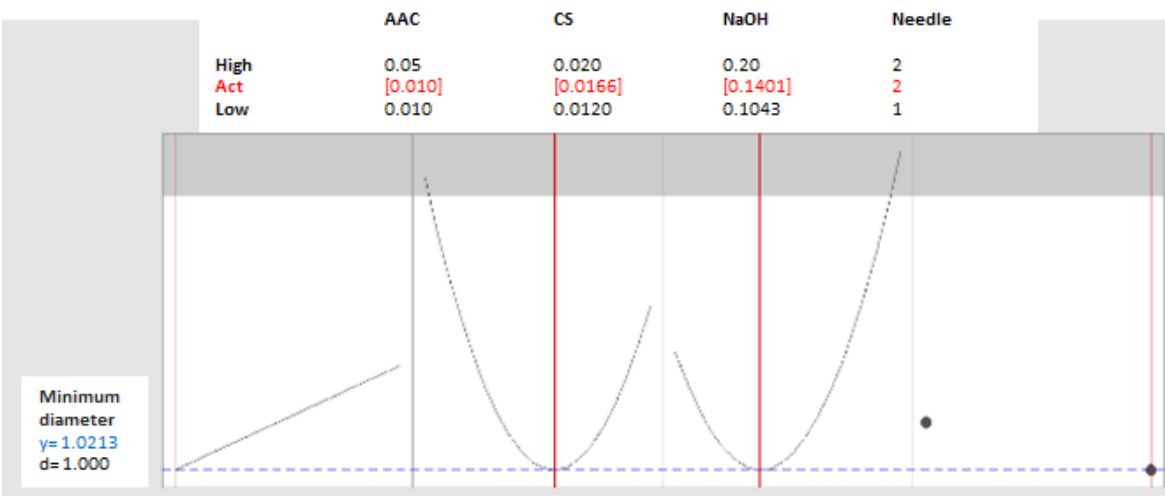


Figure S9. Optimization plot, Conditions: Needle 2, R.H. 52%±0.1%.

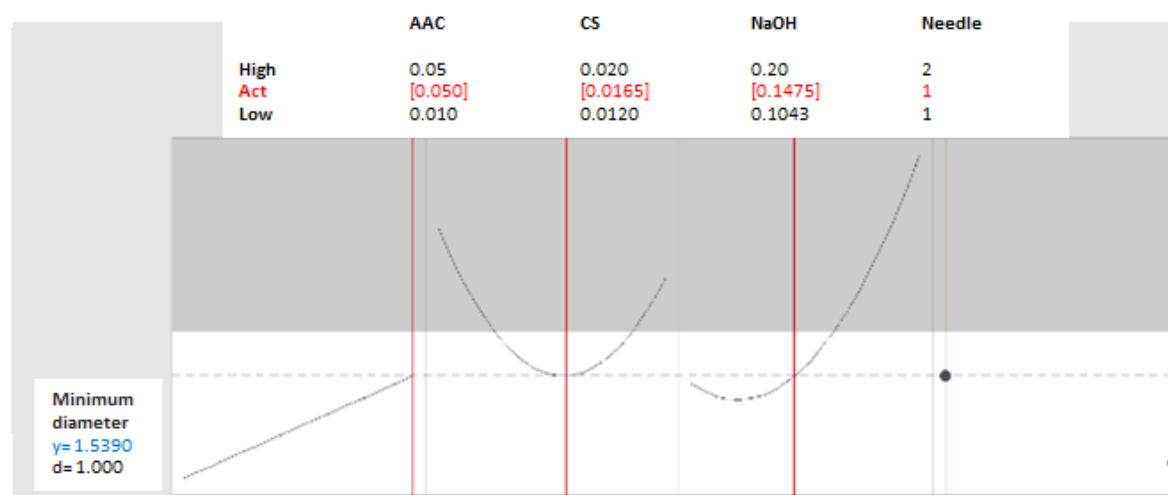


Figure S10. Optimization plot, Conditions: Needle 1, Room R.H.

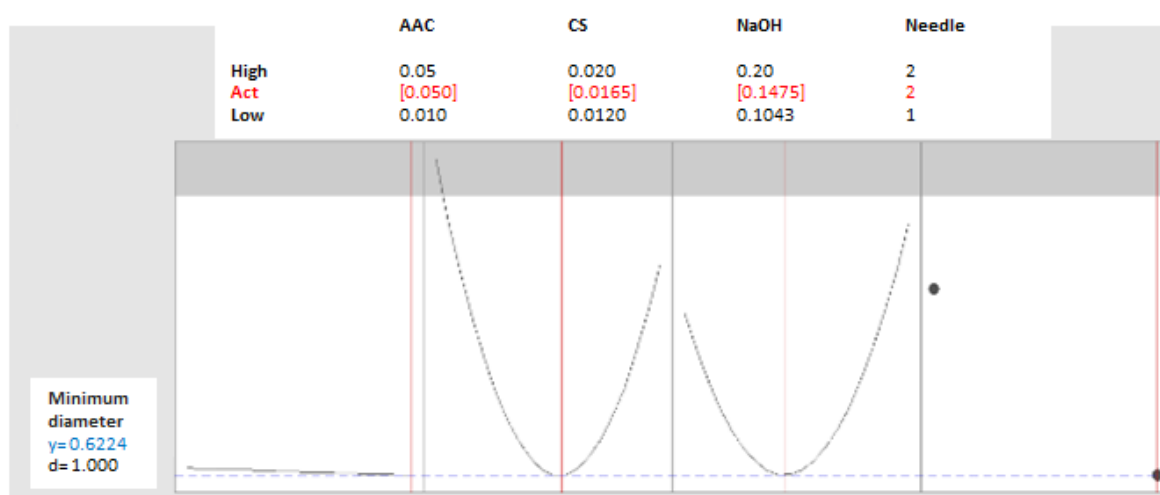


Figure S11. Optimization plot, Conditions: Needle 2, Room R.H.

Proton nuclear magnetic resonance (^1H -NMR)

Based on the chemical structure of chitosan (Figure S13) and ^1H -NMR spectrum for CS sheets and spheres (Figure S14 and S15), it could be observed peaks at 2.08 and 1.59 ppm, corresponding to the protons 3 of the methyl group of N-acetyl glucosamine. The 3.2 and 3.23 ppm peaks present in the spectrum of CS sheets and spheres, correspond to proton number 2 of D-glucosamine, that is, the deacetylated unit. The range between 3.5 and 4 ppm for both spectrums corresponds to protons 2-6. Finally, the peak at 4.60 and 4.1 ppm for CS sheets and spheres, corresponds to proton number 1 of D-glucosamine and the peak at 4.88 and 4.39 ppm for CS sheets and spheres corresponds to proton

number 1 N-acetyl glucosamine [16]. Using equation 2 and the results obtained of $^1\text{H-NMR}$, for CS sheets, the DDA was calculated as 75.01%, while for CS spheres DDA corresponds to 60.78%.

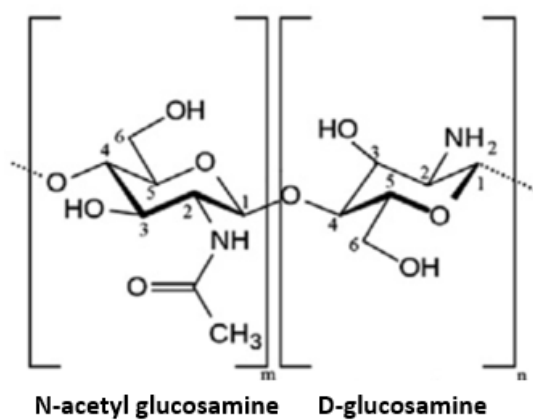


Figure S12. Chemical chitosan structure [34].

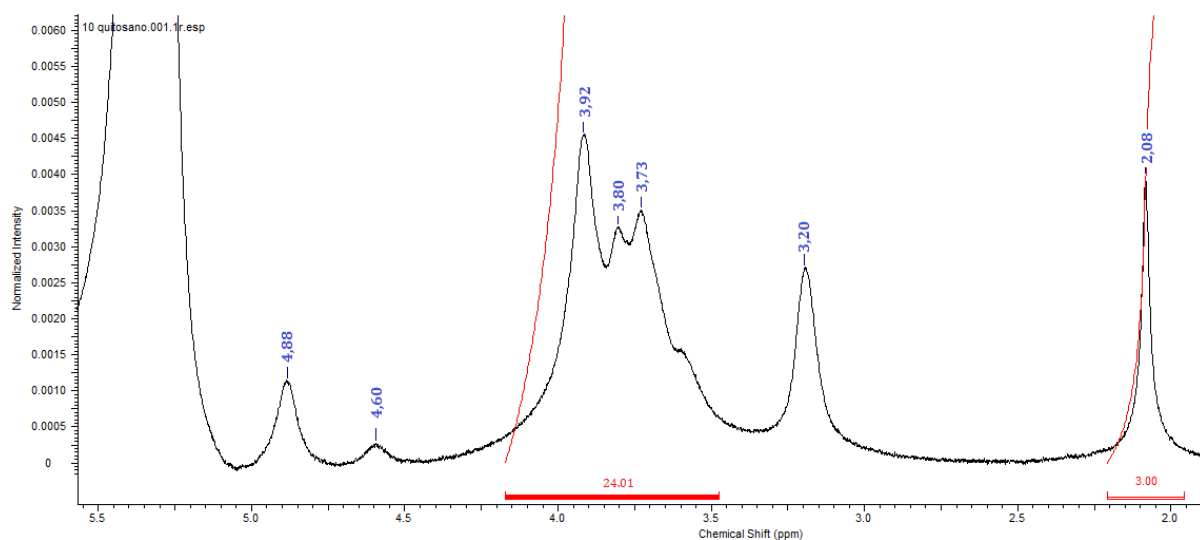


Figure S13. $^1\text{H-NMR}$ spectrum for CS sheets.

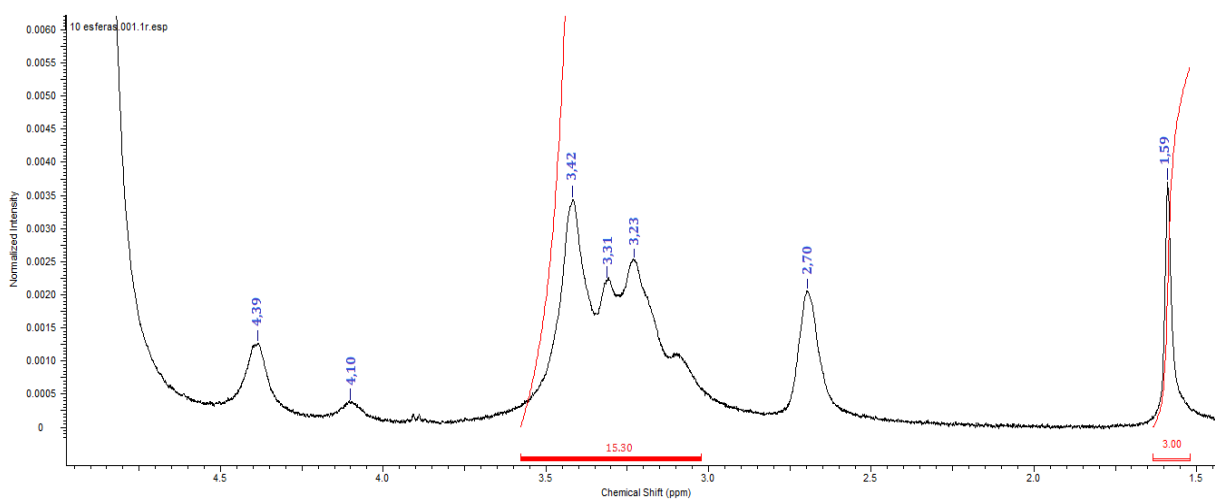


Figure S14. $^1\text{H-NMR}$ spectrum for CS spheres.

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Influence of the chitosan morphology on the properties of acrylic cements and their biocompatibility†

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Acrylic bone cements (ABC) are materials widely used in orthopedics and biomedical applications. Several active compounds have been introduced to ABC formulations to improve their mechanical properties and bifunctionality. In this research, we studied the effect of the addition of chitosan (CS) microspheres and chitosan sheets on ABC formulations. For mechanical performance optimization, the compression strength was taken as a response variable using an extreme vertices mixing design with fraction by weight of CS and poly(methyl methacrylate) (PMMA) as the variable factors. According to the statistical analysis, the control samples (without CS), samples with 7% (wt.) of CS sheets, and samples with 17% (wt.) of CS spheres presented the best compression properties: 90.6 MPa and 95.6 MPa, respectively. The study of these formulations confirmed that CS spheres allow a higher amount of loading on the formulation, maintaining comparable compression strength. By ¹H-NMR, it was observed that the residual monomer was similar in all wording. The hydrolytic degradation assay in simulated body fluid (SBF) determined that the sphere incorporation increased by 50% and 35% for the water uptake and weight loss values, respectively, when compared with the reported values with CS sheets. By morphological analysis *via* SEM, it was observed that the porosity increased considerably in the presence of CS spheres throughout the immersion time in SBF. The subdermal implant results demonstrated excellent compatibility between the cement studied and the biological environment.

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Introduction

Acrylic bone cements (ABC) have been used clinically for many years in orthopaedic surgery for the fixation of artificial joints with promising results.¹ The main function of bone cement is to transfer strains from the prosthesis to the bone, and increase the load-sharing capacity of the prosthesis.² Cements must endure considerable stresses, and thus fulfilling the

standardised mechanical properties are important for its clinical success.³

ABCs used in surgical applications are relatively weak in terms of their tensile strength, but they are resistant to compression.^{4,5} The compression strength values are in the range of 85–114 MPa, which are related to 50–70% of the cortical bone strength.⁶ However, despite the good results of implant fixation with bone cements, they still show some clinical disadvantages such as inert nature, which eventually generates aseptic loss or prosthesis deterioration.⁷ Currently, different approaches are still under investigation to overcome these limitations. Biocompatibility and functionality improvement of cements have been performed by inducing porosity with vacuum mixing devices,^{8,9} or adding bioactive compounds in the curing formulation such as nano-hydroxyapatite-based fillers.¹⁰ The bactericidal capacity was introduced using essential oil-based monomers,¹¹ graphene oxide, graphene,¹² and chitosan (CS) polymers. Chitosan has excellent mucoadhesive, hemocompatibility, biodegradability, and is not toxic. Chitosan also has anti-tumour, antioxidant, and antimicrobial properties, so it has become a very attractive biomaterial for different applications in biomedicine.^{13–15}

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Cement formulations, including CS, demonstrated physical and biological improvements.^{16,17} Several studies on the addition of CS sheets to acrylic bone cement showed a positive impact on the reduction of the maximum curing temperature and biocompatibility, with no significant effects on the tensile properties.⁸

On the other hand, Shi *et al.* investigated the use of CS nanoparticles (CS NP) as bactericidal agents in PMMA bone cement against Gram-positive strains, such as *S. aureus* and *S. epidermidis*, after a prolonged period of time.¹⁸ In addition, Meng *et al.* analysed the effects of resorbable CS microspheres into calcium phosphate cement formulations, improving their mechanical behaviour and inducing a porous morphology on the cements after immersion in simulated biological fluid (SBF) with a healthy *in vivo* recovery process.¹⁹ However, very few formulated cements have reported the effect of the CS morphology in the final properties of the material, like curing parameters, mechanical properties, or *in vitro* responses.

The purpose of the present study was to analyse the effect of adding CS in two different morphologies (sheets and microspheres) on the mechanical, physical and biological properties of ABCs by analysing the residual monomer percentage, the mechanical properties and histological results of ABCs implanted in the subdermal tissue of Wistar rats for 30 days.

Materials and methods

Materials

The solid phase of ABCs was composed of PMMA microparticles acquired from New Stetic S.A. (Medellin, Colombia), barium sulphate (BaSO₄) from Alfa Aesar (Tewksbury, MA, USA), and benzoyl peroxide (BPO). The chitosan sheets were from shrimp shells ($M_w = 190\,000\text{--}310\,000$ and a deacetylation degree of 88%) from Sigma-Aldrich, with CAS number 9012-76-4 and MDL number MFCD00161512 (Palo Alto, CA, USA). CS spheres with

a maximum diameter of 1 mm were obtained from the transformation of chitosan sheets by the coacervation method based on the methodology reported by Dias and Queiroz.²⁰ CS spheres were also used in the preparation of the solid phase.

The liquid phase was composed of methyl methacrylate (MMA), 2-(diethylamino)ethyl acrylate (DEAEA), 2-(diethylamino)ethyl methacrylate (DEAEM) (Sigma-Aldrich, Palo Alto, CA, USA), and *N,N*-dimethyl *p*-toluidine (DMPT) (Merck, Burlington, MA, USA), according to Table 1. All materials were used as received from the supplier, except for BPO, which was recrystallized from methanol.

Mixture design

The composition of experimental formulations used in this study were designed based on the mixing multi-component methodology (extreme vertices) previously reported for composites mixtures.²¹ In this case, the effect of the CS morphology added to the bone cement was studied, including two designs of mixtures of independent extreme vertices for each morphology. The following components were used for sheet design: PMMA and CS, with a process variable identified as size; and for the design of spheres, the components PMMA and CS without process variable.

Response optimization

The intervals for the experimental region of each model are given in Table 1 with compression strength as the response variable for both experimental designs.

Experimental components were used in a regression model with linear effects and significant interaction, which is represented by eqn (1):²²

$$y = \beta_0 + \beta_1x_1 + \beta_2x_2 + \beta_3x_1x_2 + \varepsilon \quad (1)$$

where Y is the response variable (compression strength), and e_i is the error in the u -exempt test. The parameter β_i represents the effect of the i -exempt pure component, β_{ij} is the impact of the interaction between components i and j , and β_{ijk} is the implication of the triple interaction components i , j , and k . The e_i errors are assumed to be independent and identically distributed usually with mean $\mu = 0$ and variance σ^2 . The parameters β_i , β_{ij} , β_{ijk} can be estimated by least-squares.

Tables S1 and S2 (ESI†) present the different proportions of each component obtained under the design of extreme vertices mixtures for sheets and spheres, respectively.

The design experiment optimization included the analysis of each optimization graph thrown by the MINITAB 18 program in the final vertices experiment designs for sheets and spheres.

The conditions for the compression strength were as follows: lower limit = 44 MPa, target = 90 MPa and upper limit = 100 MPa for the sheets design, and lower limit = 85 MPa, target = 95 MPa, and upper limit = 100 MPa for the sphere design.

Bone cement preparation

Each component proportion was considered in the designs of the sheet and sphere experiments for the acrylic bone cement

Table 1 Formulation of acrylic bone cement

Solid phase	Amount (wt%)
PMMA	88–68
BaSO ₄	10
BPO	2
CS ^a	0, 7.64 or 17 ^a
Liquid phase	Amount (wt%)
MMA	97.5
(Co-monomer 1) 2-(diethylamine)ethyl methacrylate	1.25
(Co-monomer 2) 2-(diethylamine)ethyl acrylate	1.25

^a CS was added to the solid phase as sheets (7.64 wt%) or spheres (17 wt%). Control samples without CS (0 wt%) were used as the reference group.

preparation. The cement preparation consisted of a solid–liquid phase ratio of 2 : 1 (Table 1). Both solid phase and liquid phase were weighted separately and manually mixed according to ISO 5833.²³

Physicochemical characterization of bone cement

Particle morphology and surface microstructure. The morphology of the CS sheets and spheres, and the bone cement surfaces were studied by scanning electron microscopy (SEM, JEOL JSM-6490LA, Musashino, Tokyo, Japan). Samples were gold sputtered before examination under an acceleration voltage of 20 kV.

Thermal characterization

Thermogravimetric analysis (TGA, TA Instruments Q50, United States) technique was used to study the thermal degradation of the control and experimental bone cements. The heating ramp used was 20 °C min^{−1} from room temperature to 550 °C under N₂ atmosphere.

Mechanical characterization

Mechanical compression and flexion tests were performed following ISO 5833 for acrylic bone cement.²³ The samples were prepared by compression moulding with a hydraulic press (Carver 4389, United States) of 1000 psi at room temperature, and the Teflon mould dimensions were defined by the standard.

Compression test. According to ISO 5833, 5 cylindrical specimens of each formulation of ABCs were tested 24 hours after forming. The test was performed with a universal testing machine (Tinius Olsen-H50KS, USA) at a head speed of 20 mm min^{−1} with a load cell of 50 kN.

Flexural test. According to ISO 5833, 5 rectangular specimens of each formulation of ABCs were tested 24 hours after preparation. The four-point flexion test was performed with a universal testing machine (Tinius Olsen-H50KS, USA) at a 5 mm min^{−1} head speed with a cell load of 10 kN, and a distance between internal supports of 20 mm and external supports of 60 mm. The bending module and strength values were calculated according to the international standard.²³

Hydrolytic degradation assay

ABC immersion for two months in simulated body fluid (SBF) prepared according to the Kokubo and Takadama method was used to evaluate hydrolytic degradation.²⁴ SBF was stored in plastic bottles at a temperature between 5 and 10 °C, and used within less than one month. During immersion, the pH of the solutions, absorption, and weight loss of the samples were analysed.

According to ASTM F 1635, 5 samples of each formulation of ABCs were incubated at 37 °C for 24 hours before SBF immersion, and stored in an incubator (Mettmert IN 110, Germany) at 37 °C. The buffer was replaced every week, except for the pH variation determination tests. Absorption and weight loss were calculated from water uptake (eqn (2)) and weight loss (eqn (3)), respectively:

$$\text{Water uptake (\%)} = \left(\frac{W_h - W_s}{W_s} \right) \times 100 \quad (2)$$

$$\text{Weight loss (\%)} = \left(\frac{W_s - W_i}{W_i} \right) \times 100 \times (-1) \quad (3)$$

where W_h is the wet weight of the sample, W_s is the dry weight of the sample after SBF immersion, and W_i is the initial weight of the sample.

In vivo biomodel assay

An *in vivo* biomodel assay was performed to study the compatibility between the cements and the biomodel tissues. CS cement samples with different morphologies (three replicates per formulation) and a BioMend® commercial porcine collagen as a control sample (ZIMMER BIOMET, Miami, FL, USA) were implanted. 3 samples of each formulation of ABCs of 10 mm diameter and 2 mm thickness were implanted in the subdermal tissue of three adult Wistar rats. The preparations consisted of a dorsal incision in accordance with the ISO 10993-6 standard.²⁵

After 30 days of implantation, the samples were fixed in buffered formalin, dehydrated in ascending concentration alcohol solutions (70%, 80%, 95% and 100%), diaphanized with xylol and infiltrated with paraffin. After that, the samples were cut at 4 µm using a Thermo Scientific™ Histoplast Paraffin™ and an Autotechnicon Tissue Processor™ (Leica Microsystems, Mannheim, Germany).

Hematoxylin and eosin technique and Masson's trichromacy technique were used for histological analysis of the samples. Image analysis was performed with a Leica DM 750 microscope containing a Leica DFC 295 camera, and Leica Application Suite version 4.12.0 (Leica Microsystems, Mannheim, Germany) was used. The macroscopic images were taken with a Samsung Dv150f Used 16 mp digital camera. For SEM interpretation, samples were dehydrated in ascending alcohol concentration (70%, 80%, 95% and 100%). Finally, to add conductivity, they were coated in a copper bath and analysed with a scanning electron microscope (JEOL JSM-6490LA, Musashino, Tokyo, Japan).

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of the Faculty of Medical Sciences of the Universidad del Valle, and approved by the Animal Ethics Committee of the Universidad del Valle by means of the CEAS 001-016 certificate.

Results and discussion

Optimization of the loading amount of PMMA and CS was necessary for each experimental design model

In the optimization charts (Tables S1 and S2,† notes), the best combination of input variables (X1: PMMA, X2: CS) was assessed, and an additional process variable was selected for the case of sheet design (size 1). Initially, the ratio of PMMA and CS was evaluated to maximize the compress strength, corresponding to 88% PMMA and 0% CS, which resulted in a strength of 107 MPa for both designs (Fig. S1, S2 and S3†).

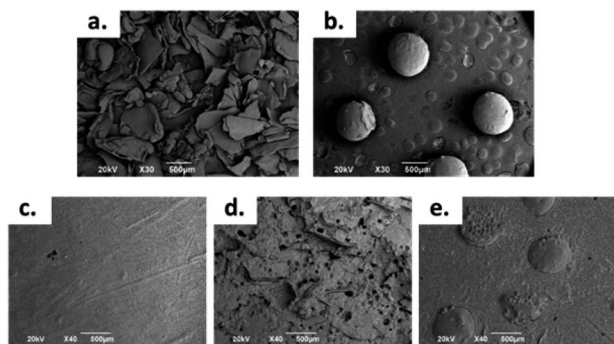


Fig. 1 SEM images at 30 $\times$: (a) CS Sheets, (b) CS spheres, and bone cement images at 40 $\times$: (c) control sample, (d) sample with 7 wt% of CS sheets, (e) sample with 17 wt% of CS spheres.

Optimization charts offer the ability to modify factor values conveniently. In the sheet design, by increasing the CS content to 0.0764 with a fraction of 0.8036 PMMA, the model predicted a compression strength of 90.60 MPa with the predictability of D-1 (Fig. S3, ESI file†). On the other hand, in the case of the sphere design, the increase in CS content to 0.17 CS and a fraction of 0.71 PMMA predicted a compression strength of 95.65 MPa with the predictability of D-1 (Fig. S4, ESI file†). The compression strength values obtained are higher than the minimum required by ISO, which is 70 MPa.²³

Chitosan itself has low mechanical strength, so when added to an acrylic matrix, mechanical strength will inevitably be compromised.²⁶

However, when working with chitosan sheets, the mechanical behaviour is drastically affected due to the angular morphology of the particles. In addition, these have a smaller particle size than CS spheres (Fig. 1a and b). Thus, it is possible for the CS sheets to disperse in a more homogeneous way, so there will be more points acting as a stress concentrator.^{17,27} When the CS spheres have a regular morphology and a particle size that is greater than 1 mm, it is possible to increase the amount of CS load without drastically compromising the mechanical behaviour with these characteristics. However, there is a maximum amount of CS spheres to add. If too many spheres are added, the matrix could be split apart by the presence of spheres, decreasing the mechanical properties.¹⁹

Therefore, the formulations for working were the control (without CS), a sample with 7 wt% of CS sheets and a sample with 17 wt% of CS spheres.

Morphology studies

The morphology of the CS sheets, CS spheres and bone cements are shown in Fig. 1. The irregular and angular shape of the CS sheets (Fig. 1a) and the regular shape of the spheres (Fig. 1b) can be appreciated. Introduction of CS into the cement bulk increased the porosity of the PMMA matrix, especially for the CS sheets (Fig. 1d) with an average pore size of 70–80 μm . Cement with CS sheets presented a homogeneous distribution (Fig. 1c), when analysed by SEM. The cement with CS spheres also showed good dispersion inside the PMMA matrix, together with small gaps around the spheres (Fig. 1d). According to Tan *et al.*,

the CS particles have a weak interaction capacity with the PMMA cement matrix, supported by hydrophobic physic interactions. For this reason, gaps can be formed between the CS particle and the polymer matrix, favouring the porosity of cement during the setting process.¹⁶

Thermal characterization

Fig. 2a shows the TGA curves for the control samples (without CS), samples formulated with sheets, and samples formulated with CS spheres. There was a weight loss of 5% around 250 $^{\circ}\text{C}$. This weight loss corresponds to the desorption of physically adsorbed water on the sample surfaces.²⁸ After this temperature, the weight loss curves become increasingly pronounced up to a temperature of 450 $^{\circ}\text{C}$ for samples with CS sheets and spheres, and at 470 $^{\circ}\text{C}$ for the control sample. Therefore, it can be said that CS decreases the thermal stability of the bone cements. Other authors have reported similar thermal behaviour by adding chitosan to ABCs.¹⁷

Besides, according to the DTGA curves (Fig. 2b), the presence of CS in the bulk induces a decrease in temperature for the maximum rate of decomposition (T_{max}) from 418 $^{\circ}\text{C}$ for the control cement to 397 $^{\circ}\text{C}$ and 399 $^{\circ}\text{C}$ for the CS sheets and CS spheres, respectively. Previous studies indicate similar behaviour relative to the control sample.²⁹

This thermal behaviour can be explained by the fact that PMMA loses stability at high temperatures of around 330 $^{\circ}\text{C}$,

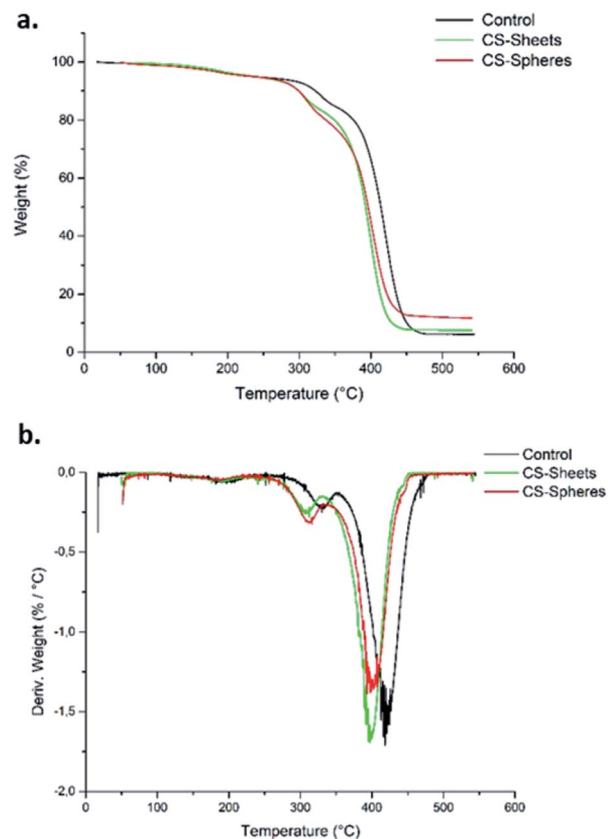


Fig. 2 Thermal analysis recorded for bone cements. (a) TGA curves, (b) DTGA curves.

Table 2 Compression and flexural modulus values $\pm$ standard deviation obtained for experimental bone cements. Control (sample without CS), (sheets) sample with 7 wt% of CS sheets, (spheres) sample with 17 wt% of CS spheres

Sample	Compressive strength (MPa)	Flexural strength (MPa)	Flexural modulus (MPa)
Control	108.68 $\pm$ 1.1	50.97 $\pm$ 2.1	2422.83 $\pm$ 215.9
Sheets	94.08 $\pm$ 1.8	34.25 $\pm$ 0.76	2113.76 $\pm$ 375.5
Spheres	93.32 $\pm$ 1.9	26.34 $\pm$ 0.72	2068.23 $\pm$ 447.1

which leads to depolymerization by chain scission at the same temperature range where CS decomposes. This produces volatile compounds, and thus accelerates the bulk decomposition and affects the thermal stability.^{28,30}

Mechanical characterization

The mechanical properties of bone cement were evaluated using compression and bending strength measurements.

Compressive test. The results of the compressive strength of experimental bone cement are shown in Table 2. The values obtained from the compression strength are in agreement of ISO 5833 standard (>70 MPa).²³ The compressive strengths obtained were 108.68 $\pm$ 1.11 MPa, 94.08 $\pm$ 1.78 MPa and 93.32 $\pm$ 1.93 MPa for the control, sample with CS sheets and sample with spheres, respectively. By adding 17 wt% of CS spheres, a strengthening in compression behaviour was obtained for cements similar to that obtained by adding 7 wt% of CS sheets. This behavior can be explained because the CS spheres have a regular morphology unlike CS sheets, which are more irregular. Therefore, CS sheets reduce the mechanical properties because the edged flake shape facilitates the propagation of cracking with a cumulative effect of the effort.¹⁷

Flexural strength test. Flexural strength values recorded for experimental bone cements are shown in Table 2. Samples containing CS did not reach the minimum value required by the ISO 5833 (>50 MPa). However, the recorded flexion module did meet the requirements (>1800 MPa).²³ It was observed that the presence of CS in the cement formulation resulted in

a reduction of 47% in the flexural strength when incorporated in the form of spheres, and almost 31% when included in the form of sheets. A similar behavior occurs when analyzing values obtained from the flexural module. The results between the cements demonstrated that the differences in the flexural strength are low, as compared to the differences in the CS content (7 wt% for CS sheets and 17 wt% CS spheres, respectively).

According to the mechanical characterization, the addition of a higher amount of CS spheres is possible without affecting the mechanical properties.

Hydrolytic degradation evaluation

A water absorption and hydrolytic degradation test was performed to determine the behaviour of experimental cements in SBF, which contained ion concentrations almost equal to those of human blood plasma.²⁴ Fig. 3 shows the water uptake capacity registered for the control and CS containing cements over time. Cements with CS spheres showed a higher absorption capacity than CS sheet samples, and CS sheets-based cements were likewise higher than the control group. This trend can be explained due to the presence of CS increasing the hydrophilicity of the bulk and partial solubility in SBF, facilitating the formation of interconnected pores and the entry of SBF.⁹ Samples with CS spheres have a higher absorption capacity because their CS weight content is higher (17%), with a superior contact surface than the content of samples containing sheets (7%). Absorption values found at week eight of

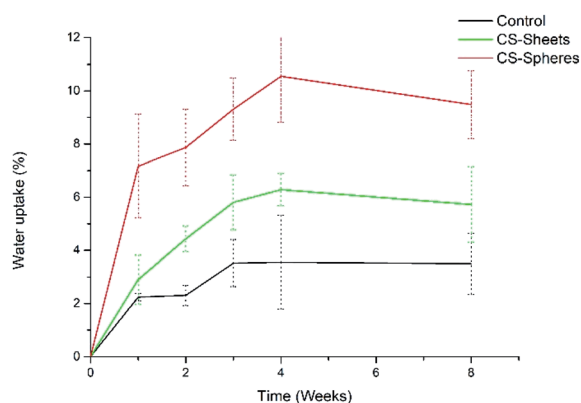


Fig. 3 Water absorption for experimental bone cements: control, cements with 7 wt% of CS sheets, and cements with 17 wt% of CS spheres. Collection time-points are represented by dots and standard deviation.

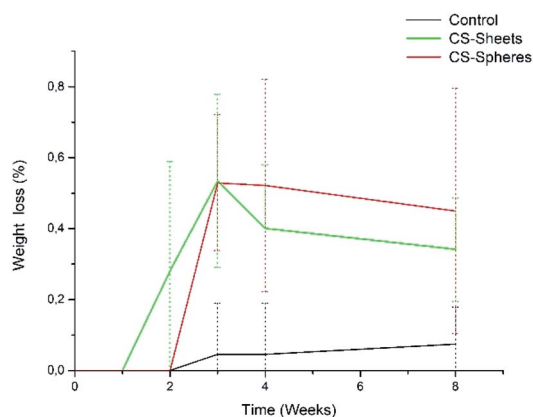


Fig. 4 Weight loss percentage for experimental bone cements: (standard) control sample (without CS), (sheets) sample cements with 7 wt% of CS sheets, and (spheres) samples with 17 wt% of CS spheres. Collection time-points are represented by dots and standard deviation.

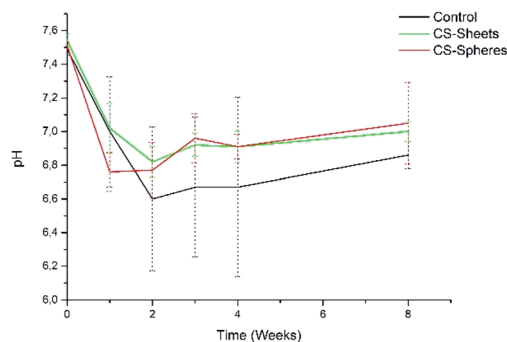


Fig. 5 pH change of bone cements after eight weeks of introduction in SBF (control), control sample (without CS), (sheets) sample with 7 wt% of CS sheets, (spheres) sample with 17 wt% of CS spheres.

immersion were 4%, 6%, and 9% for the control samples, sheets and spheres, respectively, in accordance to other authors reporting similar water absorption values for conventional acrylic cement.¹⁷

Analogously, Meng *et al.* reported new cements containing CS microspheres showing an increased internal macro-porosity, leading to a higher degradability of cements.¹⁹ Fig. 4 shows the behavior of experimental cements, where the CS spheres showed a higher weight loss than cements containing CS sheets, followed by the control group. After eight weeks of immersion in SBF, the calculated values of weight loss determined for the experimental cements were: 0.07%, 0.34%, and 0.45% for control samples, CS sheets, and CS spheres, respectively. Therefore, it is expected that a higher water uptake and enhanced degradability of CS-containing cements may facilitate the creation of a network of interconnected pores and macropores, promoting cell growth and nutrient transport,

degradation capacity, and bone formation within the bulk material.^{17,27,31}

In addition, the pH measurements in hydrolytic media showed differences between experimental groups, as observed in Fig. 5. The control group presented a pH drop from 7.4 to 6.6 during the second week. From this time, the value remained relatively stable. In a similar trend, CS-containing cements also showed a drop in the pH value, reaching values of 6.8 and 6.9 for cements prepared with CS sheets and CS spheres, respectively, after two weeks. During this stage, it is expected that chito-oligomers, which are degradation products of chitosan, will be generated, such as 1,4-linked D-glucosamine and partially 1,4-linked N-acetyl-D-glucosamine. Studies reported that the degradation products of chitosan have significant potential due their wide bioactivity, and antibacterial and antifungal activity.^{32,33} After this time, the pH values of these three groups were slightly raised up to values of 6.7, 6.9, and 7.0, for the control, CS sheets and CS spheres cements, respectively, according to ASTM F1635 and other authors for *in vivo* implantations.^{34,35}

Morphological analysis of bone cement after hydrolytic degradation. Fig. 6 shows the SEM images of experimental bone cement after SBF immersion during one, four, and eight weeks

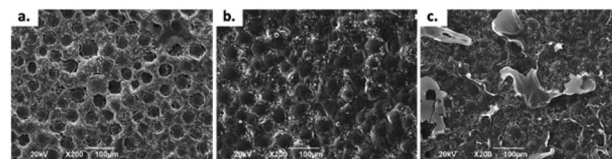


Fig. 7 Micrographs (200×) of the surface of bone cement implanted in the subdermal region of Wistar rats after 30 days of implantation: (a) control group, (b) cement with chitosan spheres, and (c) cement with chitosan sheets.

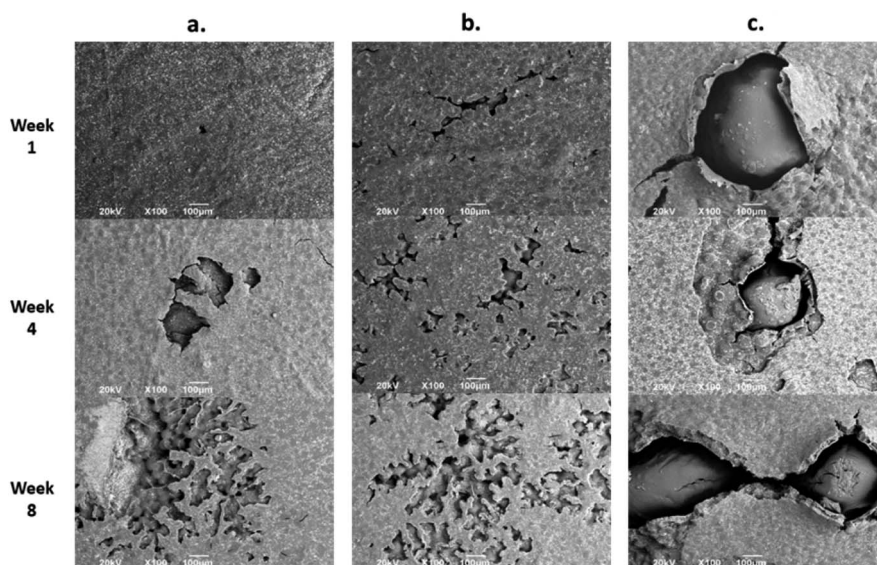


Fig. 6 SEM micrographs at 100× of experimental bone cements immersed in SBF for 1, 4, and 8 weeks. (a) Control (without CS), (b) sample with 7 wt% of CS sheets. (c) Sample with 17 wt% of CS spheres.

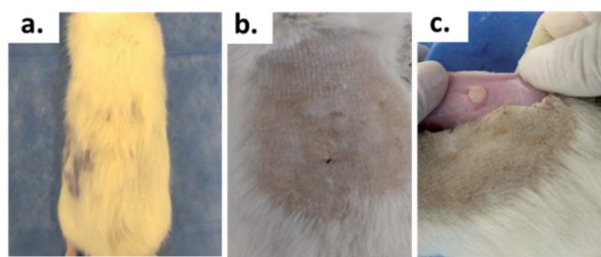


Fig. 8 Biomodel ABC implantation: (a) with hair, (b) after the shaving process, (c) encapsulated area.

of immersion. The SEM morphology of bone cement confirmed the results reported in other investigations,^{17,27} in which the incorporation of CS accelerated degradation and increased porosity from the surface to the bulk. It has been reported that this type of behavior facilitates bone integration and tissue regeneration.^{19,31}

In vivo biological studies

Fig. 7 shows the SEM images of the experimental bone cement implanted in subdermal tissues for 30 days. For CS-containing surfaces, it can be observed that the chitosan spheres were embedded (Fig. 8b), and the surface was rougher. This would facilitate tissue colonization once implanted in the spinal canal of the femur bone.

After 30 days of subdermal implantation, the implanted areas showed a normal appearance: during the macroscopic examination, the formation of new hair and the absence of scars and fistulous tracts were observed in the field of the incisions. On the other hand, after sample recovery, it was appreciated in the inner area of the skin that the material was encapsulated without allergic reaction or pus formation (complete healthy recover).

Fig. 9 shows the microscopic images of the implanted area with a soft tissue fibrous capsule surrounding the material. Collagen discs of 5 mm diameter and 2 mm thickness were

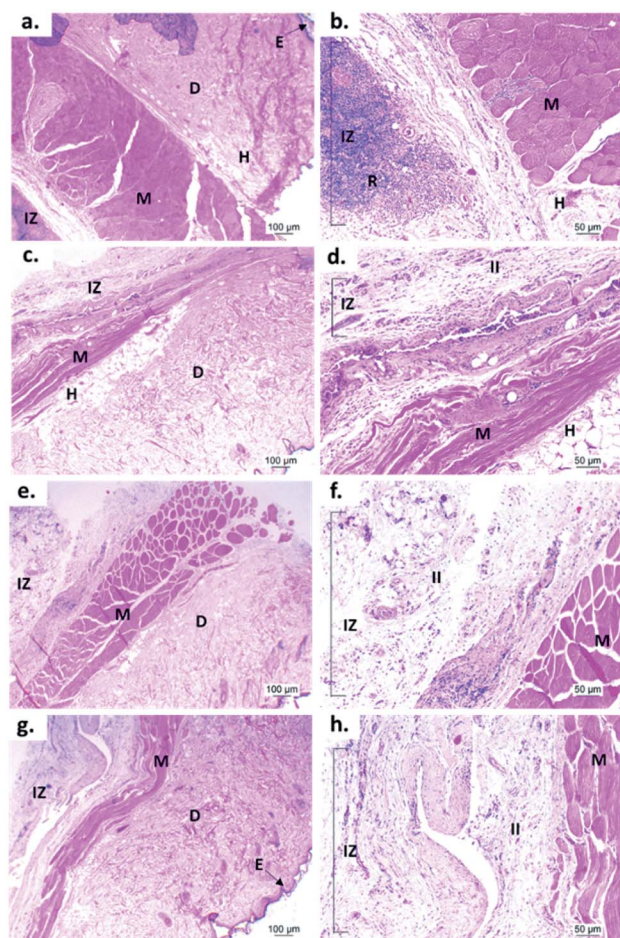


Fig. 9 Hematoxylin and eosin (H&E) staining images of samples implanted for 30 days in the subdermal tissue of Wistar rats: (a) and (b) collagen control; (c) and (d) control cement; (e) and (f) cement with CS spheres; (g) and (h) cement with CS sheets. (a), (c), (e) and (g) 4× images; (b), (d), (f) and (h) 10× images. E: epidermis. D: dermis. M: muscle. H: hypodermis. IZ: implanted zone. FC: fibrous capsule. II: inflammatory infiltrate.

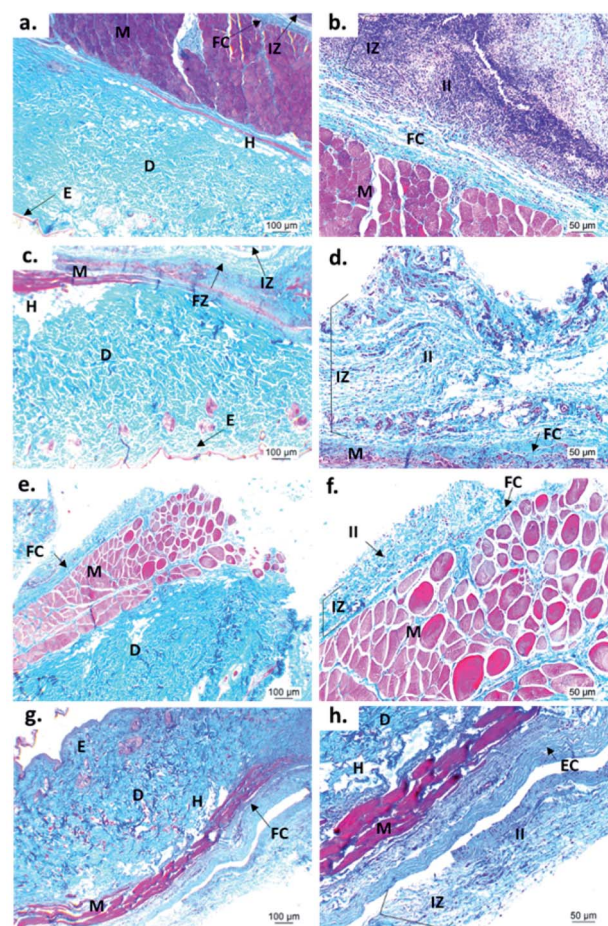


Fig. 10 Images of Masson's trichromacy staining of cement samples implanted for 30 days in the subdermal tissue of Wistar rats: (a) and (b) collagen control; (c) and (d) control cement; (e) and (f) cement with CS spheres; (g) and (h) cement with CS sheets. (a), (c), (e) and (g) 4× images; (b), (d), (f) and (h) 10× images. E: epidermis. D: dermis. M: muscle. H: hypodermis. IZ: implanted zone. FC: fibrous capsule. II: inflammatory infiltrate.

inserted as the control samples. Healthy tissue architecture was observed with no remaining material. In addition, the content was not visible and the presence of abundant inflammatory infiltrate (labeled as II in Fig. 9 and 10) was found in the implantation area (labeled as IZ in Fig. 9 and 10). Similarly, the presence of inflammatory infiltrate was observed in the area of the control cement (Fig. 9c and d) and experimental cements (Fig. 9e–h), showing a higher density of inflammatory infiltrate in the area (labeled as IZ in Fig. 9 and 10), corresponding to the control material (collagen, Fig. 10b). These images support the idea that the ABCs-incorporating CS spheres and CS sheets are not negatively affected by CS introduction, and the materials are also degraded.

On the other hand, to confirm the presence of a fibrous capsule around the cements and achieve an understanding of the composition of that fibrous capsule, trichromacy Masson's stain technique was carried out (Fig. 10). In general, it is observed that all materials were surrounded by a fibrous capsule (FC) consisting mainly of type I collagen, which is a normal response due to the body's reaction to the presence of a foreign body.³⁶ Remarkably, no immune responses were observed, demonstrating a biocompatibility of the cements in the subdermal tissues. This observation demonstrates that the introduction of CS spheres or CS sheets does not negatively affect the compatibility of cements, while the reabsorption process is like the collagen control.

Conclusions

Addition of CS sheets and CS spheres demonstrated that changes in the morphology induced changes in the cement properties. Addition of higher amounts of CS spheres (17 wt%) is comparable to the addition of CS sheets (7 wt%) in compressive and flexural modulus, which could be explained for a superior contact surface for spheres. On the other hand, morphology studies demonstrated that CS sheet incorporation introduced more porosity to the cements than CS spheres. This is probably due to a better distribution of the lower size of CS spheres particles into the cement. For the hydrolytic degradation, CS introduction promoted the water absorption and degradation. Cements including CS spheres presented higher water absorption and weight loss, indicating a faster degradation process due to a bigger CS loading in the cements, as compared to CS sheets. SEM also evidenced cement porosity on the implanted samples. The high porosity degree will facilitate the cell adhesion and colonization in tissue engineering applications. H&E and Masson trichromacy stains demonstrated that cements were protected during the implantations by type I collagen tissue, and indicative of a healthy absorption and healing of the implantation process. All of the results demonstrate that controlling the CS morphology will introduce porosity and positive changes in the reabsorption process without negatively affecting the mechanical and thermal properties of cements.

Conflicts of interest

There are no conflicts of interest to declare.

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Notes

Table S1. Design of mixtures of extreme vertices for CS sheets.

Randomization	Run Order	Type	PMMA		
			(wt. %)	CS (wt. %)	X1:Size
7	1	0	78	10	-1
9	2	1	88	0	1
2	3	1	68	20	-1
10	4	1	68	20	1
5	5	-1	73	15	-1
11	6	0	78	10	1
4	7	-1	83	5	-1
15	8	0	78	10	1
12	9	-1	83	5	1
16	10	0	78	10	1
14	11	0	78	10	1
8	12	0	78	10	-1
3	13	0	78	10	-1
6	14	0	78	10	-1
13	15	-1	0,73	0,15	1
1	16	1	0,88	0	-1

Table S2. Design of mixtures of extreme vertices for CS spheres.

Randomization	Run Order	Type	PMMA	
			(wt.%)	CS (wt. %)
7	1	0	78	10
9	2	1	88	0
2	3	1	68	20
5	4	-1	73	15
4	5	-1	83	5
8	6	0	78	10
3	7	0	78	10
6	8	0	78	10
1	9	1	88	0

Figure S1: Optimization chart (Control-Sheets)

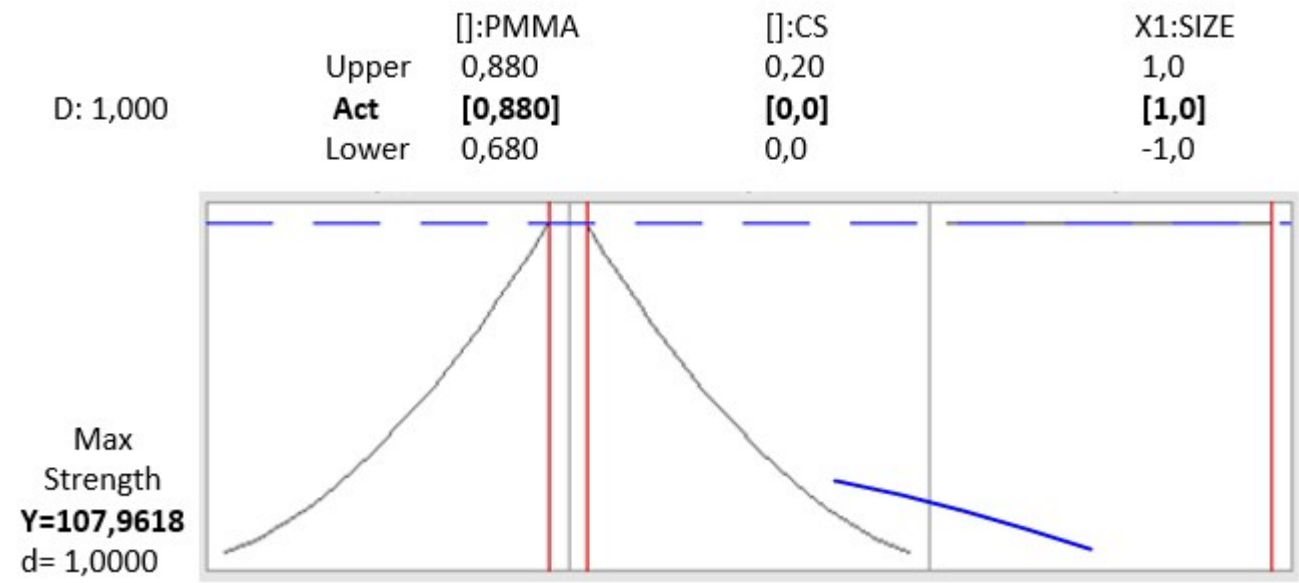


Figure S2: Optimization chart (Control-Spheres).

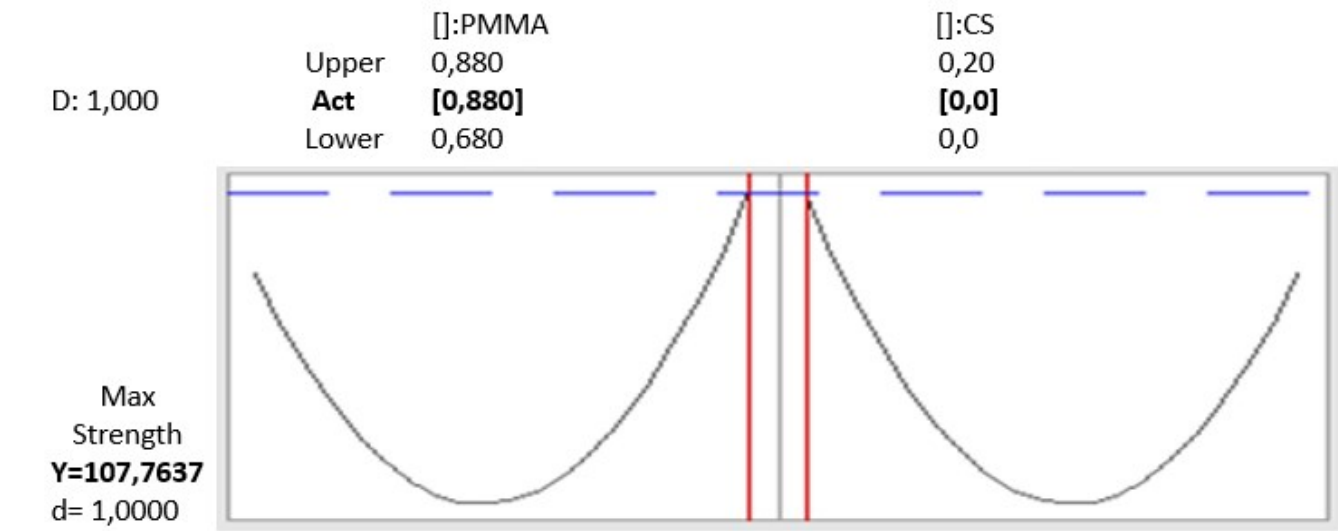


Figure S3: Optimization chart (Sheets design),

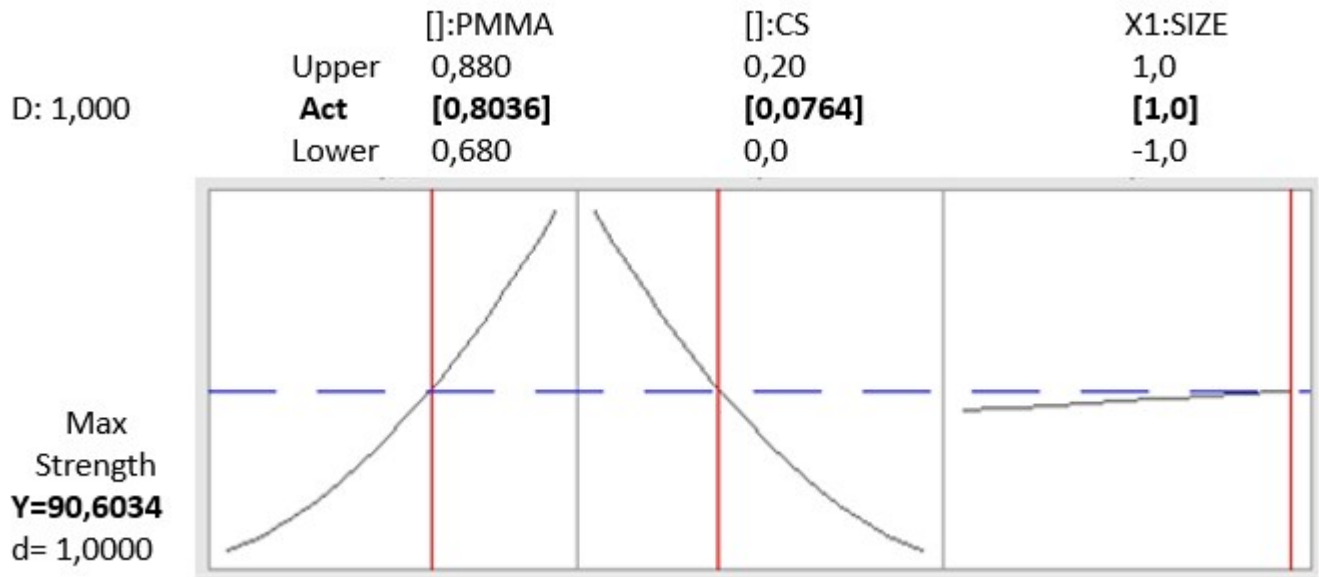
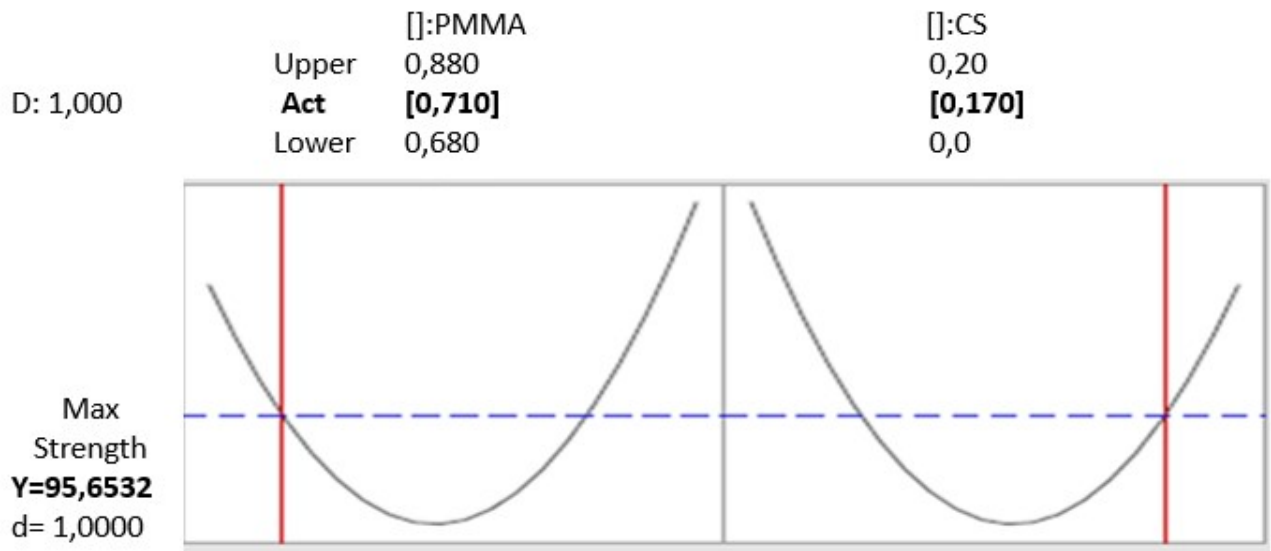


Figure S4: Optimization graphic (Spheres design).



Post-curing residual monomer

Remaining residual monomer (RM) within cured cements after 24

hours were determined by ^1H - nuclear magnetic resonance (^1H -NMR, BRUKER 400 MHz. AVANCE II, United States) as calculated from equation 2. Deuterated chloroform was used as solvent and tetramethylsilane (TMS) as internal reference.

$$MRRM_{MMA}(\%) = \frac{A_{MMA}}{A_{MMA} + A_{PMMA}}(100) \quad \text{Equation 1}$$

Where:

A_{MMA} is the signal area of methoxyl protons of MMA ($\delta=3.7$ ppm).

A_{PMMA} is the signal area of methoxyl protons of PMMA ($\delta=3.5$ ppm).

Determination of residual monomer

RM percentages of bone cement are presented in Table 3 and it can be noted that the presence of CS in cement did not induce changes in the rm percentage showing for the three materials values are within the commonly accepted range between 2-6% for orthopaedic bone cements [17,37].

Table S3. Residual bone cement monomer according to the $^1H - NMR$ spectrum

Sample	Composition (wt. %)	Residual monomer (wt. %)
Control	88% PMMA - 0% CS	2.94
Sheets	80.36% PMMA – 7.64% CS	2.94
Spheres	71%PMMA - 17% CS	3.03

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Article

Acrylic Bone Cement Incorporated with Low Chitosan Loadings

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Abstract: Despite the potential of acrylic bone cement (ABC) loaded with chitosan (CS) for orthopedic applications, there are only a few in vitro studies of this composite with CS loading ≤ 15 wt.% evaluated in bioactivity tests in simulated body fluid (SBF) for duration > 30 days. The purpose of the present work was to address this shortcoming of the literature. In addition to bioactivity, a wide range of cement properties were determined for composites with CS loading ranging from 0 to 20 wt.%. These properties included maximum exotherm temperature (T_{max}), setting time (t_{set}), water contact angle, residual monomer content, flexural strength, bending modulus, glass transition temperature, and water uptake. For cement with CS loading ≥ 15 wt.%, there was an increase in bioactivity, increase in biocompatibility, decrease in T_{max} , increase in t_{set} , all of which are desirable trends, but increase in residual monomer content and decrease in each of the mechanical properties, with each of these trends, were undesirable. Thus, a composite with CS loading of 15 wt.% should be further characterized to explore its suitability for use in low-weight-bearing applications, such as bone void filler and balloon kyphoplasty.

Keywords: acrylic bone cement; bioactivity; biocompatibility; chitosan; poly (methyl methacrylate)

1. Introduction

For years, acrylic bone cement (ABC) has played a pivotal role in orthopedic surgery. ABC is used to anchor total joint replacements (TJR), remodel areas, and in the stabilization and augmentation of osteoporosis-induced vertebral body fractures (vertebroplasty and balloon kyphoplasty) [1]. ABC is biocompatible, and, in a TJR, it provides fast fixation between the bone and the implant. However, ABC has some disadvantages, such as a high maximum exothermic temperature (T_{max}) [2], a lack of bioactivity [3–5], and monomer toxicity [6].

Bioactive materials are those that can stimulate a biological response in their environment. These are classified into three groups: Osteoconductive are materials that stimulate bone growth on the surface; osseointegration are materials that can stimulate the growth of new bone, forming a stable anchorage with the bone, and osteoinductive substances can induce bone formation in parts where the bone does not naturally grow [7,8].

Natural polymers attract attention for use in biomaterials because they possess significant similarity to biological structures, so they are readily accepted by the tissue environment and, therefore, quickly metabolized, obtaining non-toxic by-products easy to eliminate [9].

Chitosan (CS) is a copolymer of glucosamine and N-acetylglucosamine deacetylated derived from chitin [9], the second most abundant natural polysaccharide on earth (found in the shells of crabs, shrimps, and insects, for example) [7]. CS-based materials are ideal bioactive materials due to their biocompatibility, wound-healing ability, non-toxicity, antibacterial properties, anti-fungal properties, hydrophilicity, biodegradability, and ability to stimulate adherence, proliferation, and viability of cells [7,9,10]. Several reports exist on the addition of CS to ABC, resulting in improvement of various properties of ABC, such as increased setting time (t_{set}) [11], reduced maximum exotherm temperature (T_{max}) [6], increased porosity, higher antibacterial action [12], and increased bioactivity [2,5,13–15]. However, high CS loading generates considerable detriment to the mechanical properties of cements, restricting clinical uses to non-load-bearing applications. It is crucial to study lower concentrations of CS to establish an adequate balance between biological and mechanical properties that will allow it to expand its clinical usefulness to a wide range of low-weight-bearing applications such as kyphoplasty, cranioplasty, bone defect fillers, maxillofacial reconstruction, and others [16,17].

The reported bioactivity of cements CS-loaded in a simulated biological fluid (SBF) or buffered phosphate solution (PBS) was only obtained during short test times (<30 days) with cement formulations that have a high CS loading (mass of CS as a proportion of the weight of the solid component of the cement) (>20 wt.%). Information on hydrolytic degradation, bioactivity, and other properties of ABC with CS content ≤ 20 wt.%, under simulated biological fluid conditions after prolonged test times (>30 days), is lacking.

The purpose of the present study was to determine the influence of CS loading on hydrolytic degradation, bioactivity, and several handling, physical, and mechanical properties of ABC loaded with ≤ 20 wt.% CS, with testing in SBF over 28 weeks.

2. Materials and Methods

2.1. Materials

Methyl methacrylate (MMA), 2-(diethylamino) ethyl acrylate (DEAEA), 2-(diethylamino) ethyl methacrylate (DEAEM), benzoyl peroxide (BPO), and chitosan from shrimp shells with a molecular weight between 190–310 KDa and deacetylation degree of 88% were all purchased from Sigma-Aldrich (Sigma-Aldrich, Palo Alto, CA, USA). *N,N*-dimethyl *p*-toluidine (DMPT) was purchased from Merck (Merck, Burlington, MA, USA). Barium sulfate was purchased from Alfa Aesar (BaSO_4) (Alfa Aesar, Tewksbury, MA, USA). PMMA powder beads were purchased from Veracryl (New Stetic SA, Medellin, Colombia).

2.2. Preparation of ABC

ABCs with different CS loadings were prepared. The solid-phase (SP) and liquid phase (LP) were made separately by hand mixing, according to the composition shown in Table 1. LP in all formulations was comprised of 95.5 wt.% MMA, 2.0 wt.% of a 50:50 mixture of the comonomers (DEAEA:DEAEM), and 2.5 wt.% DMPT as the activator of the polymerization reaction. LP was added in SP at a solid/liquid ratio = 2 g/mL and manually mixed. The resulting dough was transferred to Teflon molds to obtain specimens to be used in the different tests.

Table 1. Composition percentage (wt.%) of the solid phase (SP) of the acrylic bone cements (ABCs) formulations.

Formulation	SP			
	PMMA	BaSO ₄	BPO	CS
ABC 0% CS	88	10	2	0
ABC 5% CS	83	10	2	5
ABC 10% CS	78	10	2	10
ABC 15% CS	73	10	2	15
ABC 20% CS	68	10	2	20

2.3. Characterization Physical, Chemical and Thermal of ABC

ABCs specimens were characterized by Fourier transform infrared (FTIR) in attenuated total internal reflectance mode (ATR) in a Spectrum One spectrometer (Perkin-Elmer, Waltham, MA, USA). Thermogravimetric analysis (TGA) was carried out in a TGA Q500 analyzer (TA Instruments, New Castle, DE, USA) in a range of 50–500 °C under nitrogen at a heating rate of 10 °C/min. Differential scanning calorimetry (DSC) was performed in a DSC 8500 (Perkin-Elmer, Waltham, MA, USA) in a range of −20–150 °C under nitrogen at a heating rate of 10 °C/min.

The water contact angle (WCA) was measured at 25 °C using a KSV CAM200 tensiometer (KSV Instruments, Helsinki, Finland). At least 10 separate measurements were taken on each sample.

Proton nuclear magnetic resonance spectra (¹H-NMR) were carried out in a Bruker Avance III HD-400 spectrometer (Bruker, Billerica, MA, USA) at 25 °C. Specimens were dissolved in deuterated chloroform (CDCl₃) one week after being prepared. Residual monomer content (RMC) in a sample was calculated by integrating the signals of the methoxy protons of the MMA and the PMMA using Equation (1).

$$\text{RMC}(\%) = \frac{A_{\text{MMA}}}{A_{\text{MMA}} + A_{\text{PMMA}}} \times 100 \quad (1)$$

where A_{MMA} is the signal area of methoxy protons of MMA ($\delta = 3.7$ ppm), and A_{PMMA} is the signal area of methoxy protons of PMMA ($\delta = 3.5$ ppm).

2.4. Determination of Handling Properties

Handling properties of the cement paste were evaluated following the protocols given in ISO 5833-02 [18]. After the mixing of the ABC components, the cement was deposited in a Teflon mold (diameter and height of 68 and 20 mm, respectively) to record time and temperature data. Temperature against time plot allowed us to calculate the maximum temperature. Setting time (t_{set}) was calculated like time required to reach setting temperature (T_{set}) calculated according to Equation (2). The test was carried out in duplicate.

$$T_{\text{set}} = \frac{T_{\text{max}} + T_{\text{amb}}}{2} \quad (2)$$

where T_{amb} is the recorded ambient temperature, and T_{max} is the highest temperature attained.

2.5. Mechanical Properties

Four-point bending and compression tests were conducted using a universal testing machine Tinius Olsen-H50KS (Tinius Olsen, Redhill, United Kingdom), following the protocols given in ISO 5833-02 [18]. For the former test, specimens measuring 75 mm × 10 mm × 3 mm were tested at a crosshead displacement rate of 5 mm/min. The bending strength (B) and modulus (E) were calculated using Equations (3) and (4), respectively.

$$B = \frac{3Fa}{bh^2} \quad (3)$$

$$E = \frac{\Delta Fa}{4fbh^3} \times (3l^2 - 4a^2) \quad (4)$$

For Equation (3), F is the force at break, b is the average measured width of the specimen, h is the average measured thickness of the sample, and a is the distance between the inner and outer loading points. For Equation (4), ΔF is the load range (50 N – 15 N = 35 N), f is the difference the deflections under loads of 15 and 50 N, and l is the distance between the outer loading points.

For the compression test, cylindrical specimens (diameter and height of 6 and 12 mm, respectively) were tested at a crosshead displacement rate of 20 mm/min. For each of these tests, a minimum of six specimens was tested for each of the cement formulations.

The morphology of the surfaces of compression test specimens, at the end of the test, were observed using an environmental scanning electron microscope (ESEM) Philips XL30 (Philips, Eindhoven, The Netherlands).

The dynamic mechanical analysis was carried out in a DMA Q800 (TA Instruments, New Castle, DE, USA) in Dual Cantilever mode to 1 Hz of frequency in a range from -100 to 200 °C at a heating rate of 3 °C/min. The test samples were prepared with the dimensions of $46 \times 6 \times 4$ mm³. At least three specimens were tested per formulation.

2.6. Assessment in a Simulated Biological Fluid (SBF)

Hydrolytic degradation of cement specimen and bioactivity (formation of an apatite-like layer on cement specimen) were determined using disc specimens (diameter = 6 ± 0.1 mm and a thickness = 2 ± 0.1 mm) and an SBF that was prepared using the methodology presented by Kokubo and Takadama [19], with its ionic concentration being similar to that of human blood plasma. On the other hand, samples to monitor compressive strength had a thickness of 12 ± 0.1 mm.

2.6.1. Hydrolytic Degradation

The protocols used followed those given in ASTM F1635-16 [20]. Cylinders were immersed in 50 mL of SBF to 37 °C in an incubator (Mettler, Schwabach, Germany) for periods between 1 and 28 weeks. At each period, at least three cylinders were evaluated. The solution was replaced with fresh SBF weekly. During the time of evaluation, the pH of the SBF was recorded using an Accumet™ AB150 pH meter (Fisherbrand, Ottawa, ON, Canada). Before immersion in the test solution, the specimen was weighed (W_0). At the end of the test period, the samples were removed from the SBF, washed with distilled water, dried superficially with a dry tissue, and weighed (W_w). They were then dried, in air, at 37 °C, for 72 h and weighed again (W_d). Hence, the weight loss (W_l) and water uptake (W_u) of the specimen were determined by Equations (5) and (6), respectively. Compressive strength was evaluated under the same conditions described in the mechanical tests.

$$W_l (\%) = \frac{W_0 - W_d}{W_0} \times 100 \quad (5)$$

$$W_u (\%) = \frac{W_w - W_d}{W_d} \times 100 \quad (6)$$

2.6.2. Bioactivity in SBF

The morphology of the surface of a specimen used in the hydrolytic degradation test was obtained using a scanning electron microscope (SEM). The attached X-ray energy dispersive spectrometer (EDS) JEOL Model JSM 6490 LV (Akishima, Tokyo, Japan) supported calculation of the amount of apatite-like deposition layer (that is, proportional to the amount of Ca and P ions on the surface).

2.7. Statistical Analysis

Quantitative property results are presented as mean (standard deviation). For each of these properties, the test of significance of the difference between means was performed using the Student t-test with the software Minitab 17 (Minitab, LLC, State College, PA, USA). The difference was considered statistically significant when $p < 0.05$. Statistical analysis was performed comparing ABCs loaded with respect to control ABC.

3. Results

Currently, PMMA-based bone cement is the most widely used type of bone cement in orthopedics [6]. In this research, ABC loaded with CS between 0–20 wt.% in its solid phase were prepared. The influence of CS content on mechanical, thermal, chemical, physical, and biological properties were analyzed.

3.1. FTIR

Figure 1 shows the FTIR spectra of the CS-loaded ABCs. The bands at 1140 and 1720 cm^{-1} corresponds to $\text{O}-\text{CH}_3$ stretching vibration and $\text{C}=\text{O}$ asymmetric stretching vibrations, respectively, corresponding to the PMMA structure [21]. The cement containing CS presented a wide overlapped band corresponding to the amine $\text{N}-\text{H}$ stretching vibration (3365 cm^{-1}) and the $\text{O}-\text{H}$ stretching vibration (3460 cm^{-1}) in the CS. Characteristics bands of PMMA and CS without shifting indicate that the preparation conditions of ABC did not promote a covalent bonding between the chitosan and the cement. Therefore, the interaction between them was only governed by physical forces.

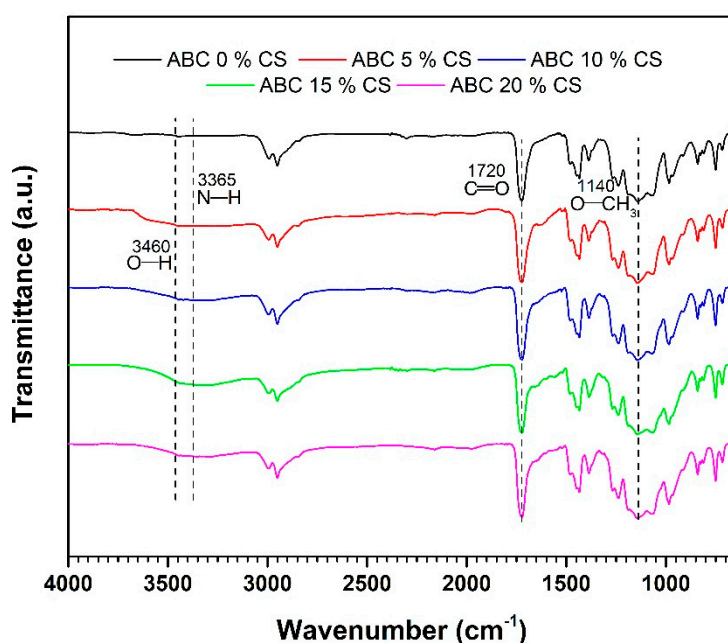


Figure 1. Fourier-transform infrared spectra in attenuated total internal reflectance mode (ATR) mode for ABCs loaded with chitosan (CS).

3.2. TGA

The thermal stability of cement is improved with the incorporation of CS (Figure 2). The temperature at which a 10% loss of weight (T_{10}) is achieved is reduced from 304 °C for ABCs without CS, to 288 °C for ABCs loaded with 20 wt.%. Additionally, T_{50} changed from 377 to 380 °C for ABCs without CS and ABC loaded with 20 wt.%, respectively. A 20 wt.% of CS introduction decreased the total loss weight percentage to 4 °C. At temperatures > 400 °C, a significant weight loss of the formulations with lower CS contents is evident. Figure 2 also shows an inset (a derivative of the thermogravimetric analysis, DTGA), in which the peak of the curve represents the temperature of the highest rate of weight loss obtained.

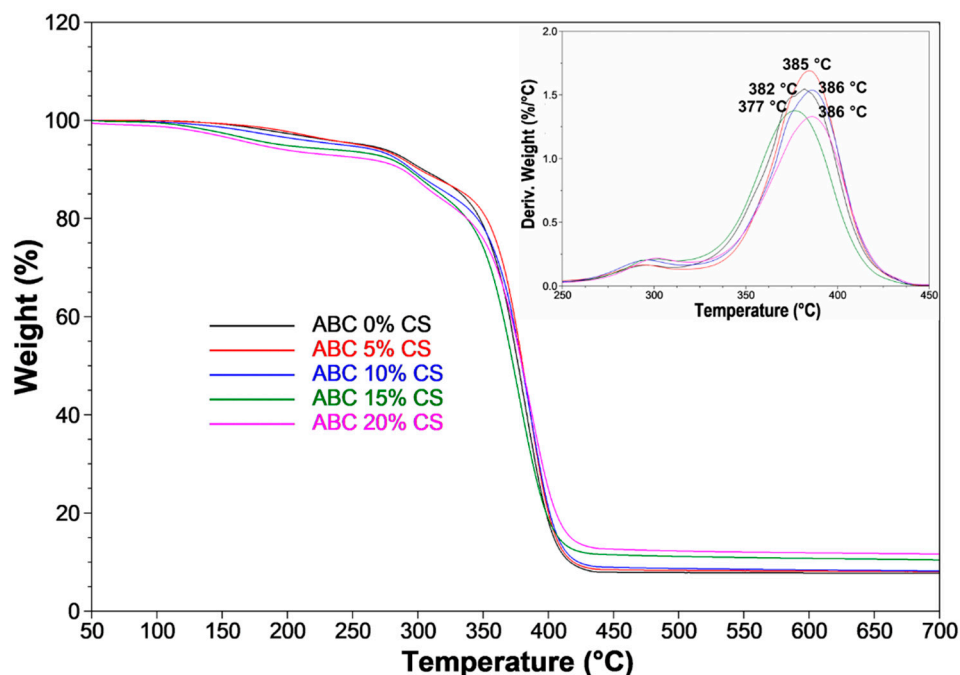


Figure 2. Thermogravimetric analysis (TGA) for ABCs loaded with CS. The inset shows the derivative TGA (DTGA) thermograms.

3.3. Handling Properties and Water Contact Angle

The handling parameters that describe the polymerization reaction are maximum temperature (T_{max}) and setting time (t_{set}). T_{max} corresponds to the maximum temperature reached during the MMA free radical polymerization reaction, which is exothermic, and the t_{set} represents the time taken to reach a temperature midway between ambient and T_{max} . From t_{set} , the significant increase of paste's viscosity caused a reduction in the handling of the cement [22].

The maximum temperature obtained during the MMA polymerization reaction shows a decrease of 13 °C with a CS loading of 20 wt.% (Table 2). The increase in t_{set} is significant for formulations with CS loading ≥ 15 wt.%. For each of the formulations, T_{max} and t_{set} values are within limits allowed by ISO 5833-02 for ABC [18].

Table 2. The maximum exotherm temperature (T_{max}), setting time (t_{set}), and water contact angle (WCA) results. Data reported as mean $\pm$ standard deviation.

Formulation	T_{max} (°C)	t_{set} (s)	WCA (°)
ABC 0% CS	59 $\pm$ 3	355 $\pm$ 30	64.9 $\pm$ 2.2
ABC 5% CS	58 $\pm$ 2	370 $\pm$ 15	65.1 $\pm$ 1.1
ABC 10% CS	54 $\pm$ 3	420 $\pm$ 15	66.4 $\pm$ 1.4
ABC 15% CS	54 $\pm$ 4	460 $\pm$ 20 *	60.3 $\pm$ 2.1 **
ABC 20% CS	46 $\pm$ 2 *	545 $\pm$ 25 *	56.8 $\pm$ 1.9 **

Significance level of * p -value < 0.05; ** p -value < 0.01.

The water contact angle showed a decrease of 7.1% and 12.5% with CS loadings of 15 and 20 wt.%, respectively ($p < 0.01$). Lower WCA is related to a more significant wetting on the ABC surface due to an increase in hydrophilicity. Other researchers have found WCA values similar to those found in this study [23].

3.4. Mechanical Properties, Residual Monomer Content and DSC

The bending strength, bending modulus, and compressive strength results are presented in Figure 3. ISO 5833-02 [18] specifies a minimum value of bending strength, bending modulus, and compressive strength for ABC as 50, 1800, and 70 MPa, respectively. Only the formulation without CS achieved the minimum bending strength. Except for the composite with CS of 20 wt.%, all other formulations produced the minimum bending modulus. Formulations with CS loadings of 15 and 20 wt.% did not achieve the minimum compressive strength.

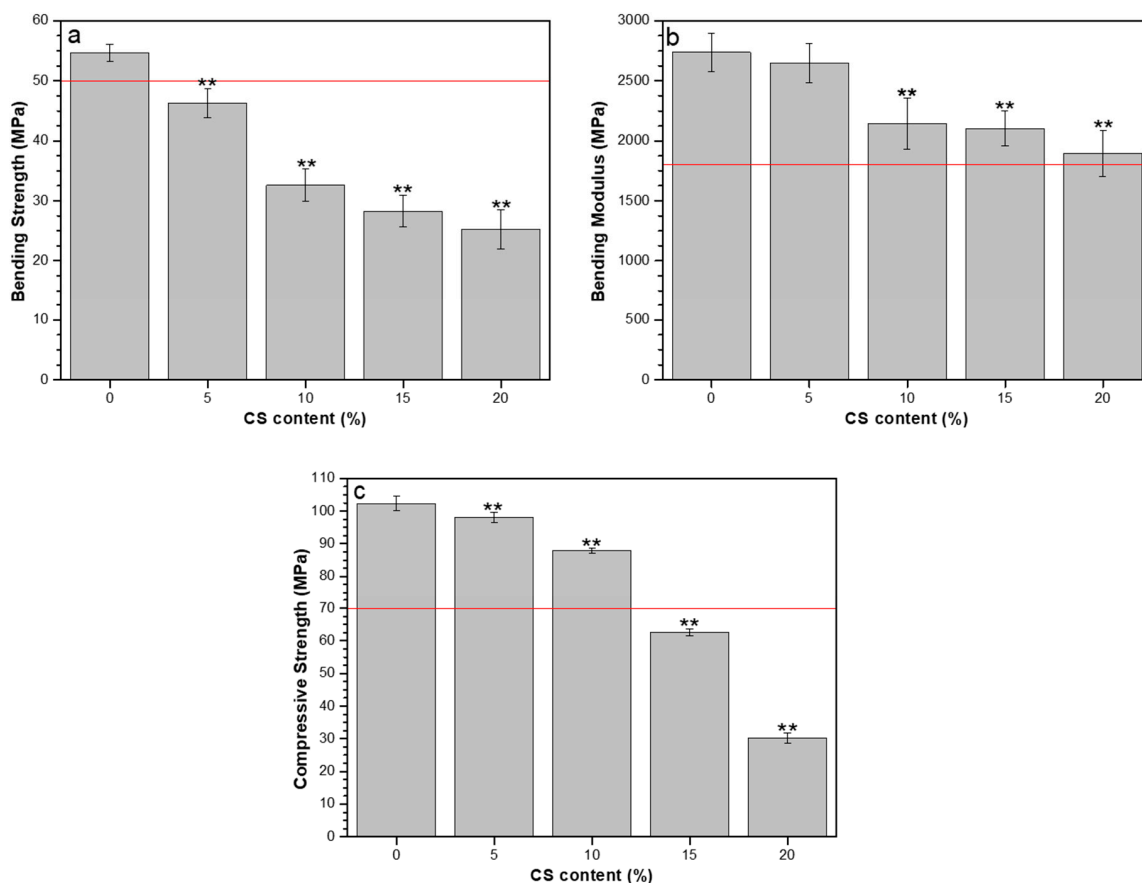


Figure 3. Mechanical properties in ABCs loaded with CS: (a) bending strength, (b) bending modulus, and (c) compressive strength. The straight red line corresponds to the minimum values specified in ISO 5833-02. Significance level of * p -value < 0.05; ** p -value < 0.01.

Both porosity and surface roughness of the compression test specimens increased with an increase in CS loading (Figure 4), indicating high potential for cell attachment for these specimens.

Storage modulus (E') obtained by DMA represents the elastic phase of the complex modulus and is equivalent to the energy stored through deformation [24]. It is related to the elastic modulus in bending; therefore, according to Figure 5, the stiffness of the cement decreased with increasing CS loading. T_g was calculated from the maxima value of $\tan \delta$ versus plotted temperature, and the trends in T_g determined by DSC and DMA were similar (Table 3).

From Table 3, it can be observed that T_g determined by DSC and DMA show similar behavior, in which CS loading ≤ 10 wt.% increases the T_g , while rates above this value generate a slight decrease in this property. There is a small increase in RMC with an increase in CS loading, except for the formulation with 20 wt.% CS, which offers an increase of more than 400% (Table 3). High RMC suggests a high risk of chemical necrosis.

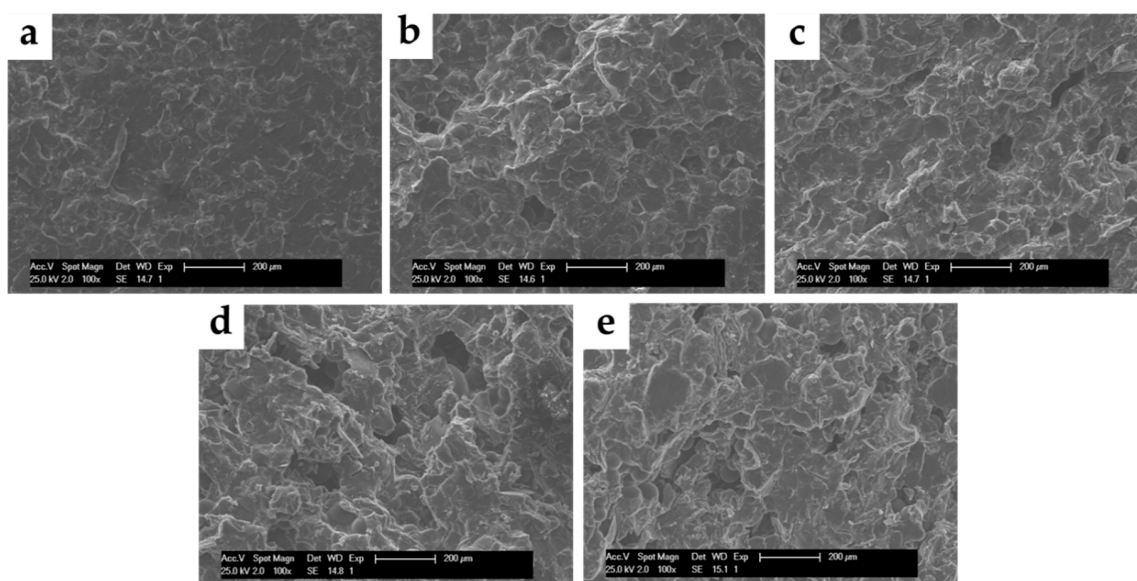


Figure 4. SEM micrographs (100×) of surfaces of ABCs loaded with CS. (a) ABC 0% CS, (b) ABC 5% CS, (c) ABC 10% CS, (d) ABC 15% CS, and (e) ABC 20% CS.

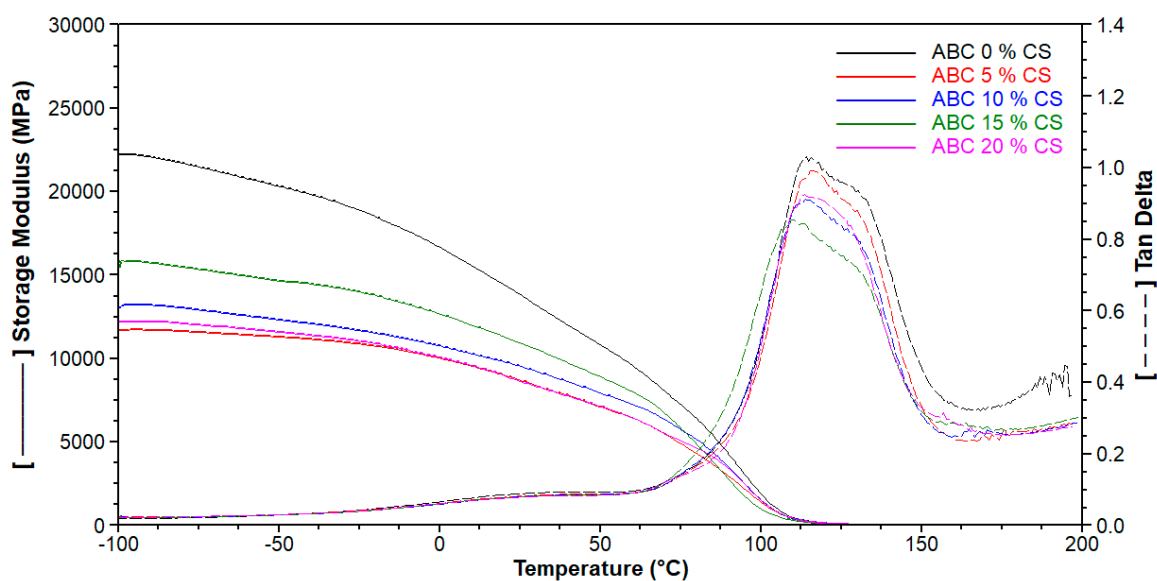


Figure 5. Dynamic mechanical analysis (DMA) of ABCs loaded with CS to 1 Hz of frequency.

Table 3. Residual monomer content (RMC) and glass transition temperature (T_g) by DSC and DMA of ABC.

Formulation	RMC (%)	T_g DSC (°C)	T_g DMA (°C)
ABC 0% CS	1.06	106	114
ABC 5% CS	1.83	108	116
ABC 10% CS	1.32	107	115
ABC 15% CS	1.57	105	110
ABC 20% CS	6.25	101	113

3.5. Hydrolytic Degradation

Figure 6 shows the change in weight, water uptake, pH, and compressive strength of ABCs with immersion time duration in SFB. The results showed that weight loss (Figure 6a) and water uptake (Figure 6b) increased with an increase in CS loading. The absorption is favored by the increase in the porosity of the cement, increasing the weight loss generated possibly by the hydrolytic degradation of CS within the cement.

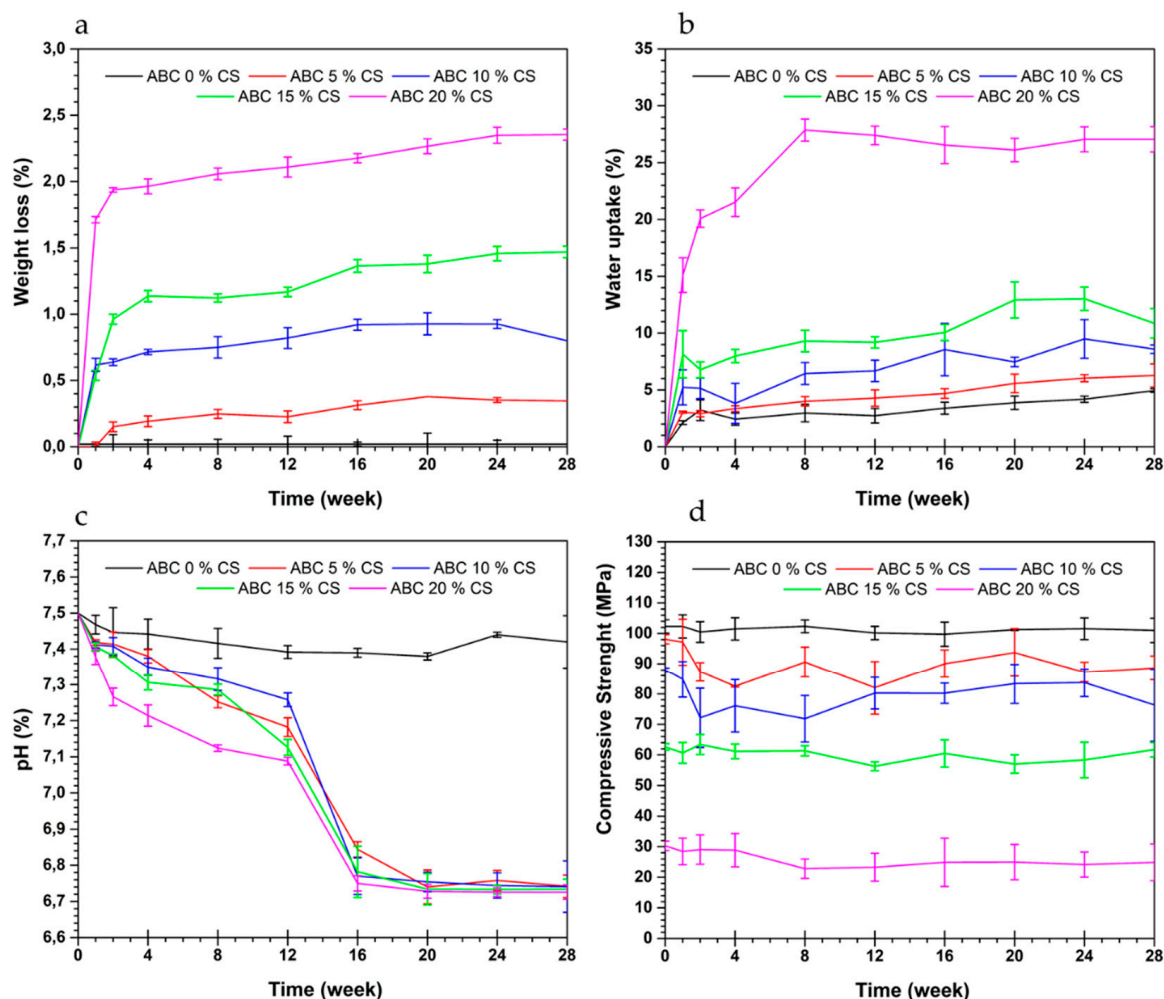


Figure 6. Effect of soaking time in a simulated body fluid of acrylic bone cements loaded with CS on (a) weight loss, (b) water uptake, (c) pH, and (d) compressive strength.

The pH of the SBF (Figure 6c) showed a decrease over time, possibly due to the CS degradation products, while when plain cement (no CS) test specimens were used, the change in SBF pH during the test was marginal. For each of the formulations, the variation of compressive strength with immersion time in SBF was negligible (Figure 6d), due to the few weight loss reported for these cement specimens (<2.5%) (Figure 6a).

3.6. Bioactivity in SBF

The presence of Ca and P on the surface of the ABCs immersed in SBF was evaluated by SEM and EDS techniques. Bioactivity in SBF was determined by monitoring the deposition of these ions on the surface of the ABC.

3.6.1. SEM

The morphologies of the surfaces of the cement specimens are shown in Figure 7. It is seen that the surface roughness increases with an increase in CS loading. For the same formulation, there are no apparent differences in surface morphology with an increase in immersion time in SBF.

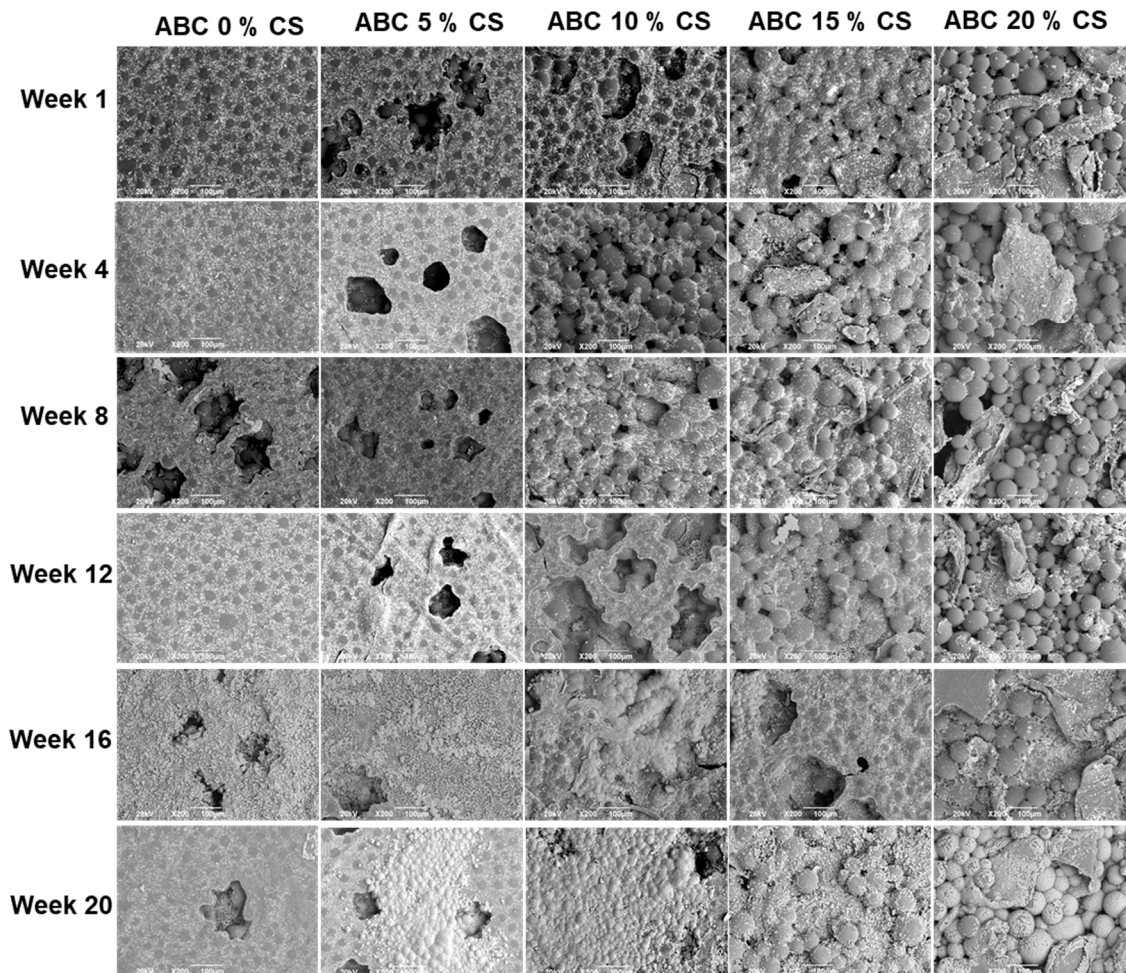


Figure 7. SEM micrographs of the surfaces of ABCs loaded with CS after different times of soaking in simulated body fluid (SBF) (200×).

3.6.2. EDS

Formulations with CS loadings of 15 and 20 wt.% CS show Ca deposition from the first week of immersion and an increase in the content of this ion until week 8, by which time the entire surface of the cement is covered (Figure 8a). On the other hand, for the same formulations, P appears from week 4 (Figure 8b). In contrast, for formulations with a CS loading < 15 wt.%, the deposition of these ions is delayed for several weeks (Figure 8a,b).

It is known that bioactivity in SBF is related to the facility of deposition of Ca and P ions on the surface of the cement, which, under in vivo conditions, promotes cell attachment of osteoblasts during the process of bone remodeling. The present results show that bioactivity in SBF improved with an increase in CS loading. The contact angle results corroborate these findings (increase in hydrophilicity with a rise in CS loading) (Table 2) and the surface morphologies (increase in the amount of surface CS with an increase of CS loading) (Figure 7).

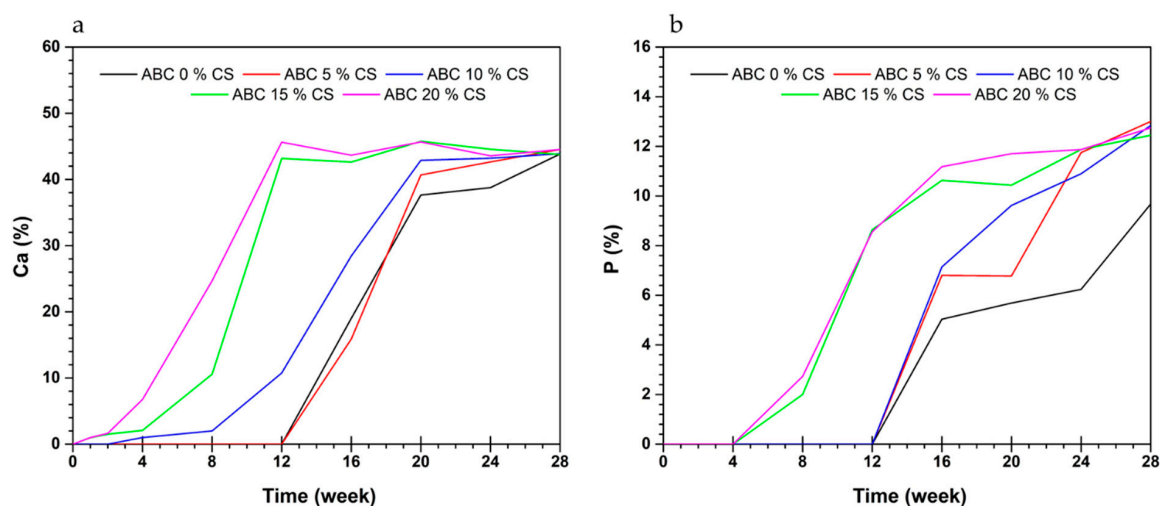


Figure 8. Content of (a) Ca and (b) P by EDS analysis of the surfaces of ABCs loaded with CS after different times of soaking in SFB.

4. Discussion

The results of the chemical, physical, thermal, mechanical, and biological characterizations of the ABCs prepared in this investigation were analyzed according to the effect of CS content.

The lack of chemical interaction between CS and PMMA, as confirmed by FTIR, shows that CS is not modified and, as such, it is expected that CS will impart some of its attractive properties, such as biocompatibility, bioactivity, and biodegradation [9,25], to the cement.

From the mechanical point of view, this lack of chemical bonding drastically affected the bending and compressive properties of the cements since the interface formed between them is weak. On the other hand, the thermal stability of the ABCs determined by TGA was improved at high temperatures, while at low temperatures, it had no significant influence.

The rigid glucosamine structure and the semi-crystalline structure of CS caused an increase in the cement viscosity [13,15]. This increased viscosity means that during the polymerization reaction, voids that are formed as entrapped air unable to escape, and the porosity of CS-loaded cement increased [6]. The increased porosity of ABCs with CS loading observed by SEM generates a loss in the mechanical properties. Still, it improves the bioactive behavior because a roughness increased with the biocompatibility favoring cell adhesion and bone tissue growth through the ABC [6,23]. It has been reported that the bone ingrowth to the ABC structure forms a strong mechanical interlocking between the cement and bone, and thus provides stability to the implant site [26].

The decrease in the maximum temperature improves the performance of the ABCs because it decreases the risk of thermal necrosis of the tissue around the cement. The increase in the setting time gives the surgeon more time available for filling and remodeling bone defects.

The trend of decrease in T_{max} with an increase in CS loading, which is the same as reported in the literature [13,15,23], is attributed to the intramolecular hydrogen bonds in CS acting as a heat sink, that is, absorbing the released heat during polymerization of the cement [3,13,15]. The reduction in temperature during the polymerization reduces the risk of bone cell necrosis and subsequent implant loosening [3]. Furthermore, the increase in t_{set} is related to the longer time available for the surgeon to manipulate the ABC during surgery.

The biocompatibility of a material is related to the surface properties, such as hydrophilicity [23]. In this research, the hydrophilicity of ABCs surfaces was determined by the water contact angle. For cement with CS loading ≤ 10 wt.%, WCA increased with an increase in CS loading, which is attributed to the surface of these cements being covered with a layer of the PMMA produced during the polymerization of MMA. In contrast, with CS loading > 10 wt.%, WCA decreases with an increase

in CS loading because of the more significant number of hydrophilic groups (NH_2 and OH) provided by the CS. The decrease of the contact angle favored the biocompatibility in SBF in ABC with 15 and 20 wt.% [3].

The significant decrease in each of the mechanical properties with an increase in CS loading may be attributed to two factors, namely (1) lack of chemical bonding, which means that the interface between the cement matrix and CS is weak; and (2) increased porosity. The low mechanical performance of CS-loaded cements concerning ISO 5833-02 [18] indicates that they should not be used for prosthesis fixation [27], but rather in applications such as remodeling areas of metastatic or osteoporotic cancer, in fractures, in the repair of cranial defects (cranioplasties) or in the restoration of vertebral fractures (vertebroplasty and kyphoplasty) [1], where less mechanical performance is required.

The T_g affects the mechanical properties of ABCs. It has been reported that the T_g of bone cements shows a direct relationship with modulus [24,28]. Our results confirm that the highest T_g was obtained for formulations with percentages ≤ 10 wt.%, which is related to the mechanical properties.

In general, the residual monomer content of formulations with percentages of CS less than 20 wt.% was low; however, the formulation with CS loading of 20 wt.% presented a high value compared to the other formulations. This increase in the RMC with the CS loading may be due to the lower diffusion capacity of the monomer during polymerization, generating more excellent heat dissipation [29]. Kühn Dieter [30] reports RMC between 1.8–4.1% for some of the available commercial formulations, so it can be assumed that the formulation with CS loading of 20 wt.% could present a risk of toxicity of the residual monomer [31].

The high weight loss and high-water uptake of ABCs with CS loading > 15 wt.% after soaking in SFB could be attributed to CS being hygroscopic and, as such, promotes the absorption of water. Also, higher porosity of the material, due to chitosan degradation, could encourage uptake water of the cement [6]. Similar weight loss and water uptake results have been reported elsewhere [6]. The pH of the SFB decreased over time, indicating that the by-products of the degradation process are responsible for the pH of the fluid [32,33]. For a given cement formulation, the change in its compressive strength over time was negligible, a trend that is consistent with the weight loss profile. In turn, this profile may be attributed to the reduced solubility of CS at $\text{pH} > 6.5$ [9,23].

Bioactivity relates to a material's ability to stimulate a biological response in its environment [7]. In this research, bioactivity in SBF was determined by Ca and P ions deposition on the cement surface. These ions are the main inorganic components of the bone, which, once deposited on the extracellular matrix material produced by the osteoblasts, generate the osteoid mineralization and, therefore, the formation of new bone [34].

The presence of Ca ions was confirmed one week later of immersion in SFB with CS loadings ≥ 15 wt.%. On the other hand, P presence was confirmed only after the fourth week. The previous results coincide with the sequence of processes reported for the formation of apatite. First, Ca^{2+} ions in SFB are selectively adsorbed onto negatively charged surfaces (OH^- groups of the CS). Second, PO_4^{3-} ions are attracted to the calcium-rich surfaces to form an amorphous calcium phosphate (ACP), and finally, the ACP is converted into apatite [9,13,17,35]. The presence of Ca and P ions in the first week of immersion of the cement specimens in SFB has been reported in the literature for cement formulations with CS loading > 20 wt.% [3,13,23].

Formulations with chitosan loading < 15 wt.% also showed bioactivity in SBF. ABCs CS loading of 10 wt.% started the deposition process after second-week soaking, while those with 0 and 5 wt.% CS registered bioactivity from week 12. For cements without CS loading, bioactivity in SBF may be attributed to the presence of alkaline comonomers [36].

The results obtained were promising for this type of cement with CS loading ≤ 20 wt.%; therefore, a more extensive biological characterization including in vitro and in vivo conditions is proposed in future research.

5. Conclusions

Formulations with CS loading ≥ 15 wt.% showed deposition of Ca from the first week following immersion in SBF and deposition of P ions from the fourth week, in contrast to cements with lower CS loading, which presented deposition between the second and twelfth week. This observation might suggest that for fast bioactivity in SBF, the minimum CS loading is 15 wt.%.

Formulation with CS loading ≥ 15 wt.% showed increased thermal stability, higher porosity, increased roughness, decreased contact angle (on water), reduced T_{max} , and increased tset. Those results were desirable, but this was accompanied by increased RMC, and significant decreases in bending strength, bending modulus, compressive strength, and storage modulus, each of which is undesirable. Thus, the cements with CS loading of 15 wt.% should be further characterized for use in low-weight-bearing applications, such as bone void fillers, vertebroplasty, and balloon kyphoplasty.

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Cementos Óseos Acrílicos Bioactivos con Aplicación en Ortopedia

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Resumen

Se formularon cementos óseos modificados, empleando un co-monómero alcalino y Quitosano como carga bioactiva. Se efectuaron ensayos de caracterización físico-química, térmica y mecánica siguiendo la norma ISO 5833. Mediante el empleo de un diseño de experimentos de superficie de respuesta, se optimizó la composición del cemento y se complementó el estudio con pruebas en condiciones *in vitro* por inmersión del material en un fluido biológico simulado e *in vivo* utilizando el cemento para fijar vástagos de titanio en el canal medular del fémur izquierdo de bio-modelos (conejos).

Palabras claves: Cemento óseo, Regeneración ósea, Co-monómeros Alcalinos, Hidroxiapatita, pruebas *in vitro*, pruebas *in vivo*.

Introducción

Los cementos óseos son materiales poliméricos tolerados por el organismo con el fin de fijar prótesis metálicas, sin embargo, no solo sirven como material de relleno, sino que también deben tener la capacidad de transferir cargas mecánicas de la prótesis al hueso y funcionar como amortiguador entre estos (Cardoso, J. 2005). Debido a que los cementos óseos convencionales exhiben alto calor de reacción, alta contracción y elevado contenido de monómero residual; generando un comportamiento mecánico inadecuado que contribuye al aflojamiento de las prótesis fijadas con los mismos; (Islas-Blancas, M. et al., 2001) se adicionan fosfatos de calcio, que son capaces de fraguar en contacto con los fluidos orgánicos sin experimentar fenómenos de descohesión ni contracción, tiempos de fraguado y endurecimiento reducidos, reacciones isotérmicas o exotérmicas limitadas, carecen de toxicidad local o general y presentan propiedades bioactivas (Hench II. 1998). También se implementa el Quitosano para mejorar la respuesta biológica de los cementos, debido a su biocompatibilidad, biodegradabilidad de productos inocuos, no toxicidad, inercia fisiológica, carácter antibacteriano, fungistático y antitumoral (Singla, AK. & Chawla, M. 2001). Adicionalmente, la incorporación de co-monómeros con grupos amino conducen a una mejora en la biocompatibilidad del material ya que las



células generadoras de hueso (osteoblastos) se depositan y proliferan mejor en superficies parcialmente hidrófilas cargadas positivamente (Islas-Blancas, M. et al., 2001). Debido a la necesidad de mejorar la zona interfacial entre el hueso-cemento-vástago y buscando incrementar el tiempo de fijación entre estos, en el presente trabajo se estudió la influencia del quitosano como carga bioactiva y un co-monómero polar con el fin de incrementar la hidrofiliidad del material, mediante el empleo de un diseño de experimentos de superficie de respuesta, dejando constante los reactivos convencionales que se utilizan para la fabricación de cementos óseos.

Metodología Experimental

Materiales Empleados

Fase sólida (S): Polimetacrilato de Metilo adquirido en Veracril®, (New Stetic). Peróxido de Benzoilo adquirido en Merck Schuchardt OHG. Sulfato de Bario, adquirido en Alfa Aesar®, (Johnson Matthey Company). Quitosano adquirido en Aldrich®.

Fase líquida (L): Metacrilato de metilo adquirido en Merck Schuchardt OHG. N, N-dimetil p-toluidina adquirido en Merck Schuchardt OHG. Metacrilato del 2 (dimetilamino etilo) (DMAEM) adquirido en Merck Schuchardt OHG.

Preparación de los Cementos Óseos

Se preparó el cemento incorporando el componente líquido (L) a la fase sólida (S), llevando a cabo un mezclado manual; en todos los casos se manejó una relación S/L constante de 2 y una temperatura de 23 ± 2 °C aproximadamente. De igual manera, se utilizó como análisis estadístico un diseño de experimentos de superficie de respuesta, tomando como factores: el contenido del co-monómero alcalino (DEAEM) y la cantidad de Quitosano.

Caracterización Térmica

La temperatura y el tiempo de curado se calcularon siguiendo la norma ISO 5833, en una muestra de 25 g de cemento.

Caracterización Mecánica

Las muestras de cemento óseo destinadas a ensayos de compresión fueron conformadas de acuerdo con la norma ISO 5833 y se evaluaron en un equipo de marca Tinius Olsen modelo H50KS, a una rapidez de desplazamiento del cabezal de 20 ± 1 mm/min.

Pruebas in Vitro

Se sumergieron 3 réplicas de cemento óseo en un Fluido Biológico Simulado (FBS), durante 7 y 30 días. El FBS fue preparado según la formulación planteada por Kim et.al., Kim, S. B., y colaboradores (2004). El análisis de la superficie del material se llevó a cabo mediante SEM y EDS.

Pruebas in Vivo

Se *implantaron vástagos* de titanio fijados con cementos óseos, en el canal medular del fémur izquierdo de bio-modelos animales (Fig. 1). El análisis e identificación de la posible formación de calcio y fósforo en las interfaces, se llevó a cabo mediante SEM y EDS.

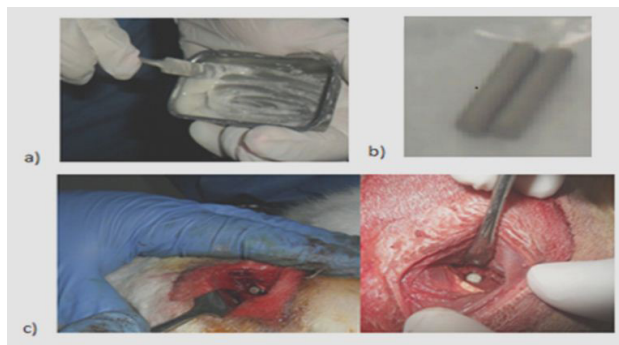


Figura 1. (a) Preparación in situ del cemento óseo, (b) vástagos de titanio, (c) Implantación del mismo.

Resultados y discusiones

El tiempo de curado del cemento óseo se encuentra influenciado tanto por el contenido de Quitosano como del DMAEM, tal como se puede observar en la Figura 2; aquí se infiere que una mayor proporción de Quitosano (aproximadamente 10%) y un bajo contenido de DMAEM (2%) generan incrementos en el tiempo de curado, a valores cercanos a 12,5 min, lo cual lo hace más útil en la manipulación del cemento durante los procedimientos quirúrgicos; cumpliéndose con la norma ISO 5833 (tiempo de curado 6-5 minutos). Por otro lado, el que el Quitosano posea un mayor tamaño de partícula que el PMMA, lo hace responsable de un tiempo de curado mayor y una menor temperatura máxima. La temperatura máxima alcanzada puede ser de 90 ± 5 °C, cumpliendo con esta condición todos los tratamientos del diseño experimental.

La cantidad de co-monómero incorporada influyó considerablemente en la resistencia a la compresión del material (Figura 2), encontrándose que para contenidos menores al 2%, se alcanza a cumplir con la resistencia

establecida por la norma ISO 5833 (70 MPa); así mismo, se pudo observar que el Quitosano incluido en la formulación del cemento tuvo poca influencia en este intervalo de trabajo.

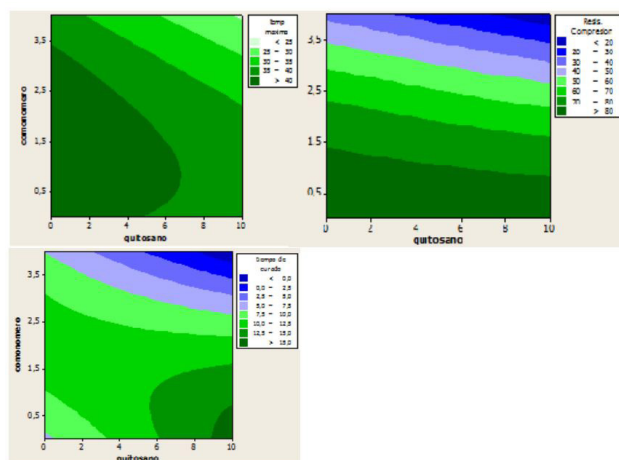


Figura 2. Graficas de contorno de la temperatura máxima de curado, tiempo de curado y la resistencia a la compresión, correspondientes a los cementos óseos modificados con DMAEM y Quitosano.

De las radiografías se puede inferir que no hubo lisis ósea, ni daño tisular ocasionado por exotermia, patología conocida como necrosis térmica, debido a que no se observa la desintegración del hueso que se encuentra en contacto con el cemento óseo, ni aflojamiento de la prótesis o pérdida del implante.

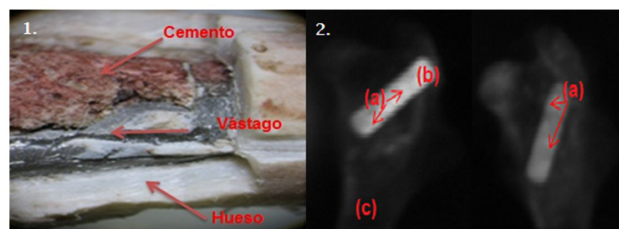


Figura 3. Interface hueso-cemento-vástago de una muestra implantada: (1). Estereoscopio, (2). Radiografía ((a) cemento óseo, (b) vástago de titanio, (c) hueso)

Conclusiones

Con la incorporación de DMAEM y Quitosano, se alcanzó una disminución en la temperatura máxima de curado de los cementos óseos modificados, además de alcanzar la resistencia a compresión especificada en la norma ISO 5833 para bajos porcentajes de comonomero.

Finalmente, algunas de las formulaciones desarrolladas en esta investigación se caracterizaron por su buen comportamiento bio-activo, según los resultados del estudio *in vitro* e *in vivo*; por lo que se puede inferir que algunas formulaciones son susceptibles de ser empleadas para la fijación de prótesis.

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Article

Novel Bioactive and Antibacterial Acrylic Bone Cement Nanocomposites Modified with Graphene Oxide and Chitosan

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Abstract: Acrylic bone cements (ABCs) have played a key role in orthopedic surgery mainly in arthroplasties, but their use is increasingly extending to other applications, such as remodeling of cancerous bones, cranioplasties, and vertebroplasties. However, these materials present some limitations related to their inert behavior and the risk of infection after implantation, which leads to a lack of attachment and makes necessary new surgical interventions. In this research, the physicochemical, thermal, mechanical, and biological properties of ABCs modified with chitosan (CS) and graphene oxide (GO) were studied. Fourier transform infrared (FTIR) spectroscopy, proton nuclear magnetic resonance (¹H-NMR) scanning electron microscopy (SEM), Raman mapping, thermogravimetric analysis (TGA), differential scanning calorimetry (DSC), compression resistance, mechanical dynamic analysis (DMA), hydrolytic degradation, cell viability, alkaline phosphatase (ALP) activity with human osteoblasts (HOb), and antibacterial activity against Gram-negative bacteria *Escherichia coli* were used to characterize the ABCs. The results revealed good dispersion of GO nanosheets in the ABCs. GO provided an increase in antibacterial activity, roughness, and flexural behavior, while CS generated porosity, increased the rate of degradation, and decreased compression properties. All ABCs were not cytotoxic and support good cell viability of HOb. The novel formulation of ABCs containing GO and CS simultaneously, increased the thermal stability, flexural modulus, antibacterial behavior, and osteogenic activity, which gives it a high potential for its uses in orthopedic applications.

Keywords: acrylic bone cement; chitosan; graphene oxide; nanocomposite

1. Introduction

Due to the increase in the aging population and the increase of sport-related trauma, the number of patients undergoing joint replacement surgery is increasing, particularly in young people [1,2]. Most

prostheses are being fixed using acrylic bone cements (ABCs) of poly(methyl methacrylate) (PMMA), which acts as a glue between the implant and the bone and is characterized as an inert material [3]. Most commercial ABCs currently available consist of two components, a solid phase composed mainly of a polymer based on methyl methacrylate (MMA) and a liquid phase based on MMA [4], which are mixed to produce the cement, due to the free radical polymerization reaction of the monomer present [1].

The main advantages of cemented prostheses are their excellent primary fixation, the uniform distribution of the load between the implant and the bone, and the fact that the technique allows rapid recovery of the patient. However, the main disadvantage is that about 10% of patients may require revision in less than ten years after implantation [5].

The observed aseptic loosening that appears over time is attributed to causes such as the lack of secondary fixation, mechanical failure of the cement, and the initial formation of fibrous tissue between the cement and the bone. This is mainly due to bone necrosis induced by the heat released during the polymerization reaction, and osteolysis caused by the foreign body reaction induced by wear particles which could come from the bone cement. The toxicity of the liquid monomer of cement is also another disadvantage. Septic loosening, produced by bacterial infections, is a common complication after a total joint replacement with infection rates up to 3% [6]. In spite of the above, ABCs still have a long way to go and a future ahead, since no material has played such an important role and has been in widespread use in orthopedics in the last 60 years [3,4].

With the aim of improving the limitations, there are studies in which ABCs imparted bioactivity through the addition of inorganic particles. These approaches led to strengthening of the cement–bone interface but had repercussions on losses in the mechanical properties of cement, which also compromises the longevity of the prosthesis. On the other hand, to prevent the formation of biofilm caused by bacteria, work has been done to control the release of antibiotics by including biodegradable polymers [7], which has achieved relatively good short-term results. However, despite the multiple strategies proposed for durable antibacterial ABCs [8,9], the appearance of resistant bacteria and the loss of mechanical properties over time persist and this makes it necessary to investigate new materials capable of combating these drawbacks.

Chitosan is a polymer of great interest for biomedical applications and implants [10], because of its intrinsic properties, such as biocompatibility, antibacterial activity [11], bioactivity [12], controllable degradation rate [13], and osteoconductive and osteoinductive activities [14]. Additionally, chitosan is obtained from renewable sources that are currently extending its applications. CS has been combined with a variety of polymeric biomaterials such as alginate, hyaluronic acid, PMMA or poly-L-lactic acid (PLLA), and inorganic bioactive compounds, like hydroxyapatite or calcium phosphate, and growth factors, for potential application in orthopedics as bone graft substitutes, intervertebral discs, and bone and cartilage tissue engineering [14,15]. Meanwhile, the use of graphene oxide (GO) nanoparticles in biomedical applications has become a hot topic of research. GO can provide mechanical reinforcement to biomaterials like chitosan [11], polycaprolactone [16] and polylactic acid [17] among others. GO possesses intrinsic antibacterial effects against Gram-positive bacteria like *Staphylococcus aureus* and Gram-negative bacteria like *Escherichia coli* that makes it a potential agent in the treatment of resistant bacterial infections [18]. Moreover, due to its low cytotoxicity, GO can be a promising candidate in orthopedic applications for regeneration of bone tissue [19], in bone cements [20], and in cartilage regeneration [21].

This work studied the unexplored effect of combined CS and GO on ABC nanocomposites and their potential to be used as antibacterial bioactive cement in orthopedic applications. The effect of the addition of each component (15% CS and 0.3% GO) was studied together and separately. The ABCs formulations were characterized by analyzing their physicochemical, thermal, and mechanical properties. Likewise, the swelling and degradation behavior of the modified cements were evaluated in vitro in simulated body conditions, their antibacterial activity assessed against Gram-negative bacteria and their biological performance studied in human osteoblast cell cultures.

2. Results and Discussion

2.1. Characterization of Graphene Oxide

Figure 1 shows the results of the GO characterization. The Raman spectrum (Figure 1a) exhibits the characteristic bands of the GO. The G band is attributed to the stretching of the bond of the carbon pairs in sp^2 hybridization in the graphite, and the D band is assigned to the breathing mode of the sp^2 carbon rings and indicates the existence of defects in the graphite network due to the presence of oxygen-rich functional groups [22]. X-ray diffraction (XRD) patterns for graphite and GO (Figure 1b) show that, in graphite, the peak corresponding to the reflection of the plane (002) appears at an angle $2\theta = 26.46^\circ$, while for the GO that peak appears at $2\theta = 10.77^\circ$ and corresponds to the reflection of the plane (001) [23,24]. Applying Bragg's law to these results, it is obtained that the interlaminar distance increases from 3.36 Å for the graphite to 7.953 Å for the GO due to the presence of the oxygen-rich functional groups in the latter [25]. The increase in the interplanar distance facilitates the dispersion of the GO sheets in different media. The values obtained in this investigation approach to those reported by other authors [11]. The GO nanosheets used in this study had an average size of 400 nm with a polydispersity (PDI) of 0.055 measured by dynamic light scattering (DLS) (Figure S1).

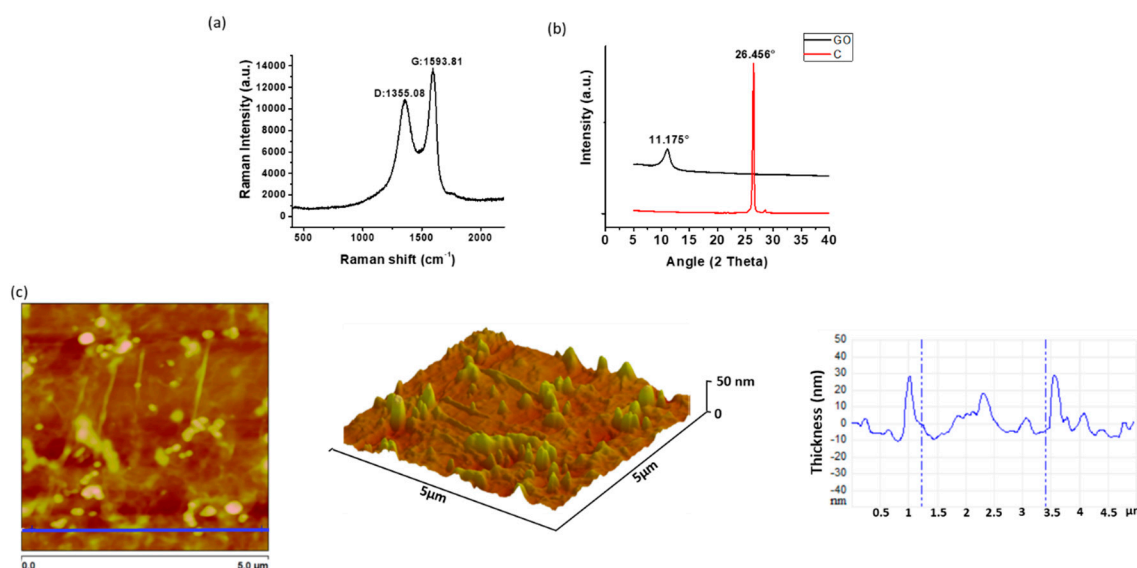


Figure 1. Characterization of GO by (a) Raman, (b) XRD (C is graphite), and (c) atomic force microscopy (AFM). Analysis of GO performed on tapping mode showing topography and thickness.

The morphology of the GO sheets was studied by atomic force microscopy (AFM), which is shown in Figure 1c. It can be observed that the thickness of the nanosheets is lower than 30 nm, confirming that there is little overlapping of GO sheets [26].

2.2. Characterization of ABC

2.2.1. Chemical Characterization

- FTIR Spectroscopy

Figure 2 shows the Fourier-transform infrared (FTIR) spectra of ABCs and those of the pure added fillers. The characteristic bands of GO [25] and CS, [27–30] were present in the ABCs spectra. In particular, the band corresponding to the amino group (3365 cm^{-1}) did not vary in the position with respect to the pristine CS, which corroborates that there is no chemical interaction between the CS and the PMMA. In addition, no variations were observed in the spectra of ABCs with GO attributable to

the incorporation of GO, mainly due to the low proportion of this component in the formulation and, also, to the fact that the main absorption bands of GO overlap with those of PMMA.

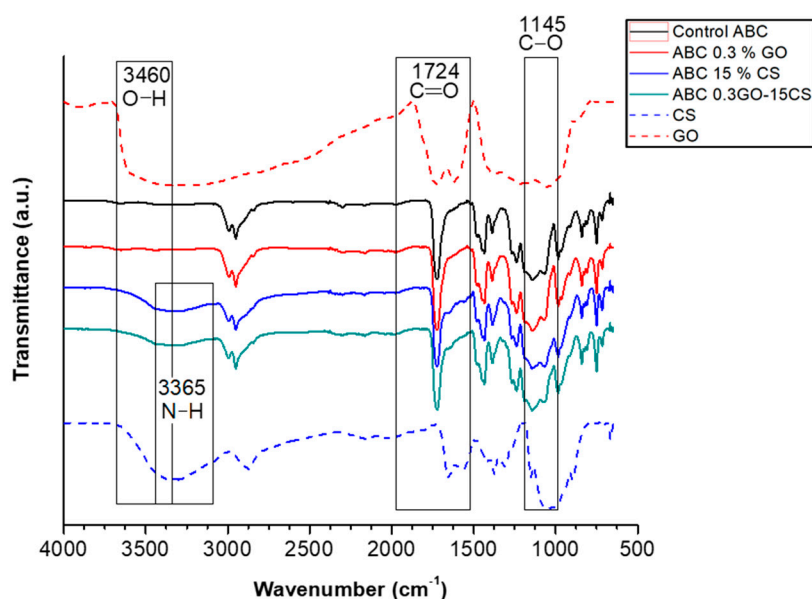


Figure 2. ATR-FTIR spectra of ABCs, CS, and GO. Control ABC corresponds to ABC without fillers.

• Residual Monomer

The amount of the residual monomer content of the ABCs nanocomposites obtained by proton nuclear magnetic resonance ($^1\text{H-NMR}$) is shown in Table 1. The results indicate that the incorporation of each component GO or CS separately hardly increased the residual monomer content of the cements. However, when both components were added together the value moderately increased. This increasing of the residual monomer with the presence of fillers can be due to a lower diffusion capacity of the monomer through the polymerizing mass during the polymerization process, leading to more moderate heat dissipation. Other authors have reported an increase in the amount of residual monomer with the incorporation of GO [20] and CS. Despite the rise, these values are lower than those reported in other studies [20,31] and are similar to those reported by Islas-Blancas et al. [32], who affirm that they are within the range observed for commercial cement [33].

Table 1. Residual monomer and glass transition temperature of ABCs.

Formulation	Residual Monomer (%)	T _g by DMA (°C)
Control ABC	1.1	114.8
ABC 0.3 % GO	1.3	119.6
ABC 15 % CS	1.6	109.5
ABC 0.3GO-15CS	3.6	124.5

2.2.2. Physical Characterization

• SEM

According to the scanning electron microscopy (SEM) images reported in Figure 3, the morphology of the ABCs nanocomposites varies significantly with the addition of fillers. Compared with the control ABC (Figure 3a) the GO (Figure 3b) generates a rough surface, as reported by Goncalves et al. [34], which is attributed to the morphology of the nanosheets. On the other hand, the presence of CS in the formulation (Figure 3c) contributes to the porosity because the higher viscosity of the paste generated

by the presence of the CS promotes the entrapment of air inside the cement during the mixing process. The formulation with both charges (CS and GO) (Figure 3d) presented a synergy in the morphology, reflecting a surface with higher roughness and porosity compared with the control.

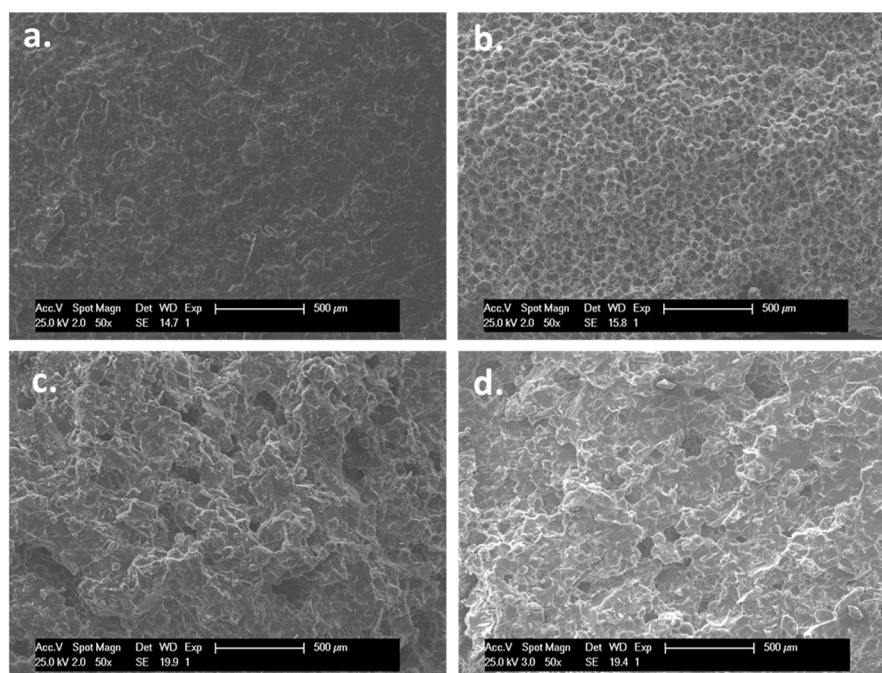


Figure 3. The SEM images (50×) of (a) control ABC, (b) ABC with 0.3% GO, (c) ABC with 15% CS, and (d) ABC with 0.3% GO and 15% CS samples.

From a biological point of view, the morphological features in terms of roughness and porosity have a major effect on the surface biocompatibility by promoting good anchoring pattern for cells. For instance, Khandaker et al. reported that an increase in the roughness of acrylic bone cements with the incorporation of polycaprolactone electrospun nanofibers generated an increase in cytocompatibility of osteoblast cells, their adhesion, proliferation, mineralization, and protein adsorption [35]. On the other hand, it has been reported that roughness plays a key role in bone contact biomaterials, such as titanium, increasing cell growth and migration and expression of proteins in the extracellular matrix [36].

- Raman Mapping

The distribution map of the GO obtained by Raman spectroscopy was used to evaluate its dispersion in the ABCs matrix. The regions used to identify the load and the matrix were determined from the characteristic bands of each component previously measured. In Figure 4a, the green color corresponds to the nanosheets of GO and the black color to the PMMA matrix, while in Figure 4b the red color corresponds to the matrix and the black color to the GO. In both figures, it is observed that the GO is well distributed within the ABCs matrix, which can be beneficial for mechanical reinforcement. Figure 4c shows the Raman spectrum obtained for the ABC sample, in which, 1324 cm^{-1} is indicated like the Raman shift used as a marker for the GO. Other authors have reported similar findings on GO dispersed in different matrices, such as inorganic silica particles [37] or polymeric substrates [38,39], where Raman spectroscopy showed a comparable surface pattern, concluding the homogeneous distribution and compatibility of GO across the respective matrices.

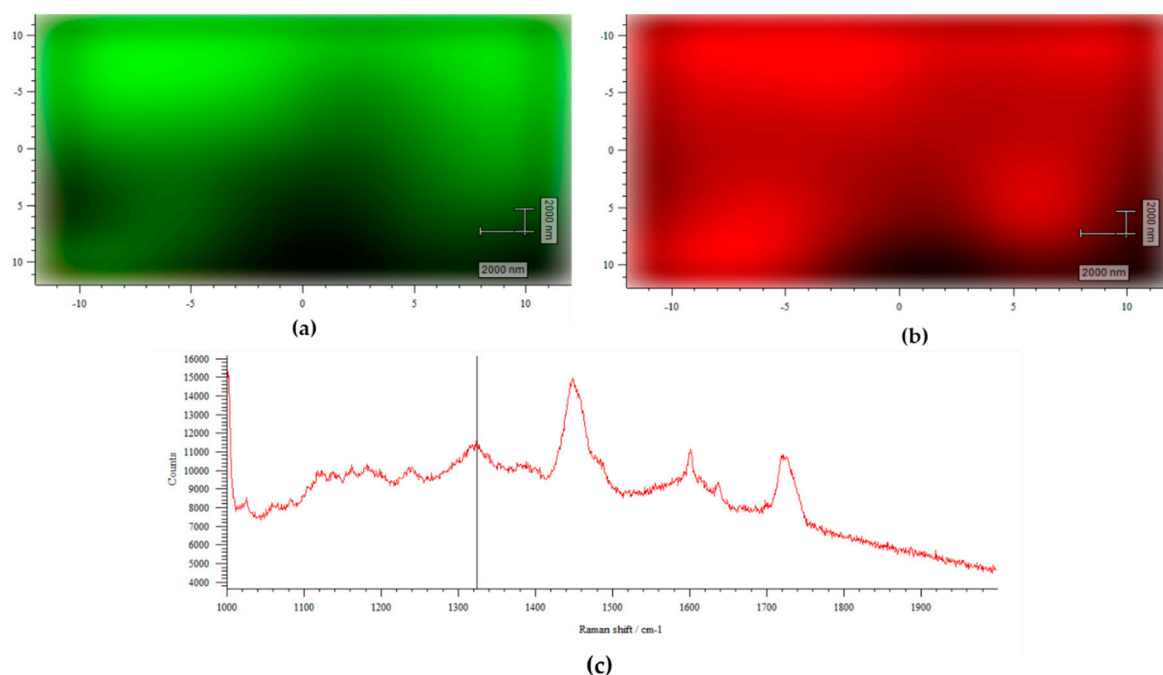


Figure 4. Raman surface mapping of ABC 0.3% GO. (a) Green corresponds to GO and black to ABC matrix; (b) red corresponds to ABC matrix and black to GO; and (c) Raman spectra to ABC 0.3% GO.

2.2.3. Thermal Characterization

- TGA

The TGA curves of the ABCs studied are shown in Figure 5. The thermal stability of the ABCs is reduced with the addition of GO or CS at temperatures below 400 °C. On the other hand, thermal stability increases with the addition of both fillers simultaneously, demonstrating a good dispersion of GO through the polymer matrix. The temperature, at which the maximum mass loss rate for formulations occurs, represented by the peaks of the graph of the first derivative of the TGA (DTGA), is increased by the presence of the GO and is decreased by the presence of the CS due to the individual thermal behavior of each component.

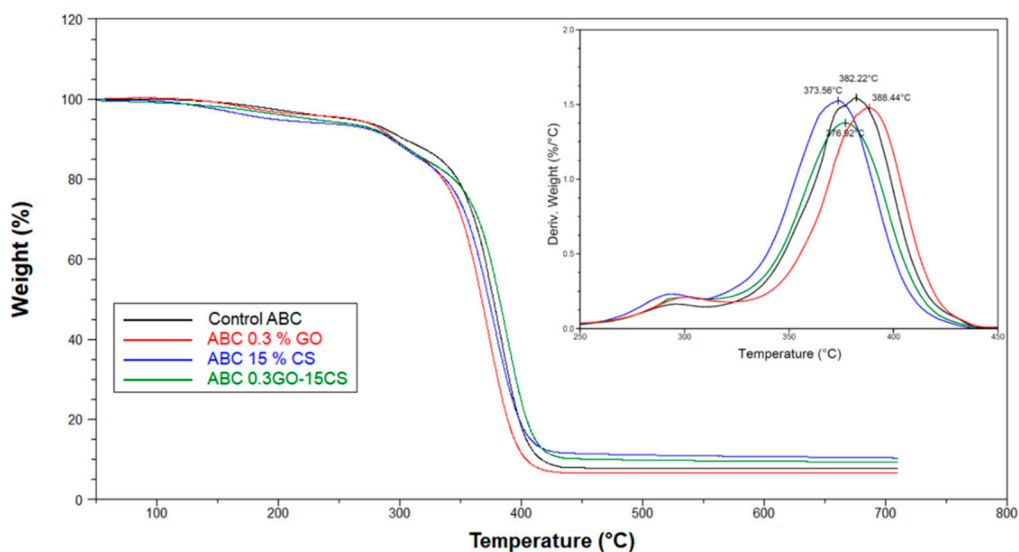


Figure 5. TGA and DTGA (inset) curves for experimental ABCs.

2.2.4. Mechanical Characterization

• Compressive Strength

Figure 6 shows that the compressive strength of the ABCs varies with composition. The incorporation of 15% of CS generates a considerable reduction of the resistance to values lower than the minimum compressive strength required in the MCS-ISO-5833 [40]. This might be due to the porosity generated by this component, the irregular shape of the sheets of CS that contain sharp edges that work as stress accumulators, and the lower amount of PMMA in the formulation. Moreover, it is possible that the presence of CS in the nanocomposite interferes with the polymerization of liquid monomer and, thus, causes significant decreases in the mechanical strength [41]. The addition of 0.3% GO to the ABCs does not present a significant difference in the compressive strength concerning the control formulation. However, when the GO is added to the formulation with 15% CS (ABC 0.3GO-15CS), the compression strength of this cement rises above the MCS ISO 5833 threshold.

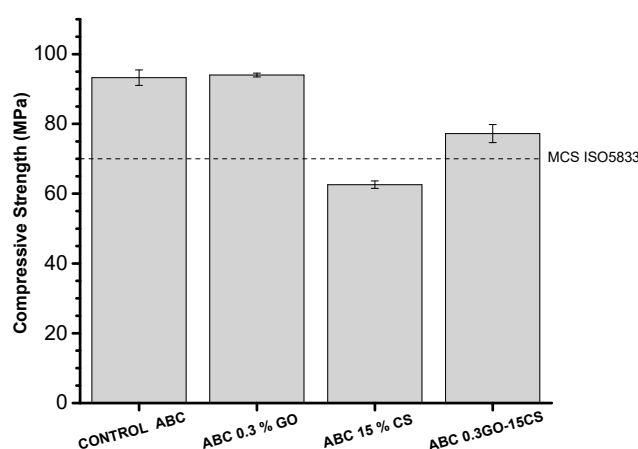


Figure 6. Compressive strength of the control ABC and experimental cements formulated with graphene oxide and chitosan. The dotted line indicates the minimum compressive strength (MCS) threshold defined by ISO5833.

The above is an indication that there is some hydrogen bonding interaction between the CS and the GO that favors the mechanical reinforcement in the cement and gives the cement a compression resistance superior to the MCS ISO 5833 threshold. Mechanical reinforcement of CS with GO has been widely reported in the literature [11,26] and is attributed to the homogeneous distribution of the GO and the interaction of the amino groups of the CS with the carboxyl groups of the surface of the GO through hydrogen bonds.

• DMA

It is known that the behavior of the storage module determined by DMA, is related to the flexural modulus of the material. From results of this parameter for ABCs it can be inferred that the addition of the fillers improves the flexural behavior of the cements, reaching the highest increases in the two formulations containing GO. The glass transition temperature (T_g) calculated from the $\tan \delta$ (Table 1 and Figure 7), shows similar behavior to the compressive strength, finding a reduction in this parameter with the incorporation of CS mainly due to the lower degree of mechanical reinforcement that is related to the hardness of the material. Marques et al. [42] reported glass transition values of materials modified with biodegradable polymers similar to those found in this investigation.

On the other hand, the content of GO increased the T_g thanks to the higher mechanical support provided by this component with nanometric size and the physical interactions between the polymer chains and the nanoparticles [43].

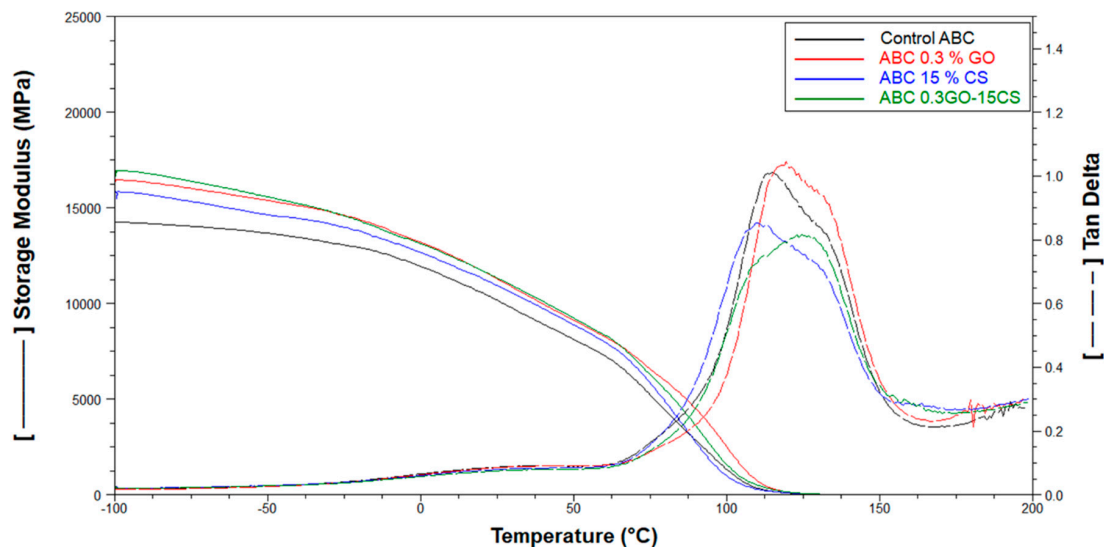


Figure 7. DMA results for the control ABC and experimental cements formulated with graphene oxide (GO) and chitosan (CS).

2.2.5. Biological Characterization

• Hydrolytic Degradation

Swelling properties are fundamental in the cell-biomaterial interactions [44]. Water absorption of PMMA ABCs is known to be very low due to their hydrophobic nature [45], which leads to the in vivo formation of a fibrous membrane around the cement. In this work, the hydrolytic behavior of the experimental ABCs will be crucial due to the incorporation of a biodegradable component which will influence the tissue interactions. From the results shown in Figure 8a, it can be observed that when the samples are immersed in Dulbecco's phosphate buffered saline (PBS), the absorption of water increases with the immersion time. The formulation containing only CS, had the highest water uptake at 14 and 21 days, mainly due to the higher porosity of this nanocomposite, observed by SEM (Figure 3c) but also, to the hydrophilicity of CS [46]. The water absorption of the ABC 0.3GO–15CS was lower which can be attributed to the formation of hydrogen bonds between CS and GO. As far as degradation behavior is concerned, according to Figure 8b, the most significant weight loss of ABCs occurs in that modified only with CS because this is a bioresorbable polymer that is hydrolyzed in aqueous media [47]. Nevertheless, weight loss of any experimental cement was not very high, which can guarantee the maintenance of their mechanical properties.

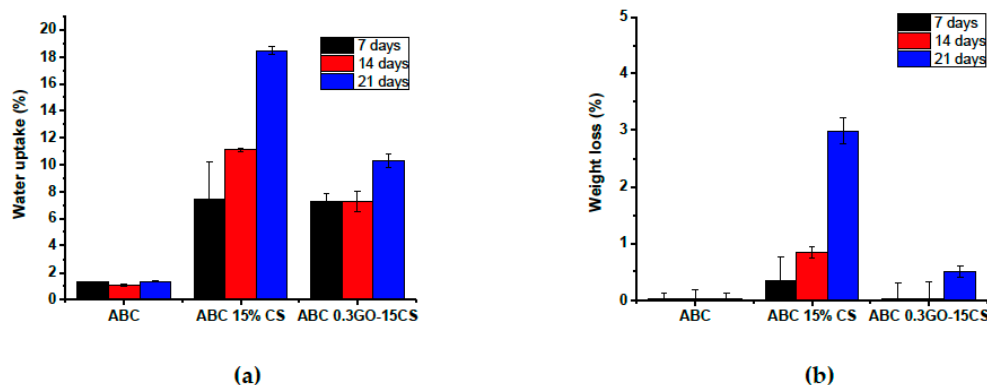


Figure 8. (a) Water uptake and (b) weight loss of experimental ABCs cements after immersion in PBS (pH 7.4) at 37 °C for 7, 14, and 21 days.

• Cell Viability

Cytotoxicity of cements was evaluated with human osteoblasts cells (HOb) using the MTT assay that measures the succinate mitochondrial dehydrogenase enzyme activity [48]. According to Figure 9a none of the studied ABCs presented cytotoxicity for human osteoblasts since, in all cases, the cellular viability in the presence of extracts taken between one and seven days increases over time and is above 90%. Figure 9b shows the cell viability results when HOb are directly seeded on the ABCs surfaces. In this case, it can be seen that the presence of CS or GO in the cement formulation enhances the capacity of HOb for surface colonization and proliferation at early stages when compared with control ABC. This observation is in agreement with other authors that have reported the benefits of GO [49] and CS [50] for cell attachment and proliferation and indicates that all the studied formulations have a high potential to be used as a biomaterial in bone treatment applications.

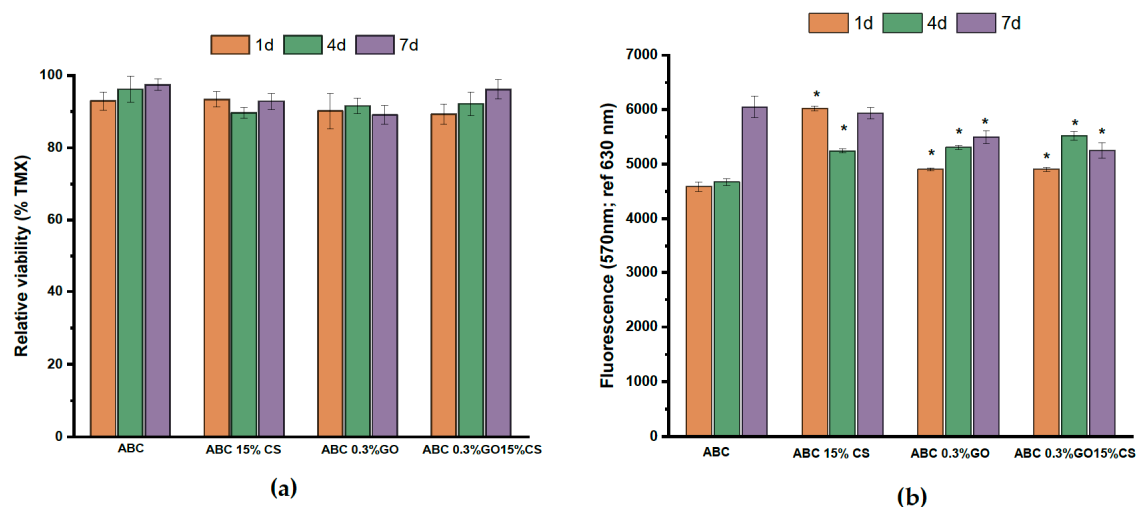


Figure 9. (a) Cell viability of human osteoblasts in the presence of extracts of ABCs taken after 1, 4, and 7 days; (b) Direct Alamar Blue results for control ABC and modified ABCs over a period of 1, 4, and 7 days. Results are the mean $\pm$ SD. Statistical analysis ($n = 12$) for each group was performed comparing experimental ABCs samples respect to ABC group at a significance level of $p < 0.01$.

• Osteogenic Properties

Biochemical detection of ALP activity after seven and 21 days of incubation was used as an indicator of osteoblast phenotype [51]. Figure 10 shows that the modification of ABCs with chitosan clearly enhanced ALP activity from the early stages, thus inducing osteoblastic activity in both experimental ABC 15% CS and ABC 0.3GO-15CS groups. Although researchers Unagolla and Jayasuriya confirmed that the GO increased the activity of ALP PCL scaffolds [49], in this research we found that the greatest effect on the ALP activity was due to chitosan. This polysaccharide has been well studied for bone tissue engineering as osteoconductive and osteoinductive material [14], since it induces the proliferation of osteoblast and mesenchymal cells, and induces in vivo neovascularization [27]. Likewise, other researchers indicated that both GO and CS have a high potential in orthopedic tissue engineering [19] thanks to the fact that they have shown an increase in the proliferation of chondrocytes [21]. They induce attachment and proliferation of mouse mesenchymal stem cells (MSCs) and stimulate expression of the osteogenic gene osteocalcin, thus leading to differentiation of MSCs into bone [50]. They are cytofriendly to rat osteoprogenitor cells, enhance differentiation of MSCs into osteoblasts, and also accelerate bridging of the rat tibial bone defect with increased collagen deposition [52]. Additionally, they promote new bone formation on cranial defects of critical size in Wistar rats [53].

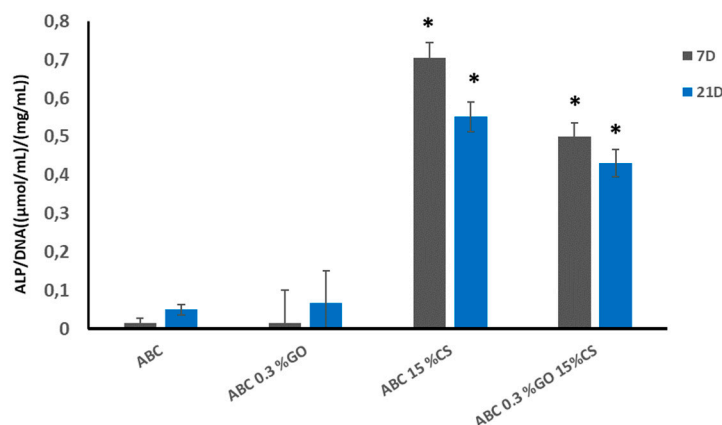


Figure 10. Variation of ALP activity expressed as the variation of p-nitrophenol concentration liberated in HOB cell cultures normalized by DNA content in the culture. Statistical analysis ($n = 8$) against control ABC group was performed at the significance level of $p < 0.01$.

- Antibacterial Activity against *E. coli*.

Immobilized CS and GO on ABC surfaces play a key role on the antibacterial effect of the experimental cements diminishing the number of viable colonies forming units on experimental cements as observed in Figure 11. However, it is clear that the main antibacterial capacity was observed in those ABCs formulated with GO. Raman surface analysis shown in Figure 4 confirmed the presence of GO sheets homogeneously distributed across the experimental ABC surfaces. Thus, this component would contribute mainly to the antibacterial activity as a result of cell membrane disruption caused by cell-surface interactions with GO [54].

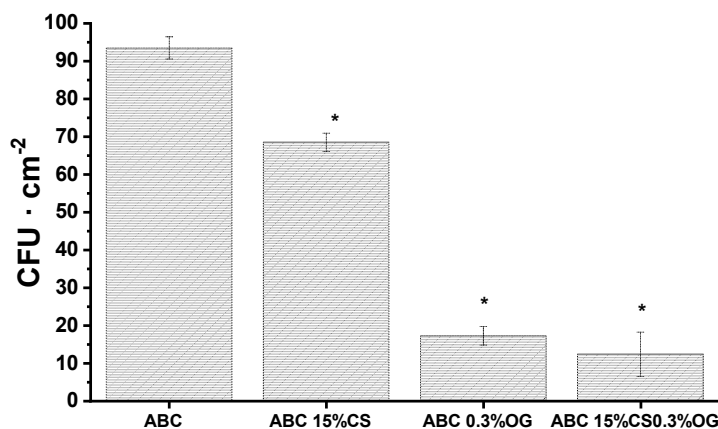


Figure 11. Number of colonies forming units recovered from ABCs surfaces after incubation of *E. coli* cultures for 24 h. Results are the mean $\pm$ SD. Statistical analysis ($n = 12$) for each group was performed comparing experimental ABCs samples respect to ABC group at a significance level of $p < 0.01$.

As mentioned above, it is clear that the presence of GO in the formulations generated the highest antibacterial effect against *E. coli* bacteria. This is confirmed by the smaller colonies forming units present in the formulations that contained GO compared with the control cement and the ABC 15% CS after 24 h of incubation. On the other hand, the antibacterial effect of the ABCs generated by CS is lower than that provided by the GO, and the presence of both GO and CS in the cement produces a synergistic effect on its antibacterial activity. It is believed that the GO antimicrobial activity occurs when the sheets come into direct contact with bacterial cells, because of their negative charge and nano-laminar structure [55]. In this process, the cell membrane seems to be the main target of GO cytotoxicity. Membrane damage has been demonstrated in bacteria exposed to GO through morphological changes

in the cellular structure, leakage of ribonucleic acid (RNA), electrolytes, intracellular absorption of membrane impermeable dyes, and changes in the transmembrane potential [56]. Additionally, oxidative stress is considered as an essential component of antimicrobial activity for bacterial cells exposed to GO [57].

3. Materials and Methods

3.1. Materials

Flake graphite (325 mesh, Alfa Aesar, Tewksbury, MA, USA), sulfuric acid (H_2SO_4), potassium permanganate (KMnO_4), hydrogen peroxide (H_2O_2), and 2-propanol (Merck, Burlington, MA, USA) were used for the synthesis of the GO. PMMA microparticles (New Stetic SA, Medellin, Colombia), barium sulfate (BaSO_4) (Alfa Aesar, Tewksbury, MA, USA), benzoyl peroxide (BPO), and chitosan from shrimp shells ($M_w = 190.000\text{--}310.000$ g/mol and a deacetylation degree of 88%, Sigma-Aldrich, Palo Alto, CA, USA) were used in the preparation of the solid phase of ABCs. Methyl methacrylate (MMA), 2-(diethylamino) ethyl acrylate (DEAEA), 2-(diethylamino) ethyl methacrylate (DEAEM) (Sigma-Aldrich, Palo Alto, CA, USA), and *N,N*-dimethyl *p*-toluidine (DMPT) (Merck, Burlington, MA, USA) were used in the liquid phase. All materials were used as received from the supplier, except for the BPO, which was recrystallized from methanol, and the CS, which was purified by dissolving in a 0.5 M solution of acetic acid, precipitation in a 2 M sodium hydroxide solution, and washing in water:ethanol solutions with decreasing concentrations (1:9, 3:7, 1:1, 2:1, 1:0, 1:0). Then it was frozen, lyophilized, and finally milled and passed through a 150 μm sieve.

3.2. Synthesis of GO

The GO synthesis was carried out using the modified Hummers method, following the methodology proposed by Mangadlao et al. with some modifications [58]. Briefly, 3 g of graphite were mixed for 10 min in a flat bottom flask, and then 3 g of KMnO_4 were added to the mixture. The reaction remained in constant agitation all the time and every 24 h, 3 g of KMnO_4 were added three more times. The reaction was stopped 24 h after the last addition of KMnO_4 by taking a third of the mixture (approximately 134 mL) and mixing it with 300 mL of an ice water/Milli Q water mixture. After 10 min 2 mL of H_2O_2 were added and stirring was continued for 10 min. The mixture was transferred to 50 mL falcon tubes and centrifuged for 10 min at 4000 rpm. The supernatant was discarded, and the sediment was washed twice with Milli Q water followed by centrifugation. The purification was then carried out using 2-propanol until reaching a neutral pH. The solution was dialyzed for three days in isopropanol using SnakeSkin Dialysis Tubing, 3.5K MWCO (Thermo Scientific, Suwanee, GA, US). Finally, two new washes were made with Milli Q water, after which the graphite oxide was frozen at -40 °C for at least 24 h and lyophilized at -51 °C and 0.12 mBar pressure for 24 h in a Freezone freeze dryer 4.5 (Labconco, Kansas City, MO, US).

3.3. Characterization of GO

The interplanar distance of the GO sheets was measured by X-ray diffractometry (XRD) in a PANalytical X'Pert PRO diffractometer (Malvern Panalytical, Jarman Way, Royston, UK), using $\text{Cu K}\alpha 1$ radiation (1.540598 Å) and $\text{K}\alpha 2$ (1.544426 Å), in a 2θ range between 5° and 40° . The Raman spectrum was measured using an Invia Raman Microscope with a wavelength laser of 514.5 nm (Renishaw, New Mills, Gloucestershire UK). The thickness of the nanosheets was determined by atomic force microscopy (AFM) in tapping mode using a Multimode AFM (Veeco Instruments, Santa Barbara, CA, USA) equipped with a Nanoscope Iva control system (software version 6.14r1). Silicon tapping probes (RTESP, Veeco) were used with a resonance frequency of ~ 300 kHz, and a scan rate of 0.4 Hz, 5×5 , 2×2 μm^2 AFM images were taken for each sample. Topography was examined by topographical AFM. Finally, the size of the particles was determined in a Zetasizer Nano ZS DLS (Malvern Panalytical, Jarman Way, Royston, UK).

3.4. Preparation of ABC

The composition of the ABCs nanocomposites is shown in Table 2. In the solid phase, BaSO₄ was used as a radiopaque agent and BPO acted as an initiator of the MMA free radical polymerization reaction. In the liquid phase, methyl methacrylate was the main monomer, and 2-(diethylamino) ethyl acrylate (DEAEA) and 2-(diethylamino) ethyl methacrylate (DEAEM) were used as comonomers to improve the bioactivity of ABCs. The DMPT was the activating agent of the polymerization reaction because it decomposes the BPO through a process of oxide-reduction by transfer of electrons to initiate the polymerization reaction at room temperature. CS and GO were added as bioactive fillers.

Table 2. Composition of ABCs formulations.

Formulation	Solid Phase (% w/w)				Liquid Phase (% w/w)			
	PMMA	BaSO ₄	BPO	CS	MMA	DEAEM/DEAEA	DMPT	GO
Control ABC	88	10	2	0	95.5	2	2.5	0
ABC 0.3 % GO	88	10	2	0	95.2	2	2.5	0.3
ABC 15 % CS	73	10	2	15	95.5	2	2.5	0
ABC 0.3GO-15CS	73	10	2	15	95.2	2	2.5	0.3

The GO was dispersed in the liquid phase for 1 h by ultrasound (J.P. Selecta, Barcelona, Spain) and the cements were prepared manually by mixing the solid and the liquid, maintaining the solid/liquid ratio in 2 g/mL. The cement paste was placed into Teflon molds with the shape required for each test.

3.5. Characterization of ABC

3.5.1. Chemical Characterization

- Infrared Spectrometry with Fourier Transform (FTIR)

Combined attenuated total internal reflectance/Fourier transform infrared (ATR-FTIR) spectroscopy spectra were recorded on a Spectrum One spectrometer equipped with an ATR accessory (Perkin-Elmer, Waltham, MA, US).

- Residual Monomer

Proton nuclear magnetic resonance spectra (¹H NMR) were recorded in a Bruker Avance III HD-400 equipment at 25 °C. ABCs were dissolved in deuterated chloroform (CDCl₃) one week after being prepared. The residual monomer (RM) was calculated by integrating the signals of the methoxyl protons of the MMA and the PMMA using Equation (1):

$$RM(\%) = \frac{A_{MMA}}{A_{MMA} + A_{PMMA}} \times 100 \quad (1)$$

where A_{MMA} is the signal area of methoxyl protons of MMA ($\delta = 3.7$ ppm), and A_{PMMA} is the signal area of methoxyl protons of PMMA ($\delta = 3.5$ ppm). A minimum of five replicates were analyzed per sample. Values were given as average $\pm$ standard deviation (SD).

3.5.2. Physical Characterization

- Scanning Electron Microscopy (SEM)

The morphology and structure of ABCs were observed using an environmental scanning electron microscope (ESEM) Philips XL30 with tungsten filament (Philips, Eindhoven, The Netherlands). ABC cylinders with the dimensions of the compression specimens were cooled with liquid nitrogen and subsequently fractured. SEM examination was performed on the fracture zone.

- Raman Spectroscopy

Surface mapping was measured with an Invia Raman microscope (Renishaw, New Mills, Gloucestershire UK) using a 785 nm diode laser. The Raman band of GO about 1324 cm⁻¹ and the band of ABC matrix about 1448 cm⁻¹ were used as wavenumber markers. The Raman mapping of ABC with GO was done over 20 × 20 μm² scanning areas.

3.5.3. Thermal Characterization

- Thermogravimetric Analysis (TGA)

Thermogravimetric analysis (TGA) curves were obtained in a thermogravimetric TGA Q500 instrument (TA instruments, New Castle, DE, US). Samples were analyzed in a range of 50–500 °C under nitrogen at a heating rate of 10 °C/min. From the thermograms, the thermal stability was obtained.

3.5.4. Mechanical Characterization

- Compressive Strength

The compression test specimens consisted of cylinders of 6 mm in diameter and 12 mm in height, and the cement was tested 24 h after mixing. The test was performed on an Instron 3366 universal testing machine (Instron, Norwood, MA, US) with a 5 kN cell at a load application speed of 20 mm/min according to ISO 5833 [40].

- Dynamic Mechanical Analysis (DMA)

The test was performed in a DMA Q800 (TA instruments, New Castle, DE, US), in dual cantilever mode. The frequency used was 1 Hz, with a working width of 20 μm. The temperature used was from −100 to 200 °C with a heating rate of 3 °C/min and an isotherm at −100 °C for 10 min. The specimens consisted of rectangular beams of 46 mm × 6 mm × 4 mm. The glass transition temperature and the storage modulus were determined from this test.

3.5.5. Biological Characterization

- Hydrolytic Degradation

The hydrolytic degradation test was carried out following the specifications of ASTM F1635-16 [59]. Cylindrical test pieces 6 mm in diameter and 12 mm in height were immersed in Dulbecco's phosphate buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO, US) for 7, 14, and 21 days. The initial weight of the samples before immersion was recorded as W_0 , the wet weight after immersion and surface drying with the absorbent paper was recorded as W_w and the weight after drying for at least 72 h in the incubator at 37 °C registered as W_d . The weight loss (W_t) and the water absorption were calculated by Equations (2) and (3), respectively. In all the experiments, a minimum of three samples were averaged:

$$W_t (\%) = \frac{W_0 - W_d}{W_0} \times 100 \quad (2)$$

$$\% \text{ water uptake} = \frac{W_w - W_d}{W_d} \times 100 \quad (3)$$

- Cell Viability

MTT Assay

Methylthiazol tetrazolium (MTT) test was used to determine the cell toxicity of the ABC in the presence of human osteoblasts cells (ECACC 06090739). Disc-shaped specimens of 10 mm in diameter

and 1.5 mm thickness sterilized were submerged in 5 mL of complete medium (Dulbecco's modified Eagle's medium (DMEM)) and incubated at 37 °C for 1, 4, and 7 days.

Human osteoblast (HOB) cells at the 8th passage were seeded in 96-well plates at a density of 9×10^4 cell/well, and 100 µL of the corresponding exposure medium were added to each well. The complete medium was used as a target. The cytotoxicity of the ABC extracts was assessed by MTT (Sigma, UK) assay. The human osteoblast cells seeded in 96 well plates were exposed to the sample extracts for 24 h. The medium was then replaced by 100 µL of 0.5% MTT solution in phosphate buffered saline (PBS), and the cells were incubated at 37 °C for another 4 h. After that, the blue precipitated formazan dye was dissolved by the addition of 100 µL of dimethylsulphoxide (DMSO).

The optical density of the wells was measured at 570 nm using a microplate reader Synergy HT (Biotek, Winooski, VT, US). Percentage of cell viability was calculated by Equation (4):

$$\% \text{ Cell viability} = \frac{DO_s - DO_T}{DO_C} \times 100 \quad (4)$$

where DO_s , DO_T , and DO_C are the optical density measurements of the sample, the target (MEM introduced into wells without cells) and the negative control (Thermanox), respectively.

Alamar Blue Assay

HOB cells were seeded at a density of 9×10^4 cell/mL and cultured for 24 h over the testing specimens placed in 24-well culture plate. After that, 2 mL of Alamar Blue dye (10% Alamar Blue solution in phenol red free DMEM medium) were added to each specimen. After 4 h of incubation 100 µL ($n = 4$) of culture medium for each test sample were transferred to a 96-well plate, and the absorbance was measured at 490 nm on a Biotek ELX808IU. The specimens were washed with PBS twice to remove the rest of the reagent, and 1 mL of culture medium was added to monitor the cells over the materials. This step was done at 1, 4, and 7 days. Results are given as mean $\pm$ SD. Analysis of variance (ANOVA) of the results for experimental ABCs was carried out with respect to control ABC at a significance level of $p < 0.01$.

- Osteogenic Properties

ALP Assay

The alkaline phosphatase (ALP) activity was evaluated using a colorimetric assay kit (Abcam, USA, ab83369) in confluent HOB cells seeded on the surface of sterilized disc-shaped specimens of 10 mm in diameter and 1.5 mm thickness (9×10^4 cell/disc) after seven and 21 days of incubation. ALP catalyzes the hydrolysis of p-nitrophenyl phosphate (pNPP) to p-nitrophenol. It has a strong absorbance at 405 nm. The rate of the increased absorbance at 405 nm is proportional to the enzyme activity. Cell-free specimens were run alongside to correct for nonspecific background activity and all the results were normalized to DNA content as quantification for the number of cells. Total DNA was measured using the PicoGreen dsDNA quantitation kit (Madrid, Spain, Fisher Scientific, P-7589, Molecular Probes). Results are given as the mean $\pm$ SD. ANOVA of the results of tested materials was performed comparing the corresponding experimental ABC with respect to control ABC at each time point ($p < 0.01$).

- Antibacterial Activity against *E. coli*

Antibacterial activity of ABCs loaded with CS and GO was evaluated versus Gram-negative bacterium *E. coli* (DH5 α). Bacterial concentration was estimated spectrophotometrically at 600 nm and adjusted with sterile PBS to an OD600 equivalent to 10^8 CFU/mL. The ability of bacteria to form biofilms was analyzed by colony-forming units (CFU) determined by standardized plate counting agar techniques. Briefly, sample discs of 10 mm diameter were placed into a separate well of a 24-well plate with the test surface facing up. The plate was introduced in a plastic container wet filter paper

beneath to maintain a relative humidity of 90%. 100 µL of test inoculums (10^5 CFU·cm⁻²) prepared in 1/500 diluted Nuria Broth medium (Sparks, MD, USA, Difco) were placed onto the substrates and incubated for 24 h at 37 °C. Afterwards, samples were washed with 1 mL of PBS, (pH 7.2) and the numbers of CFU recovered from each sample disks were determined by standardized plate counting agar techniques and referred as CFU (N)/cm². The mean reported for each concentration of filler was based on three replicates.

3.6. Statistical Analysis

Statistical analysis was performed using a Student's t-test. Values of compressive strength, degradation, cell viability and antibacterial activity were presented as the mean ± SD. Differences were considered statistically significant at $p < 0.01$.

4. Conclusions

The results of this research showed that all the ABCs developed formulations presented excellent biocompatibility, which shows their potential to be used in biomedical applications. Moreover, the presence of GO in the ABCs provided mechanical reinforcement and antimicrobial activity while the addition of CS favored the generation of pores in the cement, as evidenced by SEM, decreased the compression strength and increased the bioactivity of the cement, increasing the ALP activity.

A synergistic effect on the physical, thermal, mechanical, and antibacterial properties, along with a rise in the osteogenic activity was evidenced for the ABC nanocomposites formulated with both 15% of CS and 0.3% of GO (CS + GO). These results suggest that this formulation possesses a high potential to be used as an antibacterial bioactive cement in orthopedic applications.

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Article

Novel Bioactive and Antibacterial Acrylic Bone Cement Nanocomposites Modified with Graphene Oxide and Chitosan

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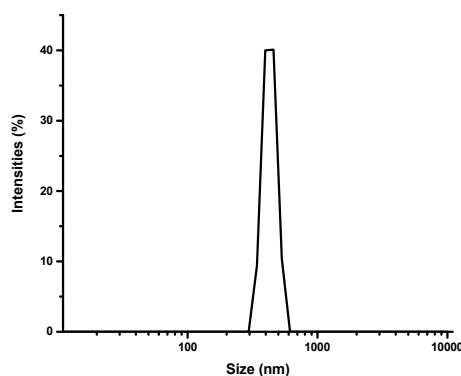


Figure 1. Particle size by Dynamic light scattering (DLS).

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CEMENTO ÓSEO ACRÍLICO BIOACTIVO Y ANTIBACTERIAL MODIFICADO CON ÓXIDO DE GRAFENO Y QUITOSANO

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RESUMEN

Los cementos óseos acrílicos (ABC) han desarrollado un papel fundamental en la cirugía ortopédica para la remodelación de huesos cancerosos, craneoplastias y vertebroplastias. Sin embargo, estos materiales presentan algunas limitaciones relacionadas con la alta temperatura de polimerización, el comportamiento inerte y la presencia de monómero residual que puede provocar la necrosis térmica y química de los tejidos circundantes alrededor del material implantado. Se han desarrollado ABCs basados en quitosano (CS) con el fin de impartir propiedades biomiméticas. Por otra parte, el uso de nanopartículas de óxido de grafeno (GO) en aplicaciones biomédicas se ha convertido en un tema de investigación muy interesante debido a su alto grado de refuerzo mecánico en biomateriales y su baja toxicidad. En este trabajo, se estudió el efecto de la combinación de CS y GO en los ABCs y su potencial para ser utilizados como cementos óseos bioactivos y antibacterianos en aplicaciones ortopédicas.

Palabras clave:

Cementos óseos acrílicos; quitosano; óxido de grafeno; bioactividad; actividad antibacterial.

ABSTRACT

Acrylic bone cements (ABC) have played a key role in orthopaedic surgery for remodeling of cancerous bones, cranioplasties and vertebroplasties. However, these materials present some limitations related to high polymerization temperature, inert behavior and the presence of unreacted monomer that can led into thermal and chemical necrosis of the surrounding tissues around the implanted material. Bioactive chitosan (CS) based ABC have been developed in order to impart biomimetic properties. Meanwhile using graphene oxide (GO) nanoparticles in biomedical applications has become a hot-topic research due to its high degree of mechanical reinforcement to biomaterials and low toxicity. This work studies the unexplored effect of combined CS and GO on ABC nanocomposites and their potential to be used as bioactive and antibacterial cements in orthopaedic applications.

Keywords:

Acrylic bone cement; chitosan; graphene oxide; bioactivity; antibacterial activity.

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I. INTRODUCCIÓN

Los cementos óseos acrílicos han tenido durante años un papel importante en la cirugía ortopédica [1] y poco a poco se extiende su aplicación en la remodelación de áreas de cáncer metastásico u osteoporótico, en fracturas, en la reparación de defectos craneales (craneoplastias) y en la reparación de las fracturas vertebrales (vertebroplastia y la cifoplastia) [1].

Se conoce como artroplastia a la cirugía de reemplazo de alguna articulación; durante esta, la mayoría de las prótesis articulares son fijadas usando un cemento óseo acrílico que actúa como un pegamento entre el implante y el hueso, cuyas funciones principales son la transferencia de las cargas de servicio y del peso corporal de la prótesis al hueso y la inmovilización inmediata de la prótesis. En las artroplastias, la más frecuente complicación a largo plazo es el aflojamiento de la prótesis articular; sin embargo, la causa exacta, no está clara, pero en muchos casos se relaciona con problemas en el cemento óseo [2].

La interfaz hueso/cemento es la clave para incrementar la longevidad de las prótesis articulares [3], y es por eso que en las últimas décadas se ha trabajado fuertemente en superar limitaciones de los cementos tales como su alta temperatura de polimerización que genera necrosis térmica del tejido próximo, su comportamiento inerte que genera la formación de un tejido fibroso a su alrededor después de ser implantado en el cuerpo, y la liberación de monómero residual que genera necrosis química del tejido alrededor; ya que todos estos problemas conllevan al aflojamiento aséptico de las prótesis; además de las posibilidades de crecimiento de bacterias en el implante.

El quitosano (CS) es un polisacárido lineal, semicristalino y el principal derivado de la quitina que es el segundo polímero natural más abundante después de la celulosa [4], además, es un polielectrolito con grupos funcionales reactivos [5]. La quitina no ha encontrado aplicaciones prácticas

debido a su pobre solubilidad en soluciones acuosas y en casi todos los solventes orgánicos [5,6]. El CS se caracteriza por ser biodegradable, biocompatible, hidrofílico, mucoadhesivo, promotor del crecimiento de células y la deposición de matriz rica en mineral por los osteoblastos, hipotóxico; además de poseer propiedades antibacterianas y antifúngicas [5,7–11].

El óxido de grafeno (GO) consiste en una hoja de grafeno con una distribución aleatoria de funcionalidades ricas en oxígeno sobre su superficie. A pesar de los defectos que estos grupos generan en las laminillas del grafeno, las hojas oxidadas conservan casi intactas sus propiedades mecánicas mostrando valores de módulos de Young tan elevados como 0,25 TPa [12]. El GO presenta excelentes propiedades antibacterianas, es biocompatible y además es mucho más soluble que el grafeno.

Por lo anterior, en esta investigación se planteó el estudio de la influencia de la adición de quitosano y óxido de grafeno sobre las propiedades físicas, mecánicas y biológicas de los cementos óseos acrílicos, con el objetivo final de desarrollar una formulación de cemento bioactivo para aplicación en artroplastia.

2. MATERIALES Y MÉTODOS

2.1. Materiales

La fase sólida estaba compuesta por perlas de polimetil metacrilato (PMMA) (New Stetic SA, Medellín, Colombia), sulfato de bario (BaSO_4) (Alfa Aesar, Tewksbury, MA, EE. UU.) Y peróxido de benzoilo (BPO) (Sigma-Aldrich, Palo Alto, CA, USA) y quitosano (CS) de mediano peso molecular. La fase líquida estaba compuesta por metacrilato de metilo (MMA), acrilato de 2- (dietilamino) etilo (DEAEA), metacrilato de 2- (dietilamino) etilo (DEAEM) (Sigma-Aldrich, Palo Alto, CA, EE. UU.), N, N- dimetil p-toluidina (DMPT) (Merck, Burlington, MA, USA) y GO sintetizado mediante el método de Hummers modificado [13]. Todos los materiales se usaron

como se recibieron del proveedor, excepto el BPO que fue recristalizado en metanol.

2.2 Preparación de los cementos

Los ABCs fueron preparados de acuerdo con las especificaciones de la ISO 5833-02 [14]. Se prepararon cuatro formulaciones de ABCs correspondientes a ABC 0% (No contiene CS ni GO), ABC con 0,3% de GO, ABC con 15% de CS y ABC con 0,3% de GO y 15% de CS cuyas composiciones se presentan en un estudio previo [13]. La fase sólida y líquida se prepararon mediante la mezcla manual de los componentes y posteriormente el líquido fue adicionado al sólido y la pasta fue mezclada manualmente y vaciada en moldes de Teflón con la forma especificada para cada ensayo.

2.3 Caracterización de los cementos

La resistencia a compresión de los cementos se evaluó en una máquina de ensayos universales Tinius Olsen-H50KS (Tinius Olsen, Redhill, Reino Unido), siguiendo las recomendaciones establecidas en la ISO 5833-02 [14]; seis muestras fueron ensayadas por cada formulación. El contenido de monómero residual en las formulaciones fue determinado mediante resonancia nuclear protónica ($^1\text{H-NMR}$). Se evaluó la degradación hidrolítica de las formulaciones sumergidas en solución de fosfato buferada (PBS), mediante el seguimiento del peso de las muestras a distintos tiempos de inmersión. La actividad antibacteriana fue evaluada frente a la bacteria Gram negativa *Escherichia Coli* y la viabilidad celular frente a osteoblastos humanos [13].

3. RESULTADOS Y DISCUSIÓN

En la tabla 1 encontramos los resultados de la caracterización de los ABCs, y en esta podemos apreciar que la resistencia a compresión de los cementos varía con la composición. La incorporación del 15% de CS genera una reducción considerable de la resistencia a valores inferiores al mínimo permitido en la ISO 5833-02 para resinas acrílicas [14] que son 70MPa. Esto puede ser debido a la porosidad que genera el CS, la forma irregular de

las láminas que contienen bordes afilados que actúan como acumuladores de esfuerzo y la menor cantidad de PMMA en la formulación. La adición de 0,3% de GO a los ABCs no presenta una diferencia significativa en la resistencia a la compresión con respecto a la formulación de control. Sin embargo, cuando se agrega el GO a la formulación con 15% de CS (ABC 0.3GO-15CS), la resistencia a la compresión el valor mínimo permitido.

Tabla 1. Caracterización de los ABCs. Resistencia a compresión (R_{com}), Contenido de monómero residual (CMR) y pérdida de peso de las muestras de cemento después de estar sumergidas en PBS [13].

Formulación	R_{com} (MPa)	CMR (%)	Pérdida de peso (%)	
			7 días	21 días
ABC 0%	93,25	1,06	0.03	0.03
ABC 0,3% de GO	94,04	1,32	---	---
ABC 15% de CS	62,60	1,57	0.34	2.98945
ABC 0,3% de GO y 15 % de CS	77,23	3,60	0.03	0.5

Los resultados de CMR permiten observar que la incorporación de cada componente por separado aumentó el contenido de monómero residual de los cementos en baja proporción; sin embargo, cuando ambos materiales fueron introducidos en la formulación se observó un valor más alto. Este aumento de CMR con la presencia de cargas se debe a una menor capacidad de difusión del monómero a través de la masa de polimerización durante el proceso de polimerización, lo que conduce a una menor disipación de calor.

Los resultados de degradación hidrolítica indican que la mayor pérdida de peso en los ABCs ocurre en la formulación con adición del 15% de CS porque este es un polímero biorreabsorbible que se hidroliza en medio acuoso.

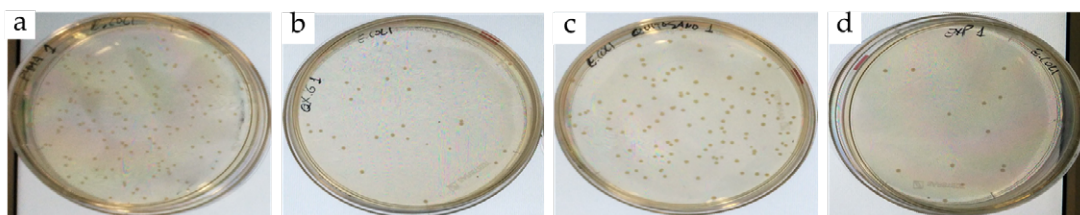


Figura 1. Unidades formadoras de colonia (UFC) de *E. coli* en contacto con: **a.** ABC 0%, **b.** ABC 0,3% de GO, **c.** ABC 15% de CS y **d.** ABC 0,3% de GO y 15% de CS.

Los resultados de la actividad antimicrobiana de los ABCs mostrados en la Figura 1, nos muestran que la presencia de GO en las formulaciones generó un marcado efecto antibacteriano contra la bacteria *E. coli*, debido a que las unidades formadoras de colonias presentes en las formulaciones que contenían GO fue mucho menor comparado con las otras formulaciones después de 24 horas de incubación de la bacteria. Por otro lado, el efecto antibacteriano generado por CS es menor que el proporcionado por GO, y la presencia tanto de GO como de CS en el cemento produce un efecto sinérgico sobre su actividad antibacteriana.

Se encuentra reportado que la actividad antimicrobiana de GO ocurre cuando las láminas entran en contacto directo con las células bacterianas, debido a su carga negativa, estructura nanolaminar y bordes afilados [15].

La biocompatibilidad de las cuatro formulaciones de cementos se evaluó mediante la prueba de MTT (3-(4,5-dimetil tiazol-2-il)-2,5-difenil tetrazolio bromuro) después de 24 h en contacto con osteoblastos humanos y en todos los casos la viabilidad celular obtenida estuvo por encima del 90%, con lo que se confirma la no citotoxicidad de las formulaciones estudiadas.

4. CONCLUSIONES

Los resultados de esta investigación confirman que todas las formulaciones desarrolladas presentaron buena biocompatibilidad, lo que demuestra su potencial para ser utilizadas en aplicaciones biomédicas. Además, la presencia de GO en los

ABCs le proporcionó refuerzo mecánico y actividad antimicrobiana.

La formulación que contiene ambas cargas estudiadas (ABC 0,3% de GO y 15% de CS) es muy prometedora para ser empleada como cemento bioactivo y biocompatible, debido a que presentó una buena viabilidad celular, la mayor actividad antibacteriana, un bajo CMR y resistencia a compresión por encima del mínimo exigido en la norma.

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Article

Osseointegration of Antimicrobial Acrylic Bone Cements Modified with Graphene Oxide and Chitosan

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Abstract: Acrylic bone cement (ABC) is one of the most used materials in orthopedic surgery, mainly for the fixation of orthopedic implants to the bone. However, ABCs usually present lack of biological activity and osseointegration capacity that leads to loosening of the prosthesis. This work reports the effect of introducing graphene oxide (GO) and chitosan (CS), separately or together, in the ABC formulation on setting performance, mechanical behavior, and biological properties. Introduction of both CS and GO to the ABC decreased the maximum temperature by 21% and increased the antibacterial activity against *Escherichia coli* by 87%, while introduction of only CS decreased bending strength by 32%. The results of cell viability and cell adhesion tests showed in vitro biocompatibility. The in vivo response was investigated using both subdermal and bone parietal implantations in Wistar rats. Modified ABCs showed absence of immune response, as confirmed by a normal inflammatory response in Wistar rat subdermal implantation. The results of the parietal bone implantation showed that the addition of CS and GO together allowed a near total healing bone–cement interface, as observed in the micrographic analysis. The overall results support the great potential of the modified ABCs for application in orthopedic surgery mainly in those cases where osseointegration is required.

Keywords: acrylic bone cement; antibacterial activity; biocompatibility; cell viability; chitosan; graphene oxide; nanocomposite; osseointegration

1. Introduction

Acrylic bone cement (ABC) is one of the most widely used materials in orthopedic surgery, and the gold standard for the fixation of orthopedic implants to bone [1]. Since ABCs are the anchoring point at the implant–bone interface, they are important for load transfer [2,3]. The main advantage of their use is a fast primary fixation with a subsequent efficient patient recovery [4].

The bone–cement interface plays a key role for the longevity of the implanted prostheses [5]. Due to this, in recent decades there has been a strong effort to overcome one of the biggest problems associated with ABCs, which is the lack of biological activity [6,7] and osseointegration [2], thus promoting ABC function based on mechanical interlocking with bone rather than adhesive chemical bonding to form a stable cement–bone interface [8]. Moreover, bone necrosis produced by high exothermic temperatures during the polymerization reaction and the susceptibility of some pathogenic bacteria [9,10] generated premature failure [7], increasing the patient complications and leading to additional interventions what is safety risky for the patient [11–13].

Bioactive fillers such as hydroxyapatite and tricalcium phosphate have been added to overcome the low prosthesis biological performance and promote direct bone apposition rather than encapsulation of the implant by fibrous tissue [14–19]. This bioactive filler addition promoted bone growth based on strong chemical interactions between cement and bone [2,20]. However, some bioactive fillers make the cement more brittle and additional modifications on the polymeric matrix are need in order to increase ductility but at expense of decreasing the modulus [12].

The presence of biodegradable substances in ABC facilitates the replacement and growth of new bone inside the cement in the pores produced by hydrolytic and enzymatic degradation in the biological medium and improves mechanical anchorage [21,22]. However, high porosity percentages in the cement produce a significant loss of mechanical properties, which is inadequate for orthopedic applications [20].

Chitosan (CS) is a linear and semi-crystalline polysaccharide consisting of β -(1→4)-*N*-acetyl-D-glucosamine and β -(1→4)-D-glucosamine units [22] widely used in bone regeneration due to its non-toxic, non-allergenic and non-immunogenic properties [23,24]. Moreover, it is mucoadhesive, biocompatible, biodegradable [25], bioactive [23], and osteoconductive, with a capacity to mimic the extracellular matrix due to the glycosaminoglycans (GAGs) similar structure [24,26]. Chitosan introduction to different biomaterials also provides very convenient antimicrobial properties in biomedical applications [24,27–30]. However, the high water affinity intrinsic property of the polysaccharide produces a serious limitation due to poor mechanical properties compared to the natural bones [22,26].

Antimicrobial fillers, such as low concentrations of graphene oxide (GO) incorporated in ABCs are interesting to promote a sepsis and mechanical reinforcement to joint prostheses [7,31]. GO can be dispersed in both aqueous and organic solvents [32], and under certain conditions it can be considered biocompatible [33] and antimicrobial [34]. In addition, it has been demonstrated that GO improves the viability and osteogenic differentiation of human bone marrow mesenchymal stem cells [35] and can be biodegraded by neutrophils [36], macrophages [37] and enzymes [33,38], suggesting its prospective for biomedical applications [39,40].

In a previous study [31], we presented the physicochemical, thermal, mechanical, and in vitro biological characterization of novel ABCs modified with CS and GO. In the present work, new research results on material setting and bending properties are reported. In addition, new in vitro assays and in vivo experiments are addressed to confirm the biocompatibility and osseointegration of the developed ABCs in the presence of cells and tissues. In particular, in vitro assays of the ABCs in the presence of human osteoblasts (HOb) and *Gramm-negative* bacteria (*E. coli*) are presented and the in vivo response of the ABCs implanted in subdermal and parietal bone tissues evaluated. The results indicate a positive synergy between CS and GO on parameters such as maximum polymerization temperature, antimicrobial activity, bioactivity, and osseointegration of ABCs demonstrating applicability in tissue engineering. Thus, it is the first report of using biodegradable and antimicrobial fillers such as GO and CS biopolymer in ABC formulations confirming the absence of immune response in Wistar rats' subdermal implantations and a practically total healing interface between bone and ACB.

2. Materials and Methods

2.1. Materials

The solid phase (SP) consisted of poly(methyl methacrylate) (PMMA) beads (New Stetic S.A., Medellin, Colombia), barium sulfate (BaSO_4) (Alfa Aesar, Tewksbury, MA, USA), CS from shrimp shells ($M_w = 190\text{--}310$ kg/mol and deacetylation degree (DD) of 88%) and benzoyl peroxide (BPO) (Sigma-Aldrich, Palo Alto, CA, USA). The liquid phase (LP) was composed of methyl methacrylate (MMA), 2-(diethylamino) ethyl acrylate (DEAEA), 2-(diethylamino) ethyl methacrylate (DEAEM) (Sigma-Aldrich, Palo Alto, CA, USA), *N,N*-dimethyl *p*-toluidine (DMPT), (Merck, Burlington, MA, USA) and GO nanosheets synthesized by the modified Hummers method (thickness < 30 nm, average size of 400 nm and interlaminar distance of 7.953 Å [31]).

2.2. Preparation of Bone Cements

Four ABC formulations were prepared (Table 1). All formulations had a solid/liquid ratio (S:L) = 2. Before manual mixing of the SP and the LP, both phases were conditioned at 23 ± 2 °C for approximately 2 h. The LP consisted of the monomer MMA, a 50:50 weight ratio of DEAEA and DEAEM, and DMPT, as an activator. Additionally, BaSO_4 , the radiopaque agent for cement radiological monitoring, was added in the SP to the PMMA beads and BPO as radical initiator.

Table 1. Composition percentage (%w/w) of the Solid Phase (SP) and Liquid Phase (LP) of the ABCs formulations.

Formulation	SP				LP			
	PMMA	BaSO_4	BPO	CS	MMA	Comonomers	DMPT	GO
Control ABC	88	10	2	0	95.5	2	2.5	0
ABC 0.3% GO	88	10	2	0	95.2	2	2.5	0.3
ABC 15% CS	73	10	2	15	95.5	2	2.5	0
ABC 0.3GO-15CS	73	10	2	15	95.2	2	2.5	0.3

2.3. Setting Properties

The setting properties of the cement paste were evaluated according to the International Standard ISO 5833-02 [41]. Right after the mixing of the ABC components, the cement was deposited in a 68 mm diameter and 20 mm high Teflon mold to record time and temperature data. The plot of the temperature against time allowed calculating the maximum temperature (T_{max}), while the setting time (T_{set}) was calculated according to Equation (1), as the time for reaching the setting temperature (T_{set}).

$$T_{set} = \frac{T_{max} + T_{amb}}{2} \quad (1)$$

where T_{amb} is the ambient temperature, T_{max} is the highest temperature recorded, the test was carried out at least three times for each formulation.

2.4. Mechanical Properties

For the four-point bending test rectangular samples of 75 mm long, 10 mm wide, and 3 mm thick were used. The test was carried out in a universal testing machine (Tinuos Olsen H50KS, Horsham, PA, USA) with a 10 kN load cell, at a rate of 5 mm/min, according to the ISO 5833-02. The bending strength and bending modulus were calculated using Equations (2) and (3), respectively.

$$B = \frac{3Fa}{bh^2} \quad (2)$$

$$E = \frac{\Delta Fa}{4fbh^3} \times (3l^2 - 4a^2) \quad (3)$$

where B is the bending strength, E is the bending modulus, F is the force at break, b is the average measured width of specimen, h is the average measured thickness of specimen, a is the distance between the inner and outer loading points, ΔF is the load range (50 N – 15 N = 35 N), f is the difference the deflections under the loads of 15 N and 50 N, l is the distance between the outer loading points. At least six specimens were tested for each formulation.

2.5. In Vitro Studies

2.5.1. Antibacterial Activity against *Escherichia coli*

Antibacterial activity of ABCs loaded with CS and GO was evaluated against the *Gram-negative* bacteria *E. coli* (DH5 α), by colony-forming units (CFU) determined by standardized plate counting agar method reported previously [31]. Briefly, bacterial concentration was estimated at 10^8 CFU/mL. An aliquot of 100 μ L of the inoculum test (10^5 CFU $\cdot$ cm $^{-2}$) prepared in 1/500 diluted Nuria Broth medium (Sparks, MD, USA, Difco) was placed onto the surface of sterile discs of 10 mm diameter and incubated for 24 h at 37 °C. Afterwards, samples were washed with 1 mL of phosphate buffer solution (PBS) (pH 7.2) and the numbers of CFU recovered from each sample disc were determined by standardized plate counting agar techniques and referred as CFU (N)/cm 2 . The mean reported for each concentration of filler was based on three replicates. Later, samples of ABCs were observed in a scanning electronic microscopy (SEM) Philips XL30 with tungsten filament (Philips, Eindhoven, The Netherlands).

2.5.2. Cell Viability

ABCs cell viability with human osteoblasts cells (HOb) (ECACC 06090739) was carried out by the methylthiazol tetrazolium (MTT) assay as previously reported [31]. Briefly, the corresponding sterilized disc, 10 mm in diameter and 1.5 mm of thickness, was submerged in 5 mL of Dulbecco's modified Eagle's medium-DMEM and incubated at 37 °C for 1, 14, and 21 days. HOb were seeded in a 96-well plate (9×10^4 cells/well) and exposed to the sample extracts for 24 h; then, a MTT solution (0.5% MTT (Sigma, St. Louis, MO, USA) in phosphate buffered saline) was added into the well. After 4 h of incubation at 37 °C, cell viability was measured using the optical density of the wells at 570 nm in a microplate reader Synergy HT (Biotek, Winooski, VT, USA). Percentage of cell viability was calculated by Equation (4):

$$\% \text{ Cell viability} = \frac{OD_s - OD_T}{OD_C} \times 100 \quad (4)$$

where OD_s , OD_T , and OD_C are the optical density measurements of the sample, the target (MEM medium introduced into wells without cells) and the negative control (Thermanox $^{\text{®}}$), respectively.

2.5.3. Cell Adhesion

HOb cells were seeded at a density of 9×10^4 cell/mL and cultured for 24 h over the testing specimens placed in a 24-well culture plate. After that, 2 mL of Alamar Blue dye (10% Alamar Blue solution in phenol red free DMEM medium) were added to each well. After 4 h of incubation, specimens were washed twice with PBS in order to remove the rest of the reagent, and 1 mL of culture medium was added to monitor cell adhesion on the materials. This step was done at 1, 7, 14, and 21 days of incubation and afterwards samples were fixed in an aqueous solution of glutaraldehyde 2.5% *v/v*. Finally, the ABCs were observed in a scanning electronic microscopy (SEM) Philips XL30 with tungsten filament (Philips, Eindhoven, The Netherlands).

2.6. In Vivo Studies

The in vivo studies followed the recommendations of ISO 10993-6 [42] related to the selection and implanted zone requirements for biomodel tests. This research was reviewed, supported, and approved

by the Institutional Ethics Review Committee with experimental animals of the Universidad del Valle (Cali, Colombia) through act 001 of 2012.

2.6.1. Subdermal Implantation

Implantation of samples corresponding to the four ABCs formulations (Table 1) was performed in subdermal tissue of nine male Wistar rats (three months old and 280 g average weight) to evaluate the *in vivo* biocompatibility. Porcine collagen membrane samples (Membracel[®], Buenos Aires, Argentina) were used as a control for biocompatible foreign membranes. The animals were supplied and hosted by the LABBIO laboratory at the Universidad del Valle, Cali, Colombia.

Each experiment consisted of an incision 1 cm long by 3 cm deep on the dorsal surface of the rats. The procedure started with sedation by intramuscular application of 70 mg/kg of ketamine and 30 mg/kg of xylazine (HOLLIDAY SCOTTS, Buenos Aires, Argentina). After 1 month of implantation, the animals were euthanized by applying pentobarbital sodium (390 mg/mL) and diphenylhydantoin sodium (50 mg/mL) (Euthanex[®], laboratory INVENT, Colombia) in a dose of 100 mg/kg with intraperitoneal application.

The recovered samples were fixed for 48 h in buffered formalin; then, the soft tissue capsule surrounding the implanted material was opened to remove the bone cement samples and analyze the morphology by SEM (JEOL Model JSM 6490 LV, Akishima, Tokyo, Japan).

Soft tissue samples were processed using a Leica TP1020 automatic tissue processor (Leica Biosystems, Buffalo Grove, IL, USA), in alcohol solutions with ascending concentration (70, 80, 95 and 100%) and xilol. Right after, the processed tissue samples were embedded in paraffin blocks using a Shandon Histocentre[™] REF8330 embedding center (Thermo Fisher Scientific[™], Waltham, MA, USA) and cut into 5 μ m sheets using a microtome (Leica Biosystems RM2245, Buffalo Grove, IL, USA). The cut tissue samples were stained with Hematoxylin-Eosin and Masson's Trichrome techniques. For the analysis of histological images, a Leica DM 750 microscope coupled to a Leica DFC camera was used, and the data were processed using the Leica Suite software version 4.12.0 (Leica Microsystems, Wetzlar, Germany).

2.6.2. Bone Implantation Study

Following the same sedation protocol of the subdermal implantation, a defect of 5 mm in diameter was made in the parietal bone of 15 Wistar rats of 8 months old and 370 g average weight. The generated defect was filled with the corresponding ABC formulation and the defect sutured as observed in the photography of the parietal bone animal zones (Figure S1).

After 3 months of implantation, animals were euthanized following the same procedure described in the subdermal implantation. Tissues of 1 $\times$ 1 cm² containing the implanted ABC and original peripheral bone were recovered. Samples were fixed in 10% buffered formalin for 48 h, washed with distilled water and dehydrated by passing through increasing ethanol solutions of 30, 50, 70 and 90% for 10 min and in pure ethanol for 1 h. Finally, the samples were stored for at least 48 h in a desiccator at room temperature and analyzed by SEM and x-ray energy dispersive spectrometry (EDS) techniques (JEOL Model JSM 6490 LV, Akishima, Tokyo, Japan). In this analysis, areas of interest at the cement, interface, and bone zones were analyzed for each sample composition as illustrated in the supporting information section.

2.7. Statistical Analysis

Values of setting and mechanical properties, cell viability, and antibacterial activity were presented as mean $\pm$ SD. Statistical analyses was performed using Student's *t*-test. Statistical analysis was performed comparing modified ABCs samples with respect to Control ABC. Differences were considered statistically significant at * $p < 0.05$ and ** $p < 0.01$.

3. Results and Discussion

3.1. Setting Properties

Table 2 demonstrates that CS and GO separately added to the cement decreased the maximum temperature (T_{max}) during polymerization and increased the setting time (T_{set}). However, the formulation containing both components presented a synergy in reducing T_{max} and increasing T_{set} by more than 21%. All the values of setting parameters in Table 2 are within the limits allowed by ISO 5833-02 [41], which sets a maximum temperature limit of 90 °C and a curing time between 3 and 15 min.

Table 2. Maximum temperature (T_{max}) and setting time (T_{set}) values recorded for ABCs formulations. Data are reported as a mean $\pm$ SD.

Formulation	T_{max} (°C)	T_{set} (min)
Control ABC	59 $\pm$ 3	355 $\pm$ 30
ABC 0.3% GO	51 $\pm$ 4	420 $\pm$ 25
ABC 15% CS	55 $\pm$ 4	460 $\pm$ 20 *
ABC 0.3GO-15CS	46 $\pm$ 3 *	440 $\pm$ 10 *

Asterisks denote significant differences of modified ABC samples with respect to Control ABC at a significance level of * $p < 0.05$.

Reduction of T_{max} is particularly important to assure the patient's healthiness, reducing the risk of tissue necrosis. Some reported cements had T_{max} up to 100 °C demonstrating a high patient risk [43].

According to Paz et al. [44], the decrease in polymerization heat observed in GO formulations, suggests that it retards and inhibits the curing reaction by interfering with the radical reaction. This was also confirmed by Ormsby et al. [45] who proposed that the chemical interaction between PMMA and carboxyl functionalized multi-walled carbon nanotubes (MWCNT-COOH) occurs through two mechanisms: A covalent bonding of the MWCNTs with the growing polymer chains via esterification reactions, or GO nanosheets acting as heat dissipaters.

On the other hand, Gonçalves et al. [46] proposed that GO acts as a radical scavenger that inhibits or delays the free radical polymerization reaction, which is consistent with the increase in setting time and the T_{max} reduction. CS addition in ABCs has also been observed as the main factor for decreasing T_{max} through a heat dissipation mechanism [9].

3.2. Mechanical Properties

According to standard ISO 5833-02 [41] compressive and bending properties of ABC are crucial for good biomechanical performance of ABCs. In a previous paper results of compression test of developed ABCs were already reported [31]. Table 3 presents results of the bending test reported in this paper. Compression and bending strengths and bending modulus were significantly reduced ($p < 0.01$) with the incorporation of 15% CS compared to Control ABC; even when 15% CS was added together with 0.3% GO compressive strength significantly decreased although it still remained higher than 70 MPa that is the minimum value allowed by the standard. However, for the ABC that contained both CS and GO bending strength was not significantly different with respect to Control ABC. On the other hand, the introduction of GO to ABCs by its own did not showed significant differences of the mechanical properties with respect to Control ABC.

Some researchers have reported improvements in tensile, compressive [7] and flexural properties [13] in bone cements with GO incorporation. The improvement is caused by a stronger interfacial interaction between GO and PMMA generated by a high amount of hydroxyl and carboxyl groups present on the GO surface [44].

On the other hand, some studies reported negative effects in the mechanical properties with CS introduction [47,48]. The interference of the polysaccharide in the polymerization of MMA and the irregular morphologies of this component that acts as an accumulator of stress are the main reasons for the detrimental effect [31]. Despite that, evidence of the reinforcing effect of GO functionalization

with CS has been reported [49–51] arguing chemical reactions between the amino groups of CS and the carboxyl groups of the GO, what indicates that the presence of both fillers is crucial for the properties improvement.

Table 3. Compressive strength (C), bending strength (B) and bending modulus (E) in ABCs formulations. Data are reported as a mean $\pm$ SD.

Formulation	C (MPa) [31]	B (MPa)	E (MPa)
Control ABC	93.3 $\pm$ 2.2	48.7 $\pm$ 4.2	2593 $\pm$ 178
ABC 0.3% GO	94.0 $\pm$ 0.5	51.8 $\pm$ 0.4	2599 $\pm$ 154
ABC 15% CS	62.6 $\pm$ 1.1 **	32.8 $\pm$ 2.9 **	2141 $\pm$ 212 **
ABC 0.3GO-15CS	77.2 $\pm$ 2.6 **	44.6 $\pm$ 4.1	2379 $\pm$ 132 *

Asterisks denote significant differences of modified ABC samples with respect to Control ABC at significance levels of * $p < 0.05$; ** $p < 0.01$.

3.3. In Vitro Studies

3.3.1. Antibacterial Activity against *Escherichia coli*

E. coli has been used in this research only as a model to evaluate the antimicrobial activity of different modifications of bone cements and prove that the biological activity of the GO and CS is maintaining after curing reaction of ABC. Previous results [31] shows that the CFU of *E. coli* for Control ABC, ABC 0.3% GO, ABC 15% CS and ABC 0.3GO-15CS were 96 ± 3 , 17 ± 2 , 69 ± 2 and 12 ± 6 , respectively. The former indicate than the effect of GO incorporation on the antibacterial activity of ABCs was much more significant compared to the introduction of only CS. ABCs loaded with GO showed a reduction in CFU by more than 80%, while those containing only CS reduced the number of colonies by 28%, as compared to the Control ABC. The result obtained for ABCs with both loadings (CS and GO) did not show statistical significance comparative with ABC loaded with 0.3% of GO.

SEM micrographs of cements with bacteria (Figure 1) confirmed a higher bacterial population with the formulations that did not contain GO. These results suggested that GO introduction is the main caused for antibacterial activity of ABCs and is promising for clinical applications devoted to avoiding bacterial infections, a common health risk during surgeries and implantations.

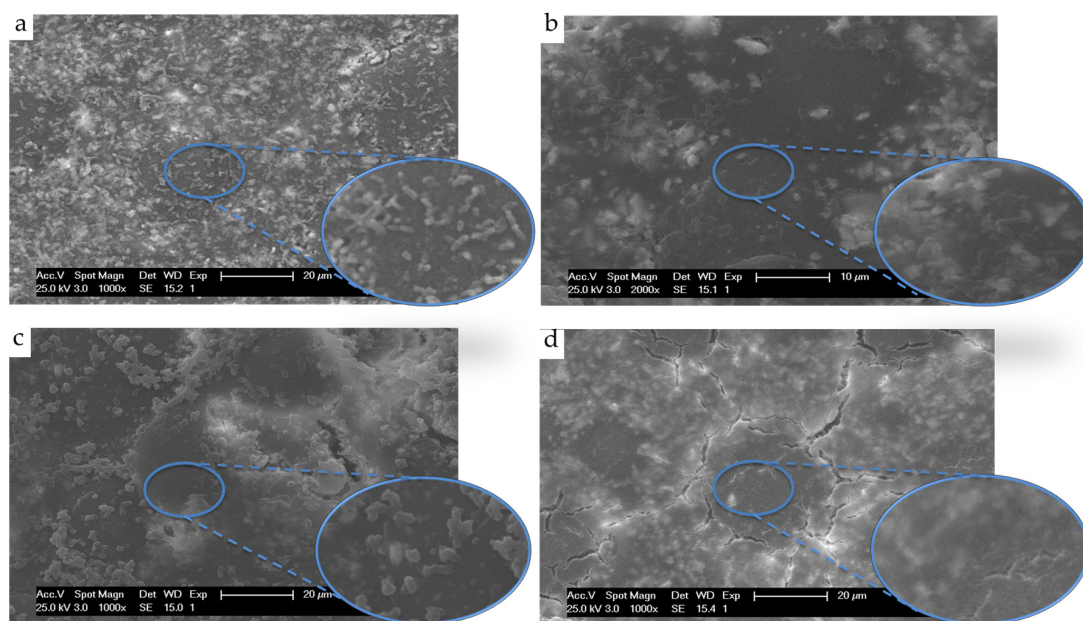


Figure 1. SEM micrographs (1000 $\times$) of ABC surfaces after incubation of *E. coli* cultures for 24 h of (a) Control ABC, (b) ABC with 0.3% GO, (c) ABC with 15% CS and (d) ABC with 0.3% GO and 15% CS.

Mori et al. [9] reported low antibacterial activity of CS in ABC formulations, suggesting the need to add other synergistic antibacterial properties. The mechanisms of the antimicrobial activity of CS involve electrostatic interactions between the positively charged chitosan chains and bacterial cell membrane, affecting cell permeability and leading to bacterial death [12]. Membrane damage, interaction with DNA/RNA, metal chelation and deposition on the microbial surface are also other possible chitosan antimicrobial mechanisms reported [52]. In this work, the antibacterial activity exhibited by the studied ABCs is enhanced by the presence of GO which presents antibacterial mechanisms due to its extremely sharp edges that cause physical damage to the bacterial membrane [49,50]. In addition, oxidative stress with production of reactive oxygen species (ROS) disrupts the balance in redox processes within the cell leading to a disruptive membrane cell mechanism [53–57] and cell lysis [53,58].

3.3.2. Cellular Behavior of Human Osteoblasts

SEM micrographs of Figure 2 show the surface morphology of the different cement samples directly seeded with human osteoblasts and cultured for 1, 7, 14, and 21 days. It is evident that cement surfaces containing CS showed a rough topography (Figure 2i–o). This topography can be caused by the high viscosity of the CS containing cement paste, which increases the porosity in the matrices [31]. At day 1, a rapid adhesion of HObs (marked with red triangles in Figure 3) with homogeneous distribution was evident across the surface of all cement formulations. After 14 days, individual cells were no longer visible; instead, it was possible to observe a continuous monolayer of extracellular matrix and cells covering all the studied samples. Remarkably, a deeper analysis of the ABC0.3GO-15CS images showed rougher surfaces for this composition revealing a qualitatively superior population of osteoblasts along with higher extracellular matrix formation. Only if cells are alive and functional healthy tissues are synthesized [59] what supports the good biocompatibility of the ABCs prepared in this study.

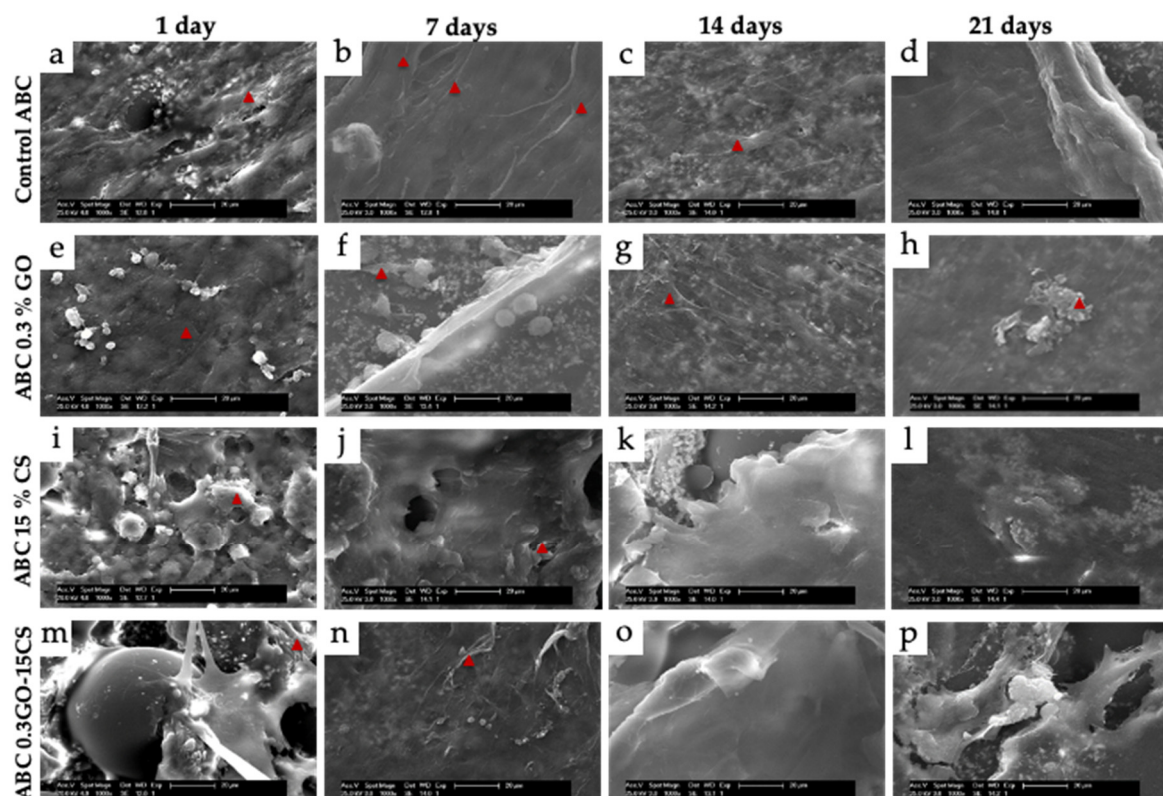


Figure 2. SEM micrographs of ABC samples seeded with human osteoblasts after 1, 7, 14, and 21 days of culture (triangles indicate osteoblasts). (a–d) Control ABC, (e–h) ABC 0.3% GO, (i–l) ABC 15 % CS and (m–p) ABC 0.3GO-15CS.

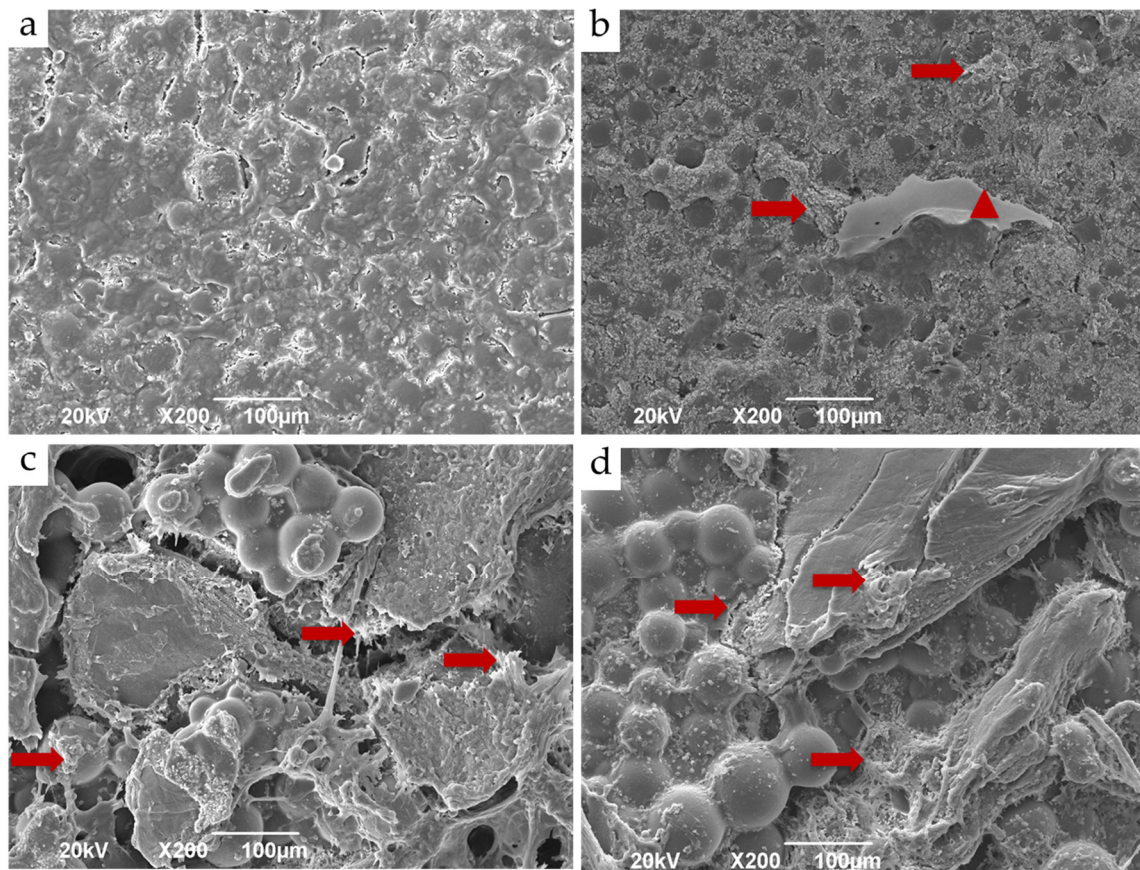


Figure 3. SEM micrographics (200×) of ABC surfaces after subdermal implantation in rats for 1 month. (a) Control ABC, (b) ABC with 0.3% GO, (c) ABC with 15% CS and (d) ABC with 0.3% GO and 15% CS samples (Triangles indicate cells and arrows indicate extracellular matrix).

The positive effects of GO and CS on the osteoblasts adhesion, proliferation and deposition of extracellular matrix have been reported by several authors [7,27,60] and they were attributed, in the case of GO introduction, to the increase in the hydrophilicity of the cement surface with hydroxyl groups, which can act as possible anchoring points for cells [7]. In the case of CS, they are related to the low toxicity and excellent ability to promote osteoblastic cell growth of this polysaccharide [27].

3.4. In Vivo Studies

In vivo biocompatibility of the developed ABCs was carried out by application of two different animal models. In particular, these studies consisted of subdermal and bone implantations. Subdermal ABCs implantation was analyzed by SEM and histology of tissues with Hematoxylin and Eosin stain and Masson-trichrome stains. For its part, implantations in parietal bones were evaluated by SEM and EDS studies at the implantation zone.

3.4.1. Subdermal Implantation

The SEM micrographs shown in Figure 3 correspond to representative images of the four cements response after 1 month of subdermal implantation in Wistar rat tissues. In Figure 3b, a structure compatible with one cell (marked with triangle in Figure 3) is observed, while Figure 3c,d showed better surface texture and abundant deposits of extracellular matrix on their surface (marked with arrows in Figure 3), compared with the Control ABC (Figure 3a). Since cells of mesenchymal origin, including fibroblasts, are rugophilic, it is valid to think that the surface of the loaded cements, being more irregular than the Control ABC, is more encouraging for the cells. Several pieces of research had demonstrated that a higher roughness of the surface of the biomaterials favors the adhesion and

proliferation of the cells [61–65]. The presence of cells and deposits of extracellular matrix on the cements confirmed their biocompatibility because according to Richards [61], the adhesion of cells to surfaces is one of the vital aspects in biocompatibility.

As far as histological analysis, the samples corresponding to the collagen control at 1 month of implantation are showed in Figure 4 which revealed the presence of remaining material (Col) in the implantation zone (IZ) in process of degradation/absorption and abundant inflammatory infiltration (II).

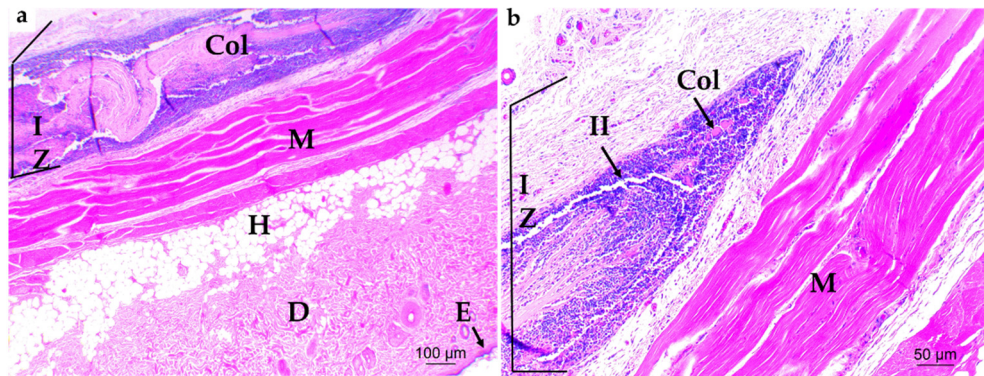


Figure 4. H&E staining of collagen membrane after 1 month of subdermal implantation in Wistar rats. (a) 4× and (b) 10×. IZ: Implantation zone. Col: Collagen. M: Muscle. H: Hypodermis. D: Dermis. E: Epidermis. II: Inflammatory infiltrate.

Figure 5 corresponds to the Control ABC formulation, showing a capsule (C) surrounding the zone of implantation (IZ) and including an inflammatory infiltrate (II).

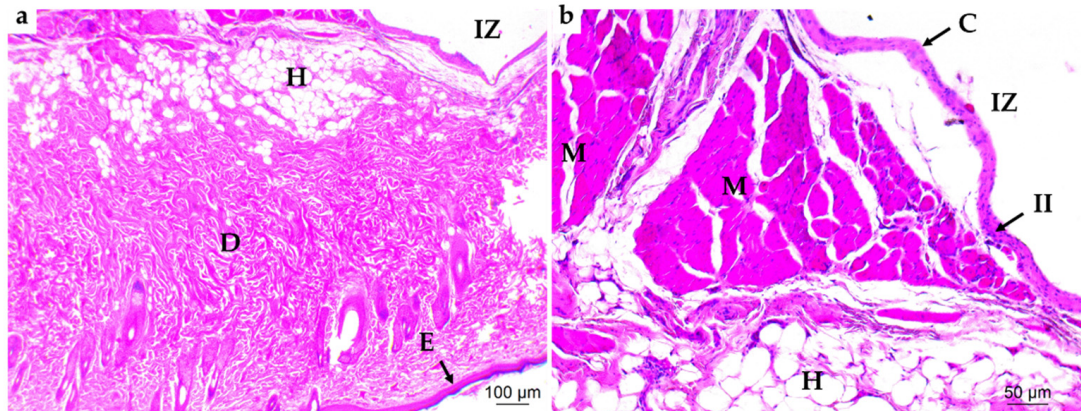


Figure 5. H&E staining of Control ABC after 1 month of subdermal implantation in Wistar rats. (a) 4× and (b) 10×. IZ: Implantation zone. Col: Collagen. M: Muscle. H: Hypodermis. D: Dermis. E: Epidermis. II: Inflammatory infiltrate and C: capsule.

The three formulations of implanted cements including GO and/or CS (Figure 6) showed similar response to that of Control ABC (Figure 5), characterized by a localized inflammatory response, surrounded by a capsule (C) with the presence of inflammatory infiltrate (II). However, compared to collagen samples (Figure 4), the latter had a more diffuse inflammatory response with abundant inflammatory infiltrate.

The tissues stained with the Masson's trichrome stain (Figure 7) showed a fibrous capsule at the IZ composed of collagen type I fibers for collagen control and the studied cements.

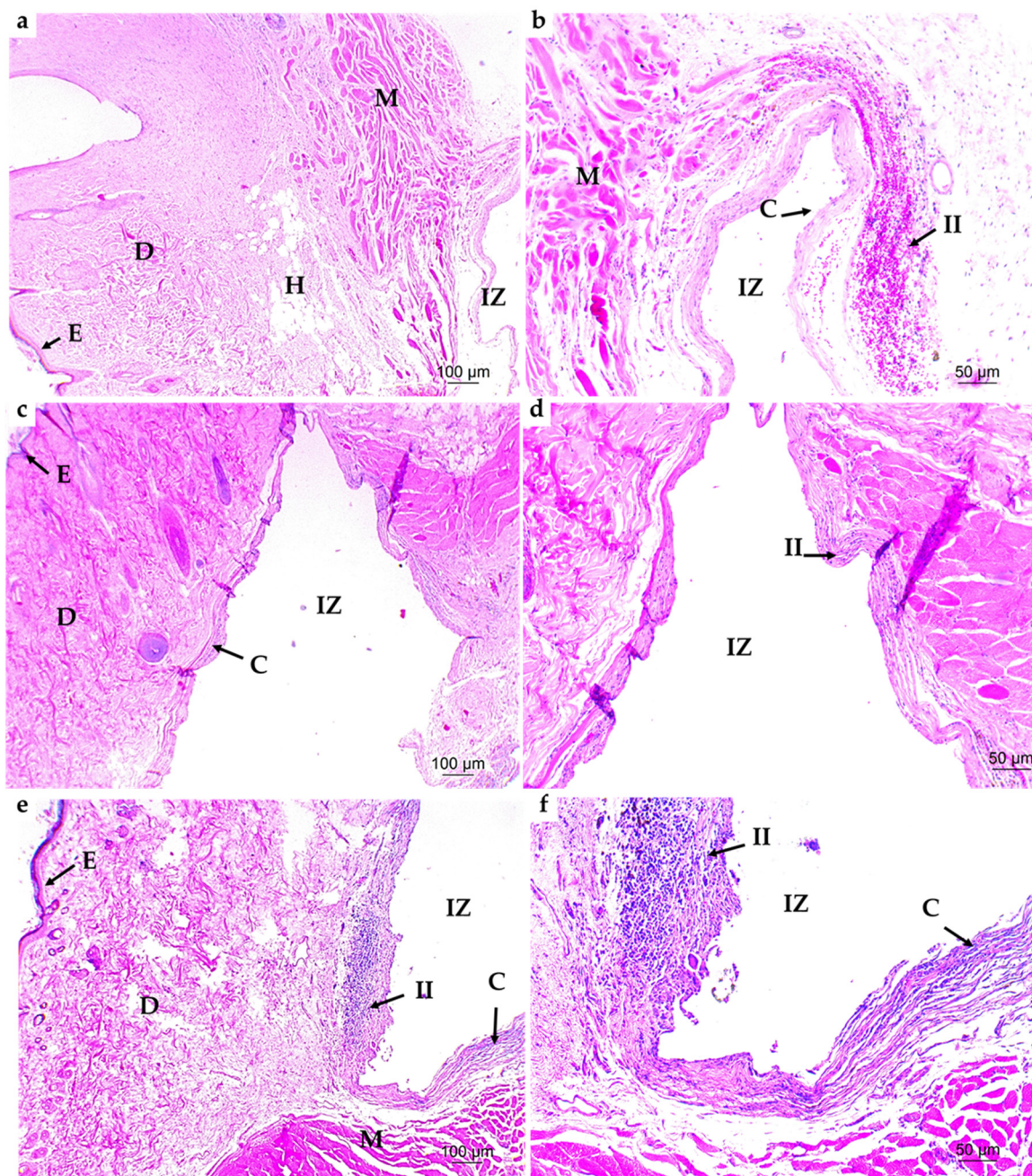


Figure 6. H&E staining after 1 month of subdermal implantation in Wistar rats. (a,b) ABC with 0.3% GO, (c,d) ABC with 15% CS and (e,f), ABC with 0.3% GO and 15% CS. (a–c,e) micrographs to 4× and 4×, and (b–d,f), micrographs at 10×. IZ: Implantation zone. M: Muscle. H: Hypodermis. D: Dermis. E: Epidermis. II: Inflammatory infiltrate and C: capsule.

The presence of the capsule and the inflammatory infiltrate surrounding the cement samples can be considered a normal *in vivo* response to materials that have been implanted in soft tissue and promote healthy wound healing [66]. For example, Maiborodin et al. [67] reported the formation of a transparent capsule surrounding samples of polyhydroxyalkanoates implanted in rats. Fibrous encapsulation has also been reported for various biomaterials, such as nano-crystalline hydroxyapatite [68] and ABCs with CS [4].

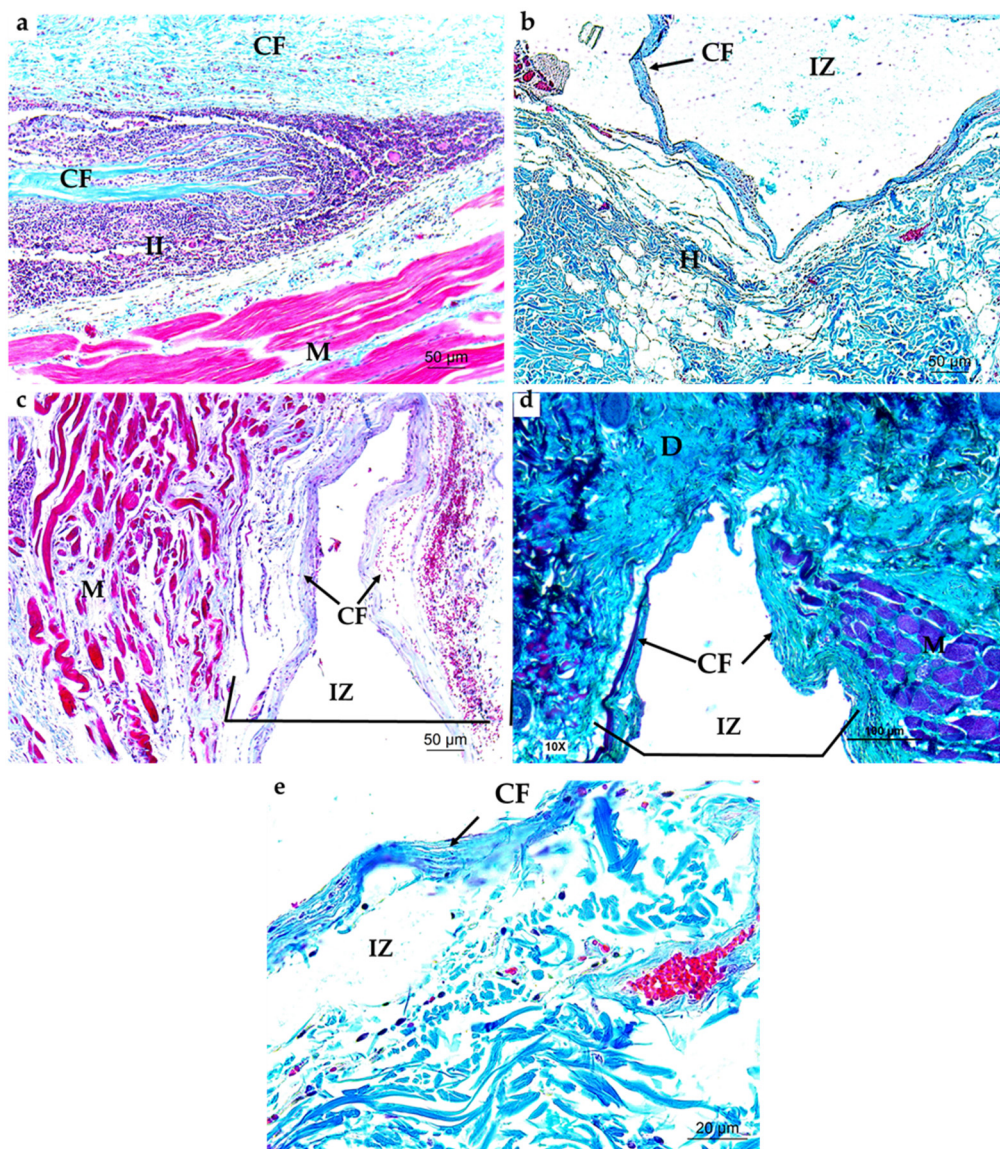


Figure 7. Micrographics of Masson's trichrome stain after 1 month of subdermal implantation in Wistar rats (10×). (a) Collagen control, (b) Control ABC, (c) ABC with 0.3% GO, (d) ABC with 15% CS and (e) ABC with 0.3% GO and 15% CS. IZ: Implantation zone. M: Muscle. H: Hypodermis. II: Inflammatory infiltrate. CF: Collagen fibers.

3.4.2. Bone Implantation of ABCs

SEM and EDS studies were performed at the interface of bone and cements to evaluate the osseointegration process through the sealing defect and to detect the presence of Ca and P, respectively.

According to the SEM micrographs illustrated in Figure 8, Control ABC implantation area in parietal bone of Wistar rats showed that after 3 months of implantation, the cement was not completely integrated with the surrounding bone tissue, since a clear separation between the two was observed (Figure 8a,b). In the area corresponding to the cement, the typical topography of the bone cement was observed with few deposits of extracellular matrix (EM).

On the contrary, the formulations modified with GO (Figure 9a,b) or CS (Figure 9c,d) and with both components together (Figure 9e,f) presented the bone–cement interface with an advanced sealing process. In addition, at the interface there were presence of osteoblasts (OB) and abundant deposits of EM that nearly completely seal the periphery of the intraosseous preparation as shown in Figure 9. The better sealing means an enhanced osseointegration or chemical affinity of the modified ABCs

with the bone. In accordance, the difference in the EM deposits between the Control ABC and the modified cements (Figures 8 and 9 respectively) is evident. Interestingly, cement with both CS and GO presented a more active interface with a group of osteoblasts growing from the interface towards the bone cement (Figure 9e,f).

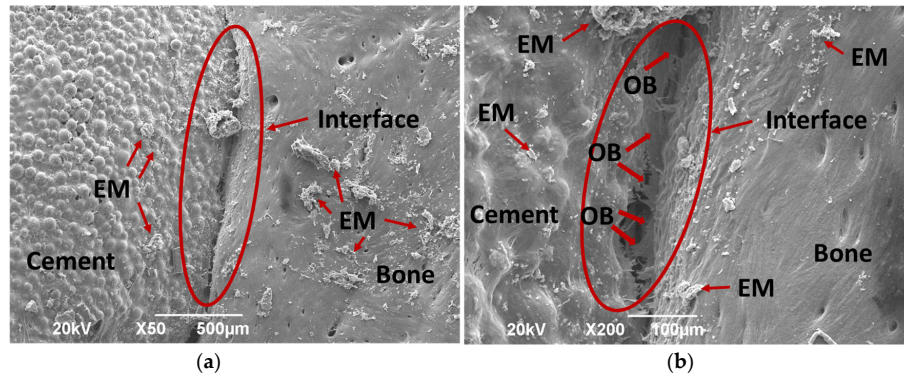


Figure 8. Micrographs of the implantation zone of Control ABC after 3 months of implantation in parietal bone of Wistar rats. (a) 50× and (b) 200×. EM: Extracellular matrix and OB: Osteoblasts.

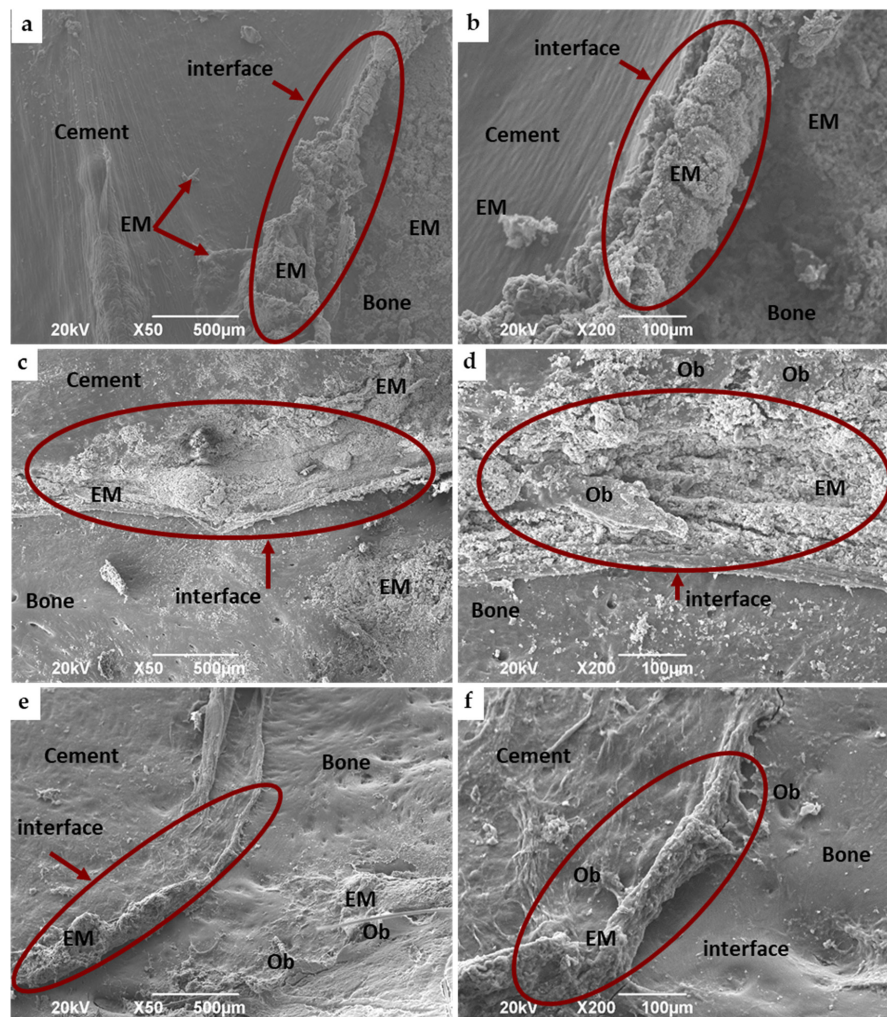


Figure 9. SEM micrographs of the implantation zone of modified ABCs after 3 months of implantation in parietal bone of Wistar rats. (a,b) ABCs with 0.3% GO, (c,d) ABCs with 15% CS and, (e,f) ABCs with 0.3% GO and 15% CS. (a,c,e) with 50× magnification. Areas surrounded by a red line are showed with deeper detail (200×) in (b,d,f) images, respectively. EM: Extracellular matrix and OB: Osteoblasts.

When the cement–bone interface of the ABC 15% CS and ABC0.3% GO-15% CS formulations were analyzed at higher magnification (500×) (Figure 10) it was appreciated that the interface of ABCs modified with only CS was not totally sealed, showing cement particles in the process of cellular colonization, while that of the ABC 0.3% GO-15% CS formulation was almost completely sealed.

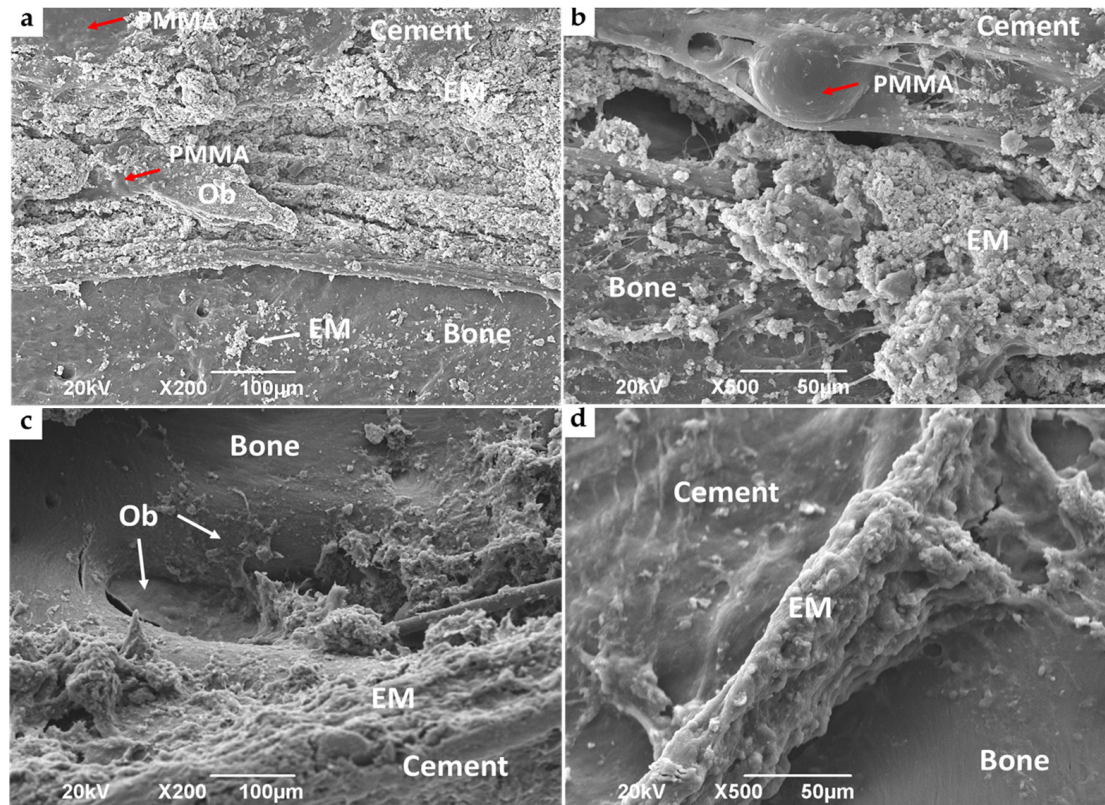


Figure 10. SEM micrographs of the implantation zone of modified ABCs after 3 months of implantation in parietal bone of Wistar rats. (a,b) ABC 15% CS, (c,d) ABC with 0.3% GO and 15% CS. (a,c) 200× and (b,d) 500×. EM: Extracellular matrix, OB: Osteoblasts and PMMA: bead of PMMA.

Analysis of EDS spectra at the interface and the bone zones (Figure 11) in all cases demonstrated the presence of bands of calcium (Ca) and phosphorous (P) in their corresponding spectra (Figure 11b,c,e,f,h,i,k,l) supporting the mineralization process and the good biocompatibility of the ABCs studied here. However, analysis of EDS spectra in the cement IZ showed the presence of the Ca signal of low intensity and absence of the that of P (Figure 11a,d,g,j).

Interestingly, increasing amounts of Ca and P were found for the formulations with 0.3% GO and/or 15% CS with respect to Control ABC, more marked for the sample containing both components (Figure S2). The deposition of Ca and P elements is a primary evidence of the osteogenesis process and the amount of elemental deposition is associated with a material biocompatibility and osteoconductive behavior [43].

These results agree with those previously obtained by SEM, since the enhanced biomineralization at the interface for the ABC with 0.3% GO and 15% CS sample correlates with a greater integration of this cement with the bone and with an improved biocompatibility compared to the other studied formulations. The foregoing indicates that this formulation is highly promising stimulating bone regeneration and therefore it has potential for the fixation of orthopedic implants to the bone, mainly in applications where osseointegration is required.

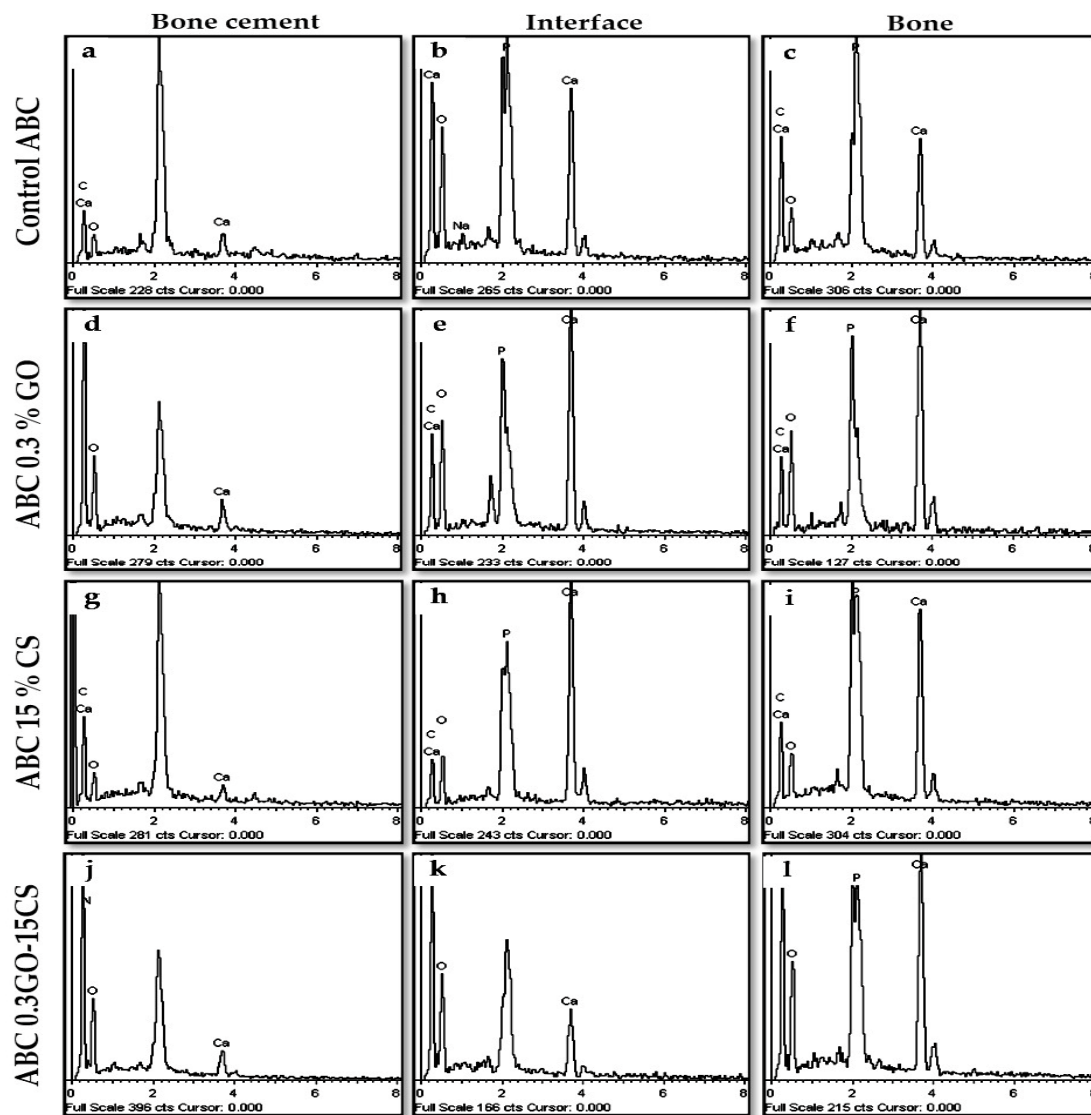


Figure 11. EDS analysis of the implantation zone of ABCs after 3 months of implantation in critically sized bone defects in the parietal bone of Wistar rats. (a,d,g,j) correspond to the ABC zone; (b,e,h,k) correspond to the interface and (c,f,i,l) correspond to the bone around the cement. (a–c) Control ABC, (d–f) ABC with 0.3% GO, (g–i) ABC with 15% CS and (j–l) ABC with 0.3% GO and 15% CS samples.

4. Conclusions

Inclusion of CS and GO separately or together in cement formulations improved setting properties and biocompatibility both *in vitro* and *in vivo*, although incorporation of only CS showed a decrease in the mechanical properties of ABCs. The synergy of the contribution of GO and CS was evident in the ABC 0.3% GO-15% CS formulation providing a significant decrease in the maximum temperature and an increase in the antibacterial activity compared to the other formulations. All formulations showed biocompatibility against human osteoblasts and in a subdermal model in rats.

Implantation of modified cements in parietal bone of rats showed that all the ABCs favored healthy sealing of the critical size defect. However, an improved process of osseointegration and biocompatibility were observed in the cements modified with 0.3% GO and 15% CS, since they show a more mature interface with a group of osteoblasts colonizing the surface of the bone cement, abundant deposits of EM and notable presence of Ca and P elements at the interface. These late corroborates the positive synergistic effect between GO and CS in the bioactivity of the ABCs and its promising use as a bioactive ABC for the fixation of orthopedic implants to the bone.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2076-3417/10/18/6528/s1>, Figure S1: Sequence of implantation of ABCs in parietal bone of Wistar rats. Figure S2: MTT assay performed with HOb in the presence of extracts of ABCs taken after 1, 14, and 21 days. Figure S3: EDS analysis of the implantation zone of ABCs after 3 months of implantation in critically sized bone defects in the parietal bone of Wistar rats.

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Article

Osseointegration in Acrylic bone cements modified with graphene oxide and chitosan

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Supplementary Materials



Figure S1. Sequence of implantation of ABCs in parietal bone of Wistar rats. **(a)** Cranial defect generated **(b)** Implanted ABC and **(c)** Suture of the implantation area.

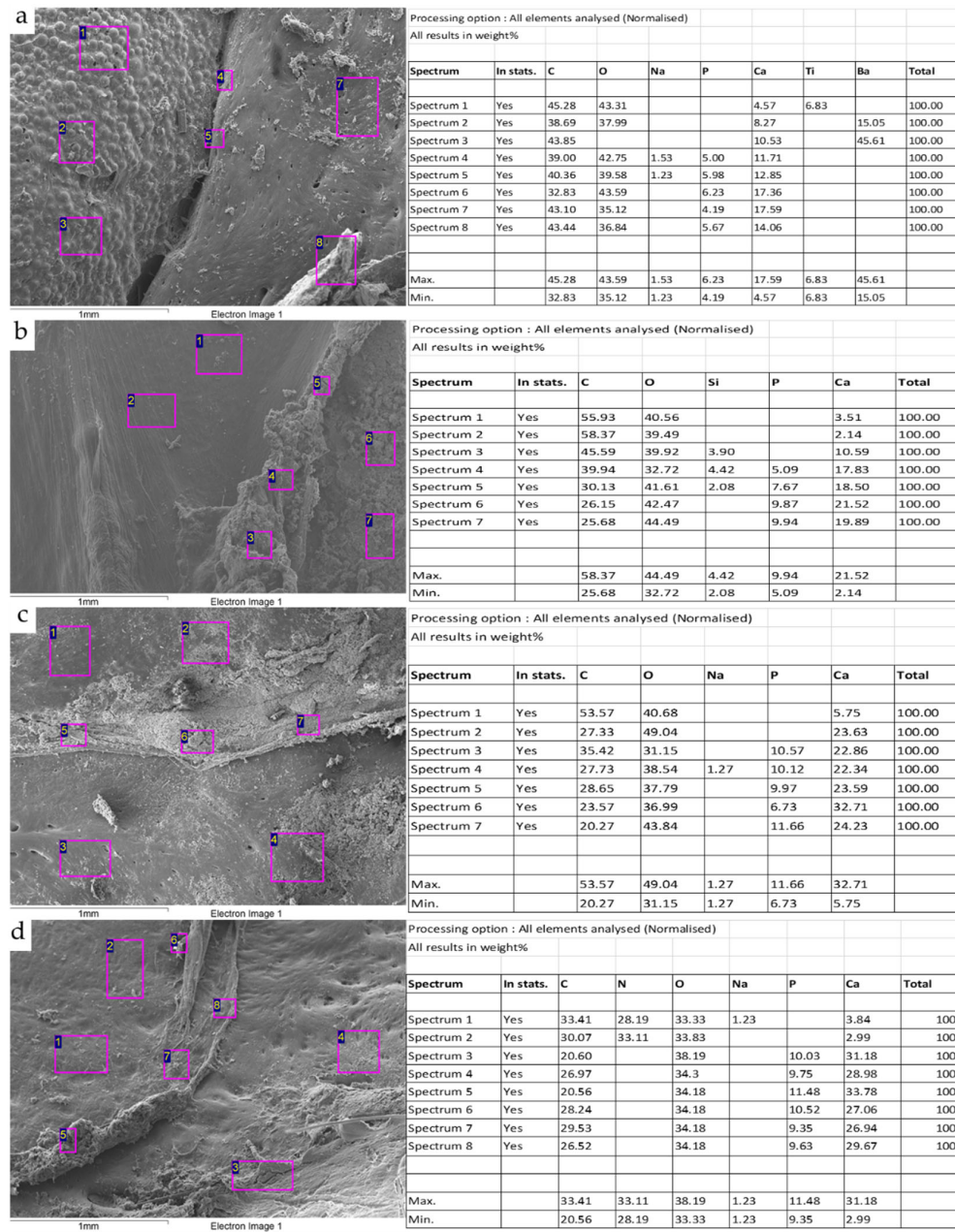


Figure S2. EDS analysis of the implantation zone of ABCs after 3 months of implantation in critically sized bone defects in the parietal bone of Wistar rats. **(a)** Control ABC, **(b)** ABC with 0.3 % GO, **(c)** ABC with 15 % CS and **(d)** ABC with 0.3 % GO and 15 % CS.

Capítulo 4. SÍNTESIS

4.1 PANORAMA GENERAL

Los resultados obtenidos en la presente investigación muestran que la adición de porcentajes mínimos de 0.3 % en peso de GO con un grado de oxidación de 68% y un tamaño lateral de 400 nm, generó inhibición total en el crecimiento de las bacterias *Bacillus cereus*, *Staphylococcus aureus*, *Salmonella enterica* y *Escherichia coli*. Este resultado es muy alentador para el tratamiento de infecciones después de una artroplastia, debido a que los métodos empleados clínicamente para tratar este tipo de infecciones consisten en el empleo de cementos óseos cargados con antibióticos que por su uso excesivo están generando bacterias resistentes y multirresistentes a los antibióticos, y, por esto, se buscan tratamientos alternativos. Adicionalmente, el uso de antibióticos genera pérdidas en las propiedades mecánicas del cemento, lo cual compromete la longevidad de la prótesis. Esta desventaja mecánica también se ve mejorada por la incorporación del GO, ya que además de la actividad antibacteriana, generó las siguientes propiedades deseables para los cementos óseos acrílicos: incremento en las propiedades mecánicas a compresión y flexión hasta en un 17%, reducción de la temperatura máxima de polimerización hasta en un 19%, incremento en la rugosidad, la humectabilidad y en el tiempo de curado en 40% con respecto a la formulación sin carga.

La falta de bioactividad de los ABC es uno de los inconvenientes a los cuales se les atribuye el aflojamiento aséptico de las prótesis, debido a que el único medio de unión hueso-cemento-prótesis es mecánico. En esta investigación se evidenció que la incorporación de CS en la formulación de los cementos en las dos morfologías estudiadas (microesferas o láminas) le confirió al cemento propiedades biológicas superiores, dado que un mayor contenido de CS promovió la fijación de los iones Ca^{2+} y P_4^{3-} sobre la superficie del cemento cuando estuvo inmerso en un FBS por diferentes periodos de tiempo. Además de la bioactividad, la incorporación de CS en la formulación del cemento favoreció la reducción de la T_{max} alcanzada durante la reacción de polimerización, incrementó el t_{set} y la biocompatibilidad frente a osteoblastos humanos; aunque, las propiedades mecánicas en los cementos disminuyeron significativamente con la incorporación de CS, por lo que el uso de este tipo de cementos estaría limitado a aplicaciones de baja carga como el relleno de defectos óseos o la cifoplastia. Un aporte importante de esta investigación consistió en el estudio de ABC con porcentajes de $\text{CS} \leq 20\%$, dado que la mayoría de las investigaciones encontradas reportan porcentajes superiores al 20%.

La presencia combinada de GO y CS en las formulaciones de ABC, mostró un efecto sinérgico sobre las propiedades de los cementos óseos, encontrándose que estos tienen un alto potencial para su uso en artroplastias, debido al incremento en la osteointegración y la biocompatibilidad generado por el CS, combinado con el reforzamiento mecánico y la actividad antibacteriana conferida por el GO.

La mayor osteointegración del cemento adicionado con CS y GO al hueso es un alcance muy importante que se logró en esta investigación, ya que se promovió una mayor fijación de tipo mecánica, biológica y química que se espera, se vea reflejada en un incremento en la longevidad de las artroplastias. Adicionalmente, dado que los cementos adicionados con 0.3% en peso de GO y 15% en peso de CS generaron una reducción del 21% en la T_{max} y un incremento en la porosidad y rugosidad del cemento con respecto al cemento sin carga, se espera que esta formulación presente menor riesgo de necrosis térmica en el tejido alrededor del implante y facilite el anclaje de células para incrementar la adhesión, proliferación y diferenciación de las células formadoras de hueso, para conseguir mayor biocompatibilidad y bioactividad en los cementos desarrollados.

4.2 CONCLUSIONES

- Se obtuvieron ABC bioactivos, gracias a la incorporación de CS en la formulación. El aumento en el contenido de CS incrementó la bioactividad del cemento, facilitando la fijación superficial de iones Ca^{2+} y P_4^{3-} sobre el cemento, al estar inmerso en FBS y asimismo favoreció la generación de poros e incrementó la actividad de la fosfatasa alcalina en estudios con osteoblastos humanos. Adicionalmente, la presencia del 20% en peso de quitosano en la fase sólida incrementó el t_{set} en 54%, redujo la T_{max} en un 22% y el ángulo de contacto en 12%, todas estas características deseables en los ABC para promover mayor afinidad biológica con el hueso. Por otra parte, el contenido de CS también disminuyó significativamente las propiedades mecánicas de los cementos e incrementó el contenido de monómero residual, estos efectos son indeseables en los ABC y limitan el uso potencial de estos cementos a aplicaciones de baja carga mecánica.
- La incorporación de GO, además de conferirle actividad antibacteriana a las formulaciones de cemento contra las bacterias *Gram-positivas* y *Gram-negativas* estudiadas, aportó los siguientes beneficios a los ABC: incremento en la rugosidad, en la humectabilidad (17%), en las propiedades de compresión y flexión hasta en un 17%, en t_{set} hasta un 40% y disminución en la T_{max} hasta un 19%. Sin embargo, este también generó una leve reducción en la estabilidad térmica y un ligero incremento en el contenido de monómero residual que podrían comprometer la estabilidad del cemento.
- La adición de 15% en peso de quitosano en la fase sólida fue seleccionada como la cantidad más adecuada en las formulaciones de los ABC, dado que representa el mínimo porcentaje que garantiza una rápida bioactividad en los cementos, sin generar un incremento considerable en el contenido de monómero residual como sucede en proporciones mayores.
- La formulación de ABC con 0.3% en peso de GO en la fase líquida tiene un alto potencial de uso en la prevención y tratamiento de infecciones en artroplastias, ya que esta presenta la mínima concentración de GO con la cual se inactivan tanto las bacterias *Gram-positivas* como las *Gram-negativas* evaluadas en esta investigación, además de mostrar los más altos grados de reforzamiento mecánico y buen desempeño de las propiedades de curado establecidas en la norma ISO 5833-02.
- Se logró obtener una formulación de cemento óseo bioactivo y antibacterial con posible uso en artroplastias, mediante la adición de 0.3% en peso de GO en la fase líquida y 15% en peso de CS en la fase sólida. La bioactividad se evidenció con una mayor osteointegración con el hueso al ser implando en un defecto óseo de tamaño crítico, ya que presentó un sellado más completo en la interfase después de tres meses

de implantación, comparado con las otras formulaciones. Por otro lado, la actividad antibacteriana de los cementos contra la bacteria *Gram-negativa E. coli* se incrementó un 88% con respecto al cemento sin adiciones y además, se favorecieron las propiedades de curado logrando una disminución de 21% en la $T_{\max}$ alcanzada durante la polimerización y un incremento en el t_{set} de 24%. Las propiedades mecánicas de esta formulación presentaron un buen desempeño comparado con los requisitos propuestos en la norma ISO 5833-02 para los ABC.

4.3 TRABAJO A FUTURO

Con el fin de ampliar los estudios realizados en esta investigación y de contar con los insumos necesarios para poder escalar los hallazgos a una fase clínica que valide estos resultados, a continuación se plantea el siguiente trabajo a futuro:

- Realizar estudios que promuevan la reducción en el contenido de monómero residual de los cementos cargados con CS y/o GO, ya que este se ve incrementado con la incorporación de ambas cargas.
- Llevar a cabo estudios en condiciones dinámicas a las formulaciones que generaron características más adecuadas en condiciones estáticas, ya que las articulaciones están constantemente sometidas a esfuerzos cíclicos que pueden generar la falla del cemento.
- Ampliar las caracterizaciones biológicas realizadas, con el fin de cuantificar el proceso de osteointegración en el hueso de los biomodelos a diferentes periodos de tiempo.
- Evaluar la actividad antibacteriana de los cementos frente a un número mayor de bacterias, principalmente aquellas que se han identificado como más recurrentes en infecciones de artroplastias (*S. epidermidis*, *S. aureus*, y *S. capitis*). Adicionalmente, es importante conocer el efecto del tiempo sobre la actividad antibacteriana de estos cementos.
- Escalar la experimentación biológica en biomodelos más grandes con el fin de realizar pruebas de uso en las que se evalúe la funcionalidad del cemento óseo en artroplastias.
- Implementar metodologías de evaluación que permitan entender el papel del mejoramiento de la zona interfacial en el tiempo de vida útil de una artroplastia, haciendo evaluaciones dinámicas en condiciones biológicas simuladas.

4.4 APORTES DE LA INVESTIGACIÓN

El desarrollo de esta investigación generó aportes desde el punto de vista **social** debido a que contribuyó en la generación de alternativas para el incremento de la longevidad de las artroplastias, lo que lleva a mejorar la calidad de vida de las personas con osteoartrosis u otro tipo de trastorno articular.

El aporte **académico** se ve reflejado en los 10 artículos que se obtuvieron como resultado de esta investigación, donde se amplió el estado del arte sobre propiedades físicas, químicas, térmicas, mecánicas y biológicas de formulaciones de cementos óseos bioactivos y antibacterianos, adicionados con bajos contenidos de quitosano y óxido de grafeno.

Esta investigación también tiene un aporte al sector **productivo**, ya que, junto con otros estudios, generó una patente de invención titulada “Kit de cemento óseos acrílico que comprende quitosano, óxido de grafeno, apatita, ácido poliláctico, cloruro de estroncio y comonomeros alcalinos”, otorgada por la Superintendencia de Industria y Comercio de Colombia, mediante Resolución N° 60312 de 2019.

4.5 LOGROS OBTENIDOS

Durante el desarrollo de esta investigación se obtuvieron los siguientes productos:

- 1 Artículo de revisión (Mostrado en la sección 2.1)
- 9 Artículos de investigación (Mostrados en la sección 3 del documento)
- 1 Patente de Invención (Sección 4.5.1)
- 15 Participaciones en eventos de divulgación científica (Sección 4.5.2)
- 1 Pasantía de Investigación (4.5.3)

4.5.1 Patente de Invención

REPÚBLICA DE COLOMBIA
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

Resolución N° 60312

Ref. Expediente N° NC2018/0004814

Por la cual se otorga una Patente de Invención

EL SUPERINTENDENTE DE INDUSTRIA Y COMERCIO

en ejercicio de sus facultades legales, en especial de las conferidas en el numeral 26 del artículo 3° del Decreto 4886 de 2011, y

CONSIDERANDO:

PRIMERO: Que mediante escrito radicado en esta Superintendencia el 5 de mayo de 2018 con el N° NC2018/0004814, por UNIVERSIDAD DEL VALLE, presentó la solicitud de patente de invención titulada “COMPOSICIÓN DE CEMENTO ÓSEO”.

SEGUNDO: Que la solicitud fue publicada en la Gaceta de la Propiedad Industrial N° 832 el 10 de julio de 2018, sin que se hubieran presentado oposiciones por parte de terceros.

TERCERO: Que realizado el examen de fondo mediante Oficio N° 5850, notificado el 5 de julio de 2019, se requirió al solicitante en los términos del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina para que presentara respuesta a las observaciones de carácter técnico, relacionadas con la patentabilidad o cumplimiento de los requisitos establecidos por esta Decisión para la concesión de la patente.

CUARTO: Que el solicitante mediante escrito radicado bajo el N° NC2018/0004814 el 3 de octubre de 2019, respondió oportunamente el requerimiento formulado y presentó las reivindicaciones 1 a 6 que reemplazan las originalmente presentadas. Se acepta este último capítulo reivindicatorio presentado, comoquiera que se ajusta a las prescripciones contenidas en el artículo 34 de la Decisión 486.

QUINTO: Que en virtud de lo dispuesto en el artículo 14 de la Decisión 486 expedida por la Comisión de la Comunidad Andina *“Los países miembros otorgarán patentes para las invenciones, sean de producto o de procedimiento, en todos los campos de la tecnología, siempre que sean nuevas, tengan nivel inventivo y sean susceptibles de aplicación industrial.”*

SEXTO: Que en el presente caso las reivindicaciones 1 a 6 incluidas en el radicado bajo el N° NC2018/0004814 el 3 de octubre de 2019, cumplen los requisitos indicados en el considerando anterior, toda vez que refieren un kit que difiere del estado de la técnica más cercano, US2012/0195848, en que contiene comonomeros alcalinos, quitosano y óxido de grafeno. Adicionalmente, estas diferencias no se encuentran sugeridas en el estado de la técnica y, como consecuencia de ello, se evidencia el efecto de mejoramiento del desempeño mecánico, actividad antimicrobiana y biocompatibilidad. Sumado a lo anterior, la materia reivindicada, es susceptible de aplicación industrial.

En consecuencia, las reivindicaciones 1 a 6 cumplen los requisitos de novedad, nivel inventivo y aplicación industrial establecidos en la normatividad citada en precedencia y este Despacho encuentra procedente conceder para las mismas la patente solicitada.

SÉPTIMO: Que de acuerdo con la modificación presentada por el solicitante en el escrito radicado bajo el N° NC2018/0004814 el 3 de octubre de 2019 y teniendo en cuenta el objeto

Resolución N° 60312

Ref. Expediente N° NC2018/0004814

concedido, se modifica el título de la invención el cual quedará de la siguiente manera: “KIT DE CEMENTO ÓSEO ACRÍLICO QUE COMPRENDE QUITOSANO, ÓXIDO DE GRAFENO, APATITA, ÁCIDO POLILÁCTICO, CLORURO DE ESTRONCIO Y COMONÓMEROS ALCALINOS”.

RESUELVE

ARTÍCULO PRIMERO: Otorgar patente de invención para la creación titulada:

“KIT DE CEMENTO ÓSEO ACRÍLICO QUE COMPRENDE QUITOSANO, ÓXIDO DE GRAFENO, APATITA, ÁCIDO POLILÁCTICO, CLORURO DE ESTRONCIO Y COMONÓMEROS ALCALINOS”

Clasificación IPC: A61L 24/00, A61L 24/02, A61L 24/08

Reivindicaciones: 1 a 6 incluidas en el radicado bajo el N° NC2018/0004814 del 3 de octubre de 2019, de acuerdo con el anexo 1.

Titular: UNIVERSIDAD DEL VALLE.

Domicilio: CALLE 13 NO 100 – 00. CALI VALLE DEL CAUCA, COLOMBIA.

Inventor(es): Carlos Humberto VALENCIA, Maria Carolina PUSTOVRH, Natalia Marcela HURTADO CHÁVEZ, Héctor Fabio ZULUAGA CORRALES, José Herminsul MINA HERNÁNDEZ, Mayra Eliana VALENCIA ZAPATA, Karen Jesenia LOZANO NIEVA, Liliana SALAZAR MONSALVE, Mario Alejandro ORTÍZ, Carlos David GRANDE TOVAR

Vigente desde: 5 de mayo de 2018.

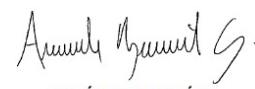
Hasta: 5 de mayo de 2038.

ARTÍCULO SEGUNDO: El titular tendrá los derechos y las obligaciones establecidos en la Decisión 486 de la Comisión de la Comunidad Andina y en las demás disposiciones legales vigentes sobre propiedad industrial, precisando que para mantener vigente la patente se deberá cancelar la tasa anual de mantenimiento, conforme lo dispone el artículo 80 de la referida norma comunitaria.

ARTÍCULO TERCERO: Notificar el contenido de la presente resolución a UNIVERSIDAD DEL VALLE advirtiéndole que contra ella procede el recurso de reposición, ante el Superintendente de Industria y Comercio, el cual podrá ser interpuesto en el momento de la notificación o dentro de los diez (10) días hábiles siguientes a ella.

NOTIFÍQUESE Y CÚMPLASE

Dada en Bogotá D.C., el 5 de noviembre de 2019



ANDRÉS BARRETO GONZÁLEZ
SUPERINTENDENTE DE INDUSTRIA Y COMERCIO

Firmado digitalmente
por: ANDRÉS
BERNARDO BARRETO
GONZÁLEZ
Fecha: 2019.11.15
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Razón: Validez jurídica.
Ubicación: Bogotá,
Colombia.



Resolución N° 60312

Ref. Expediente N° NC2018/0004814

ANEXO 1.

REIVINDICACIONES CONCEDIDAS

1. Kit de cemento óseo acrílico con una fase sólida y una fase líquida **caracterizado porque** la fase sólida se compone de un componente de matriz polimérica de polimetilmetacrilato PMMA, quitosano como componente biopolímero osteopromotor, óxido de grafeno como componente de refuerzo mecánico, un componente bioactivo seleccionado entre apatita natural, sulfato de calcio o una mezcla de los mismos y ácido poliláctico PLA como componente biopolímero osteoconductor; y la fase líquida incluye cloruro de estroncio y comonomeros alcalinos.
2. Kit de cemento óseo acrílico de acuerdo con la reivindicación 1, **caracterizado porque** el óxido de grafeno se encuentra en forma de láminas.
3. Kit de cemento óseo acrílico de acuerdo con la reivindicación 8, **caracterizado porque** la apatita natural es modificada superficialmente con agentes de acople tipo silanos.
4. Kit de cemento óseo acrílico de acuerdo con la reivindicación 4 o 7, **caracterizado porque** el óxido de grafeno está en el rango de 0.1% al 10% p/p de la composición final.
5. Kit de cemento óseo acrílico de acuerdo con las reivindicaciones 1, 5, 7 o 8 **caracterizado porque** el componente bioactivo está en el rango de 1% a 30 % p/p de la composición final.
6. Kit de cemento óseo acrílico de acuerdo con las reivindicaciones 3 o 7, **caracterizado porque** el quitosano está en el rango de 1% a 20 % p/p de la composición final.



COMPOSICIÓN DE CEMENTO OSEO

5

CAMPO DE LA TÉCNICA

La presente invención se refiere a cirugía ortopédica y traumatología,
10 materiales para prótesis o para revestimiento de prótesis, adhesivos o
cementos quirúrgicos

OBJETO DE LA INVENCION

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Proporcionar un cemento óseo con propiedades bioactivas y bactericidas,
donde el cemento óseo debe tener una buena biocompatibilidad conservando
propiedades biomecánicas adecuadas, pero procurando que se dé la
regeneración del tejido óseo en las interfaces prótesis–cemento y cemento–
20 hueso, además que tenga una temperatura de polimerización baja, liberación
de monómero residual MMA baja, que mejore la rigidez del cemento y su
acople al hueso y la prótesis y promueva el crecimiento del hueso.

25 ESTADO DE LA TÉCNICA

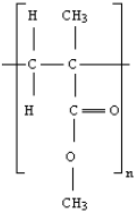
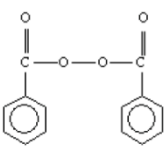
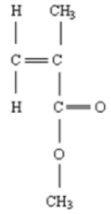
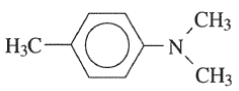
Los cementos óseos acrílicos han tenido durante años un papel importante en
la cirugía ortopédica y poco a poco se extiende su aplicación en la
remodelación de áreas de cáncer metastásico u osteoporótico, en fracturas, en
30 la reparación de defectos craneales (craneoplastias) y en la reparación de las
fracturas vertebrales (vertebroplastia y la cifoplastia) (Magnan et al. 2013).

La mayoría de los cementos óseos acrílicos (ABC) comerciales disponibles actualmente, consisten en dos componentes, uno sólido compuesto principalmente de un polímero basado en metil metacrilato (MMA) y uno líquido a base de MMA (Hendriks et al. 2004), los cuales se mezclan para producir el cemento, debido a la reacción de polimerización del monómero presente (Deb and Koller 2008b).

La principal ventaja del uso de los cementos óseos acrílicos, está en la excelente fijación primaria que se obtiene entre el implante y el hueso y la más rápida recuperación del paciente (Endogan et al. 2014).

Los componentes de los cementos óseos comerciales no varían drásticamente de una marca a otra, excepto que hay algunas modificaciones tales como la adición de copolímeros de PMMA en la fase sólida o de algunos comonómeros en el líquido, la incorporación de antibióticos, la variación de la relación sólido/líquido, el agente radiopaco y la presencia de aditivos tales como la clorofila en el cemento Palacos® o la variación de las cantidades de cada componente (Deb and Koller 2008a, 2008b). Los componentes básicos de los ABC se muestran en la Tabla 1.

Tabla 1. Componentes básicos de los cementos óseos acrílicos

COMPONENTE	COMPOSICIÓN (% APROXIMADO)	FUNCIÓN	FORMULA ESTRUCTURAL
COMPONENTE SÓLIDO			
Polimetil metacrilato (PMMA)	89	Polímero	
Sulfato de Bario o dióxido de zirconio	10	Agente radiopaco	BaSO_4 ZrO_2
Peróxido de benzoilo (BPO)	1	Iniciador de la reacción de polimerización	
COMPONENTE LÍQUIDO			
Metil metacrilato (MMA)	97	Monómero	
N,N Dimetil p-toluidina (DmpT)	2,7	Acelerante de la reacción de polimerización a temperatura ambiente	

Hidroquinona	75 ppm	Inhibidor que previene la prematura polimerización del MMA	
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Fuente: (Ginebra, Gil, and Planell 2002; Webb and Spencer 2007)

- 5 Los cementos óseos acrílicos presentan algunas limitaciones en su uso, relacionadas con:
- Temperatura de polimerización altamente exotérmica (entre 67 y 124°C) (Lewis 1997) a la que se le atribuye necrosis térmica del hueso (Fernández et al. 2002), alteración de la circulación sanguínea local y predisposición a la formación de una membrana fibrosa en la interface hueso-cemento (Endogan et al. 2014; Lopes et al. 2011).
 - Liberación de monómero residual o MMA sin reaccionar a lo que se le atribuye la necrosis química del hueso.
 - Contracción del cemento durante la polimerización.
 - La gran diferencia entre la rigidez del cemento y del hueso adyacente, lo que genera una inapropiada transferencia de carga (Boesel et al. 2007).
 - La identificación de la capa de cemento y sus interfaces con el hueso y con la prótesis, como zonas de unión débil entre el implante y el hueso.
 - Interacción de las partículas del cemento con los tejidos circundantes, lo cual produce las respuestas inflamatorias del tejido periprotésico y el aumento de la destrucción ósea.

- Falta de osteointegración debida a que el cemento óseo es un componente biológicamente inerte, por lo que normalmente no promueve el crecimiento del hueso (Dunne, Ormsby, and Mitchell 2013; Lissarrague et al. 2014)

5

Todo lo anterior se manifiesta como insuficiencia mecánica e intolerancia biológica (Duque Moran, JF et al (2011), y para solucionar estas limitaciones se han conducido investigaciones en tres sentidos, el primero consiste en generar osteointegración del cemento, lo cual es un factor clave para alcanzar

10 estabilidad en el cemento a largo plazo (Lissarrague et al. 2014), ya que se alcanza unión química entre el hueso y el cemento; el segundo consiste en mejorar las propiedades mecánicas y el tercero, consiste en proporcionar propiedades antimicrobianas a los ABC, ya que las infecciones son comunes en artroplastia y también conllevan al aflojamiento de las prótesis.

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En osteointegración se han utilizado diferentes estrategias para aumentar la resistencia interfacial entre el hueso y el cemento, y así mejorar el desempeño de la prótesis; una de estas estrategias consiste en la incorporación de cargas bioactivas en la fase sólida del cemento con el propósito de generar cementos

20 con superficie bioactiva, capaz de fomentar la aposición ósea directa en lugar de la encapsulación del implante por tejido fibroso, promoviendo el crecimiento de hueso y la formación de un enlace químico fuerte entre el cemento y el hueso (He et al. 2012; Lissarrague et al. 2014). Otras estrategias consisten en la adición de nuevos componentes en la formulación del cemento con el

25 objetivo de reducir la temperatura máxima de polimerización y el contenido de monómero residual para evitar así la necrosis térmica y química en el tejido alrededor del implante (Islas-Blancas and Cervantes 2000)(J M Cervantes-Uc, Cauich-Rodríguez, and Vázquez-Torres 2007)(Islas-Blancas et al. 2001).

30 En mejoras en las propiedades mecánicas se encuentran reportes del uso de nanotubos de carbono en los que se ha obtenido un notable incremento en las

propiedades mecánicas de la matriz (Dunne, Ormsby, and Mitchell 2013), en especial la resistencia a la fatiga y el impacto, donde los cementos óseos no han alcanzado las propiedades deseadas, a pesar de que una gran variedad de materiales se han adicionado a las formulaciones con tal fin (Marrs et al. 2006).

5

En mejoras de propiedades antibacterianas, con ccirugías abiertas siempre tienen un riesgo de contaminación, sin embargo, la presencia de biomateriales aumenta el riesgo de infección debido a su susceptibilidad a la colonización bacteriana (Van de Belt et al. 2001). Este evento depende de varios aspectos, tales como las propiedades fisicoquímicas de la superficie de los biomateriales, la estructura de la membrana celular y de los receptores de bacterias; los cuales van a definir si las bacterias que llegan a la superficie del biomaterial pueden o no adherirse fácilmente y proliferar en ella, provocando el aflojamiento séptico de la prótesis (Miola et al. 2013).

15

A continuación además se presenta una revisión de la forma como se ha tratado de mejorar las condiciones biológicas y mecánicas de los cementos óseos, por ejemplo para reducir la exotermia en la reacción de polimerización de los cementos óseos acrílicos la referencia *Incorporation of multiwalled carbon nanotubes to acrylic based bone cements: Effects on mechanical and thermal properties* Ormsby R, McNally T, Mitchell C, Dunne N.(2010) publican que la temperatura máxima de polimerización disminuye y el tiempo de curado aumenta con la incorporación de nanotubos de carbono (CNT) con el contenido de CNT; la referencia *Influencia de la incorporación de un co-monómero alcalino e hidroxí apatita en las propiedades de cementos óseos acrílicos*, Lozano K, Mina J, Zuluaga F, Valencia C, Valencia M. (2013) publican que la incorporación de una hidroxí apatita comercial y el comonómero alcalino metacrilatocrilato del 2-dimetilamino etilo), se logró la reducción de la temperatura de polimerización; la referencia *Comparative study on the properties of acrylic bone cements prepared with either aliphatic or aromatic functionalized methacrylates* Cervantes J, Vázquez H, Cauich J, Vázquez B,

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San Roman J. (2005) publican que la temperatura máxima (TP) disminuía al aumentar la concentración de monómeros 4-methacryloyloxybenzoic acid (MBA), 4-diethyaminobenzyl methacrylate (DEABM) y diethyl amino ethyl methacrylate (DEAEM).

5

Para disminuir el porcentaje de monómero residual en cementos óseos acrílicos la referencia *Efecto del contenido de BaSO₄ y DEAEA sobre las propiedades reológicas de cementos óseos para vertebroplastia* Mayra E. Valencia, José H. Mina, Fabio Zuluaga, Juan V. Cauich-Rodríguez, José M. Cervantes-U. (2012) publican que la incorporación de 4 % del Acrilato del 2- (dietilamino etilo) y 30 % de BaSO₄ presentó una reducción significativa del monómero residual comparado con el cemento comercial Zimmer Dough.

10

Para mejorar la biocompatibilidad en cementos óseos acrílicos mediante la incorporación de cargas bioactivas, la referencia *In vitro evaluation of a new polymethylmethacrylate cement reinforced with hydroxyapatite* Dalby MJ, Di Silvio L, Harper EJ, Bonfield W (1999), publican que incorporaron 17,5 % de hidroxapatita (HA) a la formulación del cemento, y encontraron que el cemento con HA era un mejor sustrato para los osteoblastos, dando como resultado una mayor proliferación y actividad de la fosfatasa alcalina (ALP). La microscopía electrónica de barrido (SEM) y la microscopía electrónica de transmisión (TEM) mostraron que los osteoblastos cultivados en el PMMA con HA anclaban preferentemente a partículas de HA expuestas en la superficie del cemento, observándose una estrecha intimidad entre los osteoblastos y la HA; la referencia *New partially degradable and bioactive acrylic bone cements based on starch blends and ceramic fillers* Espigares I, Elvira C, Mano JF, Vázquez B, San Román J, Reis RL. (2002) publican que incorporaron hidroxapatita en formulaciones de cemento con mezclas de almidón de maíz y acetato de celulosa y encontraron que la HA es responsable de inducir la bioactividad del cemento; la referencia *Influencia de la incorporación de un co-monómero alcalino e hidroxapatita en las propiedades de cementos óseos acrílicos*

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Lozano K, Mina J, Zuluaga F, Valencia C, Valencia M. (2013) publican que incorporaron hidroxapatita y un comonomero alcalino que en el compuesto generaron bajas temperaturas de polimerización, bajos módulos y resistencias mecánicas, así como la fijación de una capa similar a la apatita biológica en la superficie del material después de 30 días de inmersión en un fluido biológico simulado; la referencia *Polymethylmethacrylate composites: Disturbed bone formation at the surface of bioactive glass and hydroxyapatite* Heikkilä JT, Aho a. J, Kangasniemi I, Yli-Urpo a (1996) publican que estudiaron los efectos de la adición de hidroxapatita y biovidrio a la formulación del cemento en fémures de conejos y reportaron que la osteoconductividad de las cargas se ve perturbada por el PMMA; sin embargo, el efecto del PMMA fue menos perjudicial sobre el biovidrio; la referencia *Acrylic-phosphate glasses composites as self-curing controlled delivery systems of antibiotics* Fernández M, Méndez J a., Vázquez B, et al. (2002) publican que incorporaron biovidrios de silicato y de borato a la formulación del cemento y lograron la interacción del cemento con el medio del fosfato buferado mediante la deposición de orto y pirofosfatos sobre los espacios disponibles después de la disolución del biovidrio; la referencia *Preparation and study of in vitro bioactivity of PMMA-co-EHA composites filled with a $\text{Ca}_3(\text{PO}_4)_2\text{-SiO}_2\text{-MgO}$ glass* Lopes PP, Leite Ferreira BJM, Almeida N a F, Fredel MC, Fernandes MH V, Correia RN (2008) publican que la fase sólida del cemento fue modificada con diferentes porcentajes del vidrio $\text{Ca}_3(\text{PO}_4)_2\text{-SiO}_2\text{-MgO}$. Los resultados obtenidos confirmaron el crecimiento de una capa similar a la hidroxapatita (HA) en la superficie de los materiales compuestos preparados. Como era de esperar, la formación de la capa de HA fue más rápida para compuestos preparados con mayor contenido del biovidrio; la referencia *Silicate and borate glasses as composite fillers: A bioactivity and biocompatibility study* Lopes PP, Leite Ferreira BJM, Gomes PS, Correia RN, Fernandes MH, Fernandes MH V (2011) publican que los vidrios de silicatos demostraron un efecto inductivo sobre la proliferación de osteoblastos humanos; la referencia *Acrylic formulations containing bioactive and biodegradable fillers to be used as bone cements:*

Properties and biocompatibility assessment Lopes PP, Garcia MP, Fernandes MH, Fernandes MH V (2013) publican que la fase sólida de los cementos adicionados con biovidrios se modificó con la adición de los polímeros biodegradables poli (3-hidroxibutirato) (PHB) y su copolímero con hidroxivalerato (PHBV), la inclusión del PHBV mejoró enormemente la respuesta biológica en ambos cementos rellenos con vidrio de silicato de borato, en comparación con la inclusión del PHB; la referencia *A bone substitute composed of polymethylmethacrylate and α -tricalcium phosphate: Results in terms of osteoblast function and bone tissue formation*. Fini M, Giavaresi G, Nicoli Aldini N, et al (2002) publican que las propiedades biológicas de compuestos de PMMA y fosfato tricalcico alfa (α –TCP) fueron ensayadas en cultivos de osteoblastos (MG 63) demostrando que el compuesto con la carga afectó positivamente la viabilidad celular y en los implantes en conejos mostró mayor osteointegración y velocidad de formación de nuevo hueso comparado con el PMMA solo; la referencia *A new bioactive bone cement: effect of glass bead filler content on mechanical and biological properties* Shinzato S, Nakamura T, Kokubo T, Kitamura Y (2001) publica que estudiaron las propiedades biológicas de un nuevo cemento óseo bioactivo (designado GBC), que consiste en perlas de vidrio bioactivo como un relleno inorgánico y polimetilmetacrilato (PMMA) como una matriz orgánica en tibias de ratas. Reportan que el índice de afinidad se incrementa con el contenido de la carga y con el tiempo de implantación y a partir de los resultados concluyen que el GBC con 60 % de la carga es la formulación más adecuada; ya que muestra una excelente osteoconductividad y suficiente fuerza mecánica para uso clínico; la referencia *Mechanical performance and in vivo tests of an acrylic bone cement filled with bioactive sepia officinalis cuttlebone* García-Enriquez S, Guadarrama HER, Reyes-González I, et al. (2010) publican que para promover la osteointegración, se usaron partículas bioactivas de hueso de tibia que contenían colágeno como relleno en un cemento acrílico, variando la concentración de la carga entre 0-50 % en peso. Las pruebas In vivo en huesos parietales de conejos adultos sanos arrojaron mayor osteointegración en los

cementos que contenían la carga en comparación con aquellos que no; la referencia *Porous Surface Modified Bioactive Bone Cement for Enhanced Bone Bonding* He Q, Chen H, Huang L, et al (2010) publican que la formulación de cemento óseo fue modificada con la adición de micropartículas de carboximetilcelulosa, alginato y gelatina para promover el crecimiento de tejido óseo en un defecto creado en el condilo femoral de conejos. El grado de adhesión, proliferación y diferenciación osteogénica de los preosteoblastos, además de la unión interfacial con el hueso fue mejorada con las micropartículas debido a la formación de la capa de apatita y la osteointegración después de la implantación en el defecto óseo; la referencia *The in vitro bioactivity of two novel hydrophilic, partially degradable bone cements* Boesel LF, Cachinho SCP, Fernandes MH V, Reis RL. (2007) publican que desarrollaron cementos bioactivos, biodegradables e hidrofílicos que contenían mezclas basadas en almidón y biovidrios; los cuales presentaron una excelente combinación de propiedades mecánicas, de curado, degradación y bioactivas.

Para mejorar la resistencia biomecánica en cementos óseos acrílicos, la referencia *Chapter 8. Carbon Nanotubes in Acrylic Bone Cement* Dunne N, Ormsby R, Mitchell CA (2013) publican que se obtuvo un notable incremento en las propiedades mecánicas de la matriz, en especial la resistencia a la fatiga y el impacto; la referencia *The mechanical study of acrylic bone cement reinforced with carbon nanotube* Nien Y-H, Huang C. (2010) publican que se reemplazó el 30% de PMMA en la fase sólida del cemento óseo comercial OSTEOBOND, por perlas compuestas de PMMA y nanotubos de carbono multipared (MWCNT) en diferentes proporciones con la finalidad de obtener mejor dispersión de los MWCNT y así lograron disminuir la rigidez y la Tg del cemento y aumentar las propiedades a compresión y a tensión; la referencia *Augmentation of acrylic bone cement with multiwall carbon nanotubes*. Marrs B, Andrews R, Rantell T, Pienkowski D. (2006) publican que la incorporación de 2% en peso de MWCNT en la fase sólida de un cemento comercial, mejoró

- notablemente las propiedades a flexión y la resistencia a fatiga del ABC comparado con el cemento sin MWCNT; la referencia *Influence of multiwall carbon nanotube functionality and loading on mechanical properties of PMMA/MWCNT bone cements* Ormsby R, McNally T, Mitchell C, Dunne N. (2010b) publican que con adiciones al cemento de MWCNT funcionalizados con grupos carboxilos y aminos en una proporción $\leq 0,25$ % generaron un aumento significativo en las propiedades a compresión, fractura y flexión; aunque, la mala dispersión de los CNT afectó las propiedades de los cementos con mayor contenido de estos; la referencia *Incorporation of MWCNTs to PMMA Bone Cements: Effects on Fatigue Properties* Lin Y, Xu YZ (2014) publican que las propiedades a fatiga se ven significativamente mejoradas con el aumento en la proporción de CNT hasta 0,25%, atribuido esto a que los CNT retardan la propagación de las grietas mediante un efecto de puente; la referencia *Fatigue and biocompatibility properties of a poly(methyl methacrylate) bone cement with multi-walled carbon nanotubes* Ormsby R, McNally T, O'Hare P, Burke G, Mitchell C, Dunne N (2012) publican que las propiedades a fatiga se ven mejoradas con el aumento en la proporción de CNT en el cemento.
- Para mejorar la actividad antimicrobiana en cementos óseos acrílicos la referencia *Gentamicin release from modified acrylic bone cements with lactose and hydroxypropylmethylcellulose* Virto, M. R.Frutos, P. Torrado, S. Frutos, G. (2003) publican que la incorporación de Lactosa, Hidroxipropil-metilcelulosa incrementó la liberación de sulfato de gentamicina en el cemento comercial CMW1; la referencia *Antibiotic-loaded acrylic bone cements: An in vitro study on the release mechanism and its efficacy* Miola, M., Bistolfi, A., Valsania, M. C., Bianco, C., Fucile, G., Verné, E. (2013) publican que investigaron las propiedades antibacterianas de los cementos comerciales Palacos® R + G y Palacos® LV + G los cuales vienen adicionados con gentamicina y de los cementos Palacos® R y Palacos® LV a los cuales se les adicionó manualmente la gentamicina y se encontró que hay muy poca diferencia en la

- velocidad de liberación, actividad antimicrobiana y en la resistencia a compresión del cemento comercialmente cargado con el antibiótico y el manualmente mezclado; la referencia *Release of gentamicin sulphate from a modified commercial bone cement. Effect of (2-hydroxyethyl methacrylate) comonomer and poly(N-vinyl-2-pyrrolidone) additive on release mechanism and kinetics* Frutos, P., Diez-Peña, E., Frutos, G., & Barrales-Rienda, J. M. (2002) publican que la incorporación de poli (n-vinil-2-pirrolidona) incrementó la liberación de sulfato de gentamicina en el cemento comercial CMW1®; la referencia *Nanostructured material formulated acrylic bone cements with enhanced drug release* Shen, S.-C., Ng, W. K., Dong, Y.-C., Ng, J., & Tan, R. B. H. (2016) publican que la incorporación de porcentajes de hidroxapatita mayores al 10 %, de nanotubos de carbono o de nanopartículas de sílica nanoporosa incrementó la liberación de gentamicina en los cementos comerciales CMW, Smart GHV y Simplex P; la referencia *Acrylic-phosphate glasses composites as self-curing controlled delivery systems of antibiotics* Fernández M, Méndez J a., Vázquez B, et al. (2002) publican que incorporaron biovidrios y antibiótico (vancomicina) a la formulación del cemento para mejorar la liberación del fármaco; la referencia *Antibiotic-releasing porous polymethylmethacrylate / gelatin/antibiotic constructs for craniofacial tissue engineering* Shi, M., Kretlow, J. D., Spicer, P. P., Tabata, Y., Demian, N., Wong, M. E., ... Mikos, A. G. (2011) publican que la incorporación de micropartículas de gelatina en la formulación del cemento comercial mejoró la liberación de sulfato de colistina.
- De acuerdo con la anterior revisión se puede observar que aún se está en la búsqueda del material cementante ideal, por ejemplo, la adición de cargas de hidroxapatita y fosfatos de calcio sintético si bien mejoran la biocompatibilidad afectan las propiedades biomecánicas. De acuerdo con los resultados de los trabajos antes mencionados, se puede establecer que la incorporación de hidroxapatita (HA) a formulaciones de cemento óseo mejora la biocompatibilidad y bioactividad de estos materiales, pero generalmente reduce

sus propiedades mecánicas. Esto está relacionado no sólo a la falta de unión interfacial entre la matriz polimérica y la carga inorgánica, sino también debido a la aglomeración de Hidroxiapatita (es decir, partículas inorgánicas hidrófilas no se dispersan fácilmente en una matriz hidrofóbica) que actúa como

5 concentradores de esfuerzos. En este mismo sentido, se reporta que la sustitución parcial de las perlas de PMMA por la combinación de diferentes partículas de rellenos bioactivos y/o biodegradables (quitosano, vidrio a base de silicato o de borato, nanopartículas de plata, polímeros biodegradables, entre otros), a pesar de que inducen una respuesta biológica positiva, retienen

10 el comportamiento rígido y frágil del material cementante. Por otro lado, si bien la incorporación de fibras de diferente naturaleza en la mayoría de las investigaciones mejora las propiedades mecánicas tales como la resistencia al desgaste, esfuerzo y dureza, no se reporta que éstas mismas estimulen un comportamiento biocompatible y/o osteoconductor de la matriz hidrofóbica de

15 MMA. Finalmente, la incorporación de comonómeros en diferentes proporciones a la fase líquida del compuesto acrílico, puede contribuir en el decrecimiento de la exotermia de polimerización y aumento de la respuesta bioactiva, pero en algunos casos trae consigo un decrecimiento en la resistencia y el módulo elástico debido a los copolímeros generados entre éstos

20 y el MMA.

La patente US 4,910,259 muestra una composición de cemento óseo de acrilato preparada a partir de un componente A (met) acrilato monomérico líquido y un componente B de polvo de polímero, donde el componente B

25 comprende polímeros o copolímeros de (met) acrilato o una mezcla de los mismos que tiene una temperatura de transición vítrea en el intervalo de 37 De 0 a 90 ° C; donde el componente A comprende una mezcla de C1-C2 metacrilato de alquilo, un (met) acrilato de cadena larga lineal o ramificada que tiene un peso molecular de al menos 168, seleccionado del grupo que consiste

30 en metacrilato de etilhexilo, metacrilato de isodecilo, metacrilato de n-decilo y metacrilato de laurilo, y un (met) acrilato cíclico que tiene una molécula peso de

al menos 168, teniendo dicha mezcla una temperatura de transición vítrea cuando se polimeriza, en el intervalo de 23°C a 70°C; por lo que, cuando el componente monomérico líquido A se mezcla con el componente polimérico B, la composición de cemento óseo resultante tiene una temperatura de transición
5 vítrea en el intervalo de 37°C a 70°C.

La patente ES 2 373 975 muestra un cemento óseo acrílico se caracteriza porque alcanza una viscosidad de al menos 500 Pascal-segundos dentro de los 180 segundos siguientes al inicio de la mezcla de un componente monomérico
10 y un componente polimérico y se caracteriza por una suficiente biocompatibilidad para permitir el uso in vivo. Para la preparación de emplean proporción en peso de $\approx 2,2:1$ y $2,5:1$, respectivamente. En general, el aumento de la proporción en peso de polímero con respecto a monómero produce un cemento que alcanza una mayor viscosidad en menos tiempo. No obstante,
15 existe un límite más allá del cual no hay suficiente monómero para humectar todas las perlas de polímero.

La solicitud internacional de patente WO 2004/103420 muestra un desarrollo de formulaciones acrílicas portadoras de un componente bioactivo, vidrios
20 bioactivos o nanopartículas de sílice con diferentes tamaños y morfologías tales como aerosil, y de un fármaco antiinflamatorio portador de grupos fosfato, que sean inyectables y de curado 'in situ'. Este tipo de formulaciones están diseñadas para su aplicación en vertebroplastias percutáneas, defectos óseos producidos tras recesión de tumores o de hipertrofias, y estabilización
25 biomecánica en cuadros de osteoporosis con pérdida de masa ósea.

La patente ES 2 420 861 muestra un cemento óseo inyectable para el llenado de huesos con unas propiedades mecánicas equivalentes a las del hueso esponjoso vertebral que comprende entre el 70% y el 99% en peso de un
30 polímero acrílico combinado con un compuesto radiopaco de tipo inorgánico y que comprende entre el 1% y 30% en peso de partículas sólidas flexibles

hidrófilas calibradas (gelatina y/o polisebacato de glicerol) que pueden absorber los líquidos fisiológicos sin crear porosidad interconectada. El cemento óseo según la presente invención es de calidad médica y encuentra aplicaciones particularmente ventajosas en la vertebroplastia, la cifoplastia o cementoplastia.

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La solicitud internacional de patente WO 11/039905 muestra un cemento óseo que comprende al menos un componente polimerizable hidrófilo, al menos un componente hidrófilo y material biodegradable, que comprende partículas biodegradables, no mezclándose componente hidrófilo e hidrófobo, y estando
10 contenidas las partículas biodegradables en el componente hidrófilo al menos parcialmente, caracterizado por que el componente hidrófilo, junto con el material biodegradable, está configurado como pasta, y presentándose el material biodegradable en forma de nanopartículas.

15 La patente ES 2 285 663 muestra un cemento acrílico utilizado en la reparación de los traumatismos de los huesos y de las articulaciones. Tiene como objetivo un cemento fluido de uso médico en la reconstrucción ósea, particularmente para rellenar cuerpos vertebrales, así como una composición binaria destinada a la preparación de dicho cemento. Otro objetivo de la presente invención es un
20 dispositivo de envasado de dicha composición binaria. Se reivindica asimismo un procedimiento de preparación de un cemento óseo a partir de una composición binaria.

De acuerdo con la revisión de las patentes no se encontraron formulaciones
25 que afectaran significativamente la capacidad inventiva del producto que se está presentando; la patente US 4,910,259 muestra algunos rangos de temperatura y unos porcentajes de monómero residual similares a los mostrados en los resultados de esta invención, sin embargo ellos lo logran a partir de modificar principalmente la fase líquida con unos reactivos y aditivos
30 que no están contemplados en la formulación propuesta, de igual manera ocurre con la solicitud de patente WO 2004/103420, en la cuál incorporan

componentes bioactivos de una naturaleza diferente a los empleados en la fase sólida de ésta invención y además se informa que está diseñado para emplearse en aplicaciones en las cuales no se demande alta resistencia biomecánica, otro aspecto a resaltar es que en esta invención se preparan los
5 cementos óseos teniendo cuidado de incorporar la fase a la fase líquida tal como lo reportan las invenciones US 4,910,259, ES 2 373 975, WO 2004/103420, ES 2 420 861 y ES 2 285 663; sin embargo, los rangos de las cantidades empleadas para cada uno de los componentes de ambas fases de la presente invención, difiere significativamente de las mencionadas
10 anteriormente.

En conclusión de esta consideración del estado de la técnica es que si bien de alguna forma las diferentes invenciones trataron de solucionar algunos de los problemas ya mencionados como por ejemplo la temperatura de reacción
15 exotérmica, en este trabajo se enfrentó en forma integral la tolerancia biológica y la suficiencia mecánica partiendo del precepto que las dos están directamente relacionadas y un buen cemento óseo debe tener una buena biocompatibilidad conservando propiedades biomecánicas adecuadas, pero procurando que se dé la regeneración del tejido óseo en las interfaces
20 prótesis–cemento y cemento–hueso.

DESCRIPCIÓN DE LA INVENCION

5 La presente invención revela una composición de cemento óseo acrílico mejorado, con aplicación en ortopedia y traumatología con uso para prótesis articulares endóseas y material de osteosíntesis de aplicación intraósea con cualidades osteoconductoras, bioactivas, osteopromotoras y bacteriostáticas, así como relleno óseo.

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DESCRIPCIÓN DETALLADA DE LA INVENCION

La invención desarrollada se caracteriza por estar conformada por dos fases, una sólida y una líquida, donde a la fase sólida se le han incorporado componentes bioactivos como una apatita natural modificada superficialmente para lograr una mejor la interacción con la matriz polimérica de PMMA, un componente biopolímero osteopromotor como el quitosano, un componente biopolímero osteoconductor como el ácido poliláctico y un componente bioactivo definido por Williams en 1987 como *"Material que induce una actividad biológica específica"*, bacteriostático y un componente de refuerzo mecánico como el óxido de grafeno.

En la fase líquida se incorporó cloruro de estroncio el cual además de reforzar la radiopacidad del cemento mejora la resistencia mecánica y los comonomeros alcalinos que reducen la temperatura de la polimerización, comonomeros alcalinos como metacrilato de dimetil amino etilo DMAEM.

La formulación presentada, con los componentes mencionados en el párrafo anterior, permite tener un cemento óseo acrílico con componentes hidrofílicos e

hidrofóbicos que permiten mejorar la relación del cemento con el tejido óseo, con unas características de biocompatibilidad mejoradas que crean un ambiente adecuado para el crecimiento celular y una menor respuesta inflamatoria; conservando unas propiedades biomecánicas adecuadas con una

5 disminución significativa en los valores de la temperatura de reacción exotérmica y en los porcentajes de monómero residual.

A continuación, se presentan los principales resultados que se obtuvieron al incorporar los componentes: apatita natural modificada superficialmente con

10 agente de acoplamiento silano, quitosano y óxido de grafeno.

Resultados de la incorporación de una apatita natural modificada superficialmente, la apatita natural utilizada como carga bioactiva, se obtuvo de hueso compacto femoral de ganado bovino. Los huesos se sometieron a un

15 proceso de limpieza, cortado y molienda, obteniéndose de esta manera partículas a las que posteriormente se le realizó un tratamiento térmico a 800°C, tras realizar pruebas biológicas mediante cultivo de osteoblastos, los resultados mostraron adhesión, crecimiento celular y presencia de depósitos compatibles con matriz ósea extracelular con contenido orgánico e inorgánico.

20 Además, la caracterización fisicoquímica permitió identificar que este fosfato de calcio natural obtenido, además de cumplir química y térmicamente con los estándares necesarios para comportarse de manera óptima al ser utilizado como material de relleno en los cementos óseos, tiene muy buenas propiedades desde el punto de vista morfológico.

La modificación superficial de las partículas de apatita natural con el uso del agente de acoplamiento silano, 3-Aminopropiltietoxisilano, repercutió positivamente la zona interfacial de la apatita natural con la matriz orgánica de PMMA, contribuyendo al mejoramiento del desempeño mecánico. Ya que la

30 incorporación de apatita natural a la formulación de cemento óseo acrílico en porcentajes entre 1 y 20%, aumento desde 80 MPa la resistencia a la

compresión y en la medida que esta interaccionó en presencia del agente de acoplamiento silano, alcanzó los 120 MPa. Así mismo, la resistencia a la flexión del cemento óseo aumentó con la incorporación de apatita natural desde 40 MPa hasta los 50MPa cuando se realizó tratamiento superficial a las partículas minerales con el aminosilano.

En condiciones in vivo, a partir de la implantación del cemento óseo modificado con la incorporación de hasta 14% de apatita natural silanizada en el tejido conectivo de biomodelos, se observó recuperación del pelo en las zonas intervenidas y cicatrización normal con ausencia de bridas cicatrízales. Al incidir los tejidos se encontró que el material estaba rodeado de una capsula fibrosa, sin presencia de exudados purulento o inflamatorios y el resto de los tejidos blandos con apariencia normal. El análisis histológico evidenció un proceso cicatrizal normal con recuperación de la arquitectura tisular normal de epidermis, dermis y tejido subcutáneo, con condiciones tisulares normales en el resto de la muestra incluyendo las capas de epidermis y dermis donde se incidió para introducir el material.

Resultados de la incorporación del óxido de grafeno, la presencia de óxido de grafeno (GO) en la formulación de cemento óseo acrílico en porcentajes entre 0,001 a 2 % de la fase sólida generó un refuerzo mecánico en presencia de polímeros biodegradables como el quitosano (CS), alcanzándose un incremento en la resistencia a compresión de la formulación con 15 % de CS de 62,7 MPa a 77,2 MPa con la adición de 0,3 % de GO, con los resultados anteriormente obtenido se puede extrapolar una mejor resistencia a compresión que podrían incluir porcentajes de óxido de grafeno (GO) hasta el 10% p/p de la composición final en la práctica.

El incremento en el porcentaje de óxido de grafeno reduce significativamente la temperatura máxima de polimerización alcanzando valores inferiores a 50 °C.

La presencia de óxido de grafeno en la formulación del cemento óseo aumenta la actividad antimicrobiana frente a la bacteria gramnegativa *Escherichia coli* (*E. coli*) en un 81 % aproximadamente comparado con el cemento sin adición. Cuando se combina GO y CS en la formulación del cemento se alcanza un

5 aumento por encima del 86 % en esta propiedad con respecto a la formulación sin adición.

La resistencia a compresión y flexión de los cementos óseos con quitosano y fosfato de calcio fue 82.25MPa y 42.64 MPa (Tabla 2) (Figura 1 y 2),

10 respectivamente, hallándose que la adición de quitosano y fosfato de calcio reducía estas propiedades respecto a los cementos óseos sin adiciones. En cuanto al cumplimiento de los estándares presentados en la norma ISO 5833 se encontró que el cemento óseo con quitosano y fosfato de calcio cumplía con el requerimiento de resistencia a la compresión (Mínimo 70 MPa) pero estuvo

15 por debajo de la resistencia a flexión establecida (Mínimo 50 MPa).

A pesar de la reducción en la resistencia a la flexión, la adición de quitosano se compensa por las propiedades biológicas de este componente, como son la baja reacción a cuerpo extraño, la biodegradabilidad, hidrofiliidad, no

20 toxicidad, actividad anti-microbiana, bioadherencia y afinidad celular.

Es ampliamente conocido que la reacción de polimerización del cemento óseo acrílico es exotérmica, debido al rompimiento del doble enlace carbono-carbono del metacrilato de metilo, por esto es muy importante la medición de

25 las propiedades de curado, debido a que al ponerse este biomaterial en contacto con el tejido vivo es necesario que cumpla ciertas cualidades, entre las más importantes la temperatura de curado (que se encuentra directamente relacionada con la temperatura máxima alcanzada en la polimerización). Algunos autores indican que es necesario controlar la temperatura máxima

30 para minimizar la necrosis del tejido circundante y para alcanzar esto han

empleado la adición de cargas como los fosfatos de calcio, quitosano y monómeros, entre otros.

El tamaño de partícula del quitosano (412.8 μm) influyó en la temperatura de curado, respecto a esto algunos investigadores han evidenciado que partículas con tamaños de partícula $> 300 \mu\text{m}$ causan reducción en la transferencia de calor conllevando a una menor entrega de calor al cuerpo. Adicionalmente. Las partículas de quitosano incorporadas al cemento óseo permiten una reducción en la temperatura de curado ya que trabajan dentro del compuesto como sumideros de calor.

La reducción de la temperatura es un hecho beneficioso desde el punto de vista biológico y la adición de quitosano generó que la temperatura máxima alcanzada se redujera desde 83.5 a 79°C.

Tabla 2. Caracterización mecánica y térmica de los cementos óseos con adición de 10% quitosano – 20% fosfato de calcio.

Muestra	σ_c , Mpa	E_c , Mpa	σ_f , Mpa	E_f , Mpa	Tiempo de Curado, min	Temperatura de Curado, °C
10% Quitosano- 20% Fosfato de Calcio	84.25 ± 2.79	2061.46 ± 76	42.64 ± 3.58	4331.18 ± 572.38	6.05 ± 0.2	52 ± 0.71
Sin adiciones	101.46 ± 9.16	2083.91 ± 75	60.32 ± 6.61	3820.67 ± 292.28	4.67 ± 0.07	54.25 ± 0.35

tóxicas y generan resultados confiables siendo una alternativa poco costosa, sencilla y rápida. Este bioensayo es aplicable a compuestos con estructuras y modos de actividad diversos, lo que le otorga la característica de ser de amplio espectro, de ahí que sea usado como método de análisis para detectar

5 residuos de pesticidas, micotoxinas, anestésicos y compuestos de tipo morfínico entre otros, es considerado tóxico el producto que induce una mortalidad, en las Artemias, mayor o igual a 30%.

En este ensayo se observó que el cemento óseo con adición de quitosano y

10 fosfato de calcio no generó citotoxicidad, Tabla 3.

Tabla 3. Resultados de bioensayo con Artemia salina en cementos óseos con quitosano.

Cemento	# de Artemias muertas	# de Artemias vivas	%Mortalidad
Control(Agua de mar)	1	19	5
Blanco(Etanol)	20	0	100
10%Quitosano- 20%fosfato de calcio	0	20	0

La pérdida de peso y absorción de agua (Tabla 4) no fueron significativas, en los 60 días de inmersión en fluido biológico simulado no se evidenció una degradación efectiva del quitosano o fosfato de calcio.

Tabla 42. Resultados de la absorción de agua y pérdida de peso de los cementos óseos con quitosano después de 60 días de inmersión.

Cemento	Absorción de Agua (%)	Pérdida de Peso (%)
10% Quitosano- 20%fosfato de calcio	3.81 ± 0.74	0.33 ± 0.25
Sin adiciones	5.92 ± 1.36	0 ± 0.07

Se evidenció que la inmersión en el fluido biológico tuvo un efecto más significativo en el módulo que en la resistencia a compresión atribuyendo este comportamiento a una posible absorción de agua, lo que generó un efecto plastificante en los cementos óseos. Este hecho se observa en la Tabla 5, donde se muestra que los cementos óseos presentaron una absorción de agua de 5.92% para los cementos sin adiciones y 3.81% para los cementos que contenían 10% quitosano y 20% fosfato de calcio.

La cantidad de monómero residual se encontró entre lo recomendado por varios autores para los cementos óseos acrílicos, ya que se encontró por debajo de un 6% (Tabla 5).

Tabla 53. Porcentaje monómero residual en formulaciones de cementos óseos con quitosano.

Cemento	Monómero residual (%)
0% polímeros biodegradables- 0% fosfato de calcio	1,67
10% de quitosano – 20% fosfato de calcio	1,94

Para la realización de los ensayos en condiciones in vivo se implantaron 4 muestras de cementos con quitosano y 4 muestras para los cementos sin adiciones teniendo en cuenta los resultados obtenidos en los ensayos mecánicos y en condiciones in vitro que se realizaron previamente. Las
5 formulaciones seleccionadas presentaron un bajo contenido de monómero residual < 3% y un comportamiento mecánico aceptable.

Ninguno de los biomodelos murió en el periodo de estudio. Todos los biomodelos presentaron apetito normal. Pasados los 30 días de implantación,
10 se procedió a retirar los implantes después de practicar la eutanasia. Se observó que macroscópicamente los biomodelos no presentaron ningún comportamiento anormal, además el tejido alrededor de la zona de implantación había cicatrizado normalmente y se encontraba ya cubierto de pelo (Figura 5).

15 Al retirar el implante, se encontró que internamente no presentaba ningún contenido purulento que indicará una respuesta adversa del organismo hacia el material implantado (Figura 5), el tejido se observaba sano. Se evidenció la formación de una cápsula alrededor del implante siendo esta una respuesta
20 normal del organismo al encontrar un cuerpo extraño. Esta baja respuesta adversa se esperaba ya que los componentes en su mayoría son considerados biocompatibles y/o bioactivos.

La muestra de tejido retirada de la zona de implantación se empleó para
25 analizar la histología del mismo por medio de tinción con hematoxilina y eosina y poder establecer cambios a nivel microscópico en los biomodelos por interacciones con el material implantado, mediante la toma de fotografías con un microscopio óptico.

30 Las imágenes histológicas mostraron una arquitectura normal con los componentes habituales de este tipo de tejido, con ausencia de bridas

cicatrizales y alteraciones patológicas en las zonas intervenidas; en la figura 6, se observan las fotografías tomadas a 40X de una muestra de piel que estuvo en contacto con cemento óseo con adición de 10% de quitosano-20% de fosfato de calcio, mostrando la zona de la epidermis, dermis e hipodermis, donde se evidenció la presencia de fibroblastos, colágeno, glándulas sebáceas, células adiposas y escasas células inflamatorias, en su mayoría linfocitos.

El proceso cicatrizal en el caso de este tipo de procedimientos, en el cual además del trauma quirúrgico se implanta un material parcialmente degradable y reabsorbible, está determinado por una reacción a cuerpo extraño; en partículas de tamaño grande, ante la imposibilidad de ser fagocitado las células inflamatorias las rodean de una capsula fibrosa en un intento por limitar el daño y permitir que la cicatrización siga su proceso normal.

En el caso de la totalidad de las muestras utilizadas en esta investigación se observó que el material implantado estaba rodeado de una capsula fibrosa lo que permitió que el proceso cicatrizal continuara normalmente, lo que se confirma por las imágenes histológicas que muestran parámetros de normalidad con restauración de la arquitectura histológica típica de este tejido.

Se encontró que en los cementos con adición de quitosano se presentaba una porosidad en el cemento óseo de entre 64-150 μm , en este sentido un tamaño de poro de 150 μm es bueno para el crecimiento de hueso.

El quitosano es un polielectrolito con grupos funcionales reactivos, alta capacidad de absorción y biodegradabilidad, biocompatible y no tóxico para tejidos vivos, además presenta propiedades antibacteriales, antifúngicas y antitumorales. Con la adición de quitosano se esperaba un incremento en la adhesión celular, ya que este material se ha demostrado presenta buena adherencia a los tejidos epiteliales como también a fibroblastos favoreciendo la proliferación celular.

La anterior descripción solo puede ser tomada como referencia y no limitativa en sus componentes ni en su relación explícita, sino que han sido descritos para proporcionar una idea clara sobre la conformación general de la materia de la invención reclamada.

5

DESCRIPCIÓN DE LAS FIGURAS

5

Para complementar la descripción y con objeto de ayudar a una mejor comprensión de las características técnicas del invento, se acompaña a la presente memoria descriptiva como parte integrante de la misma, un conjunto de figuras definidas así:

10

Figura 1. Resistencia a la compresión cementos óseos con adición de 10% Quitosano- 20% fosfato de calcio.

15

Figura 2. Resistencia a la flexión cementos óseos con adición de 10% Quitosano- 20% fosfato de calcio.

20

Figura 3. Propiedades mecánicas de los cementos óseos con adición de 10% quitosano- 20% fosfato de calcio a diferentes tiempos de inmersión en fluido biológico simulado: 0, 15, 30, 45 y 60 días. Resistencia a la compresión.

25

Figura 4. Propiedades mecánicas de los cementos óseos con adición de 10% quitosano- 20% fosfato de calcio a diferentes tiempos de inmersión en fluido biológico simulado: 0, 15, 30, 45 y 60 días. Resistencia a la flexión.

30

Figura 5. Fotografías del estado macroscópico de los biomodelos después de 30 días de implantación, extracción de la muestra.

Figura 6. Fotografías del tejido en biomodelos implantados con la formulación 10% quitosano- 20% fosfato de calcio. (a) Epidermis 40X (b) Dermis 40X (c) Hipodermis 40X. Técnica H&E.

5 Figura 7. Microscopía electrónica de barrido 100X de cemento óseo después de 30 días de implantación subcutánea en biomodelos ratas 10% quitosano- 20% fosfato de calcio.

REIVINDICACIONES

1. Composición de cemento óseo con una fase sólida y una fase líquida **caracterizada porque** la fase sólida tiene un componente de matriz polimérica orgánica, un componente biopolímero osteomotor, un componente de refuerzo mecánico, un componente bioactivo o mezcla de componentes bioactivos y un componente biopolímero osteoconductor y la fase líquida incluye cloruro de estroncio y comonómeros alcalinos.
2. Composición de cemento óseo de acuerdo con la reivindicación 1, **caracterizada porque** el componente de matriz polimérica orgánica es polimetilmetacrilato PMMA.
3. Composición de cemento óseo de acuerdo con la reivindicación 1, **caracterizada porque** el componente biopolímero osteomotor es quitosano.
4. Composición de cemento óseo de acuerdo con la reivindicación 1, **caracterizada porque** el componente de refuerzo mecánico es óxido de grafeno en forma de láminas.
5. Composición de cemento óseo de acuerdo con la reivindicación 1, **caracterizada porque** el componente bioactivo es seleccionado entre apatita natural, sulfato de calcio o una mezcla de los mismos.

6. Composición de cemento óseo de acuerdo con la reivindicación 1, **caracterizada porque** el componente biopolímero osteoconductor es ácido poliláctico PLA.
7. Composición de cemento óseo con una fase sólida y una fase líquida **caracterizada porque** la fase sólida tiene un componente de matriz polimérica de polimetilmetacrilato PMMA, quitosano, óxido de grafeno, un componente bioactivo, ácido poliláctico y la fase líquida incluye cloruro de estroncio y comonomeros alcalinos.
8. Composición de cemento óseo de acuerdo con la reivindicación 7, **caracterizada porque** el componente bioactivo es seleccionado entre apatita natural, sulfato de calcio o una mezcla de los mismos.
9. Composición de cemento óseo de acuerdo con la reivindicación 8, **caracterizada porque** la apatita natural es modificada superficialmente con agentes de acople tipo silanos.
10. Composición de cemento óseo de acuerdo con la reivindicación 4 o 7, **caracterizada porque** el óxido de grafeno está en el rango de 0.1% al 10% p/p de la composición final.
11. Composición de cemento óseo de acuerdo con las reivindicaciones 7 y 8 **caracterizada porque** el componente bioactivo está en el rango de 1% a 30 % p/p de la composición final.
12. Composición de cemento óseo de acuerdo con la reivindicación 7, **caracterizada porque** el quitosano está en el rango de 1% a 20 % p/p de la composición final.

RESUMEN

COMPOSICIÓN DE CEMENTO OSEO

La presente invención revela una composición de cemento óseo con una fase sólida y una fase líquida donde la fase sólida tiene un componente de matriz polimérica orgánica, un componente biopolímero osteomotor, un componente de refuerzos mecánicos, un componente bioactivo o mezcla de componentes bioactivos y un componente biopolímero osteoconductor y la fase líquida incluye cloruro de estroncio y comonómeros alcalinos.

FIGURA 1

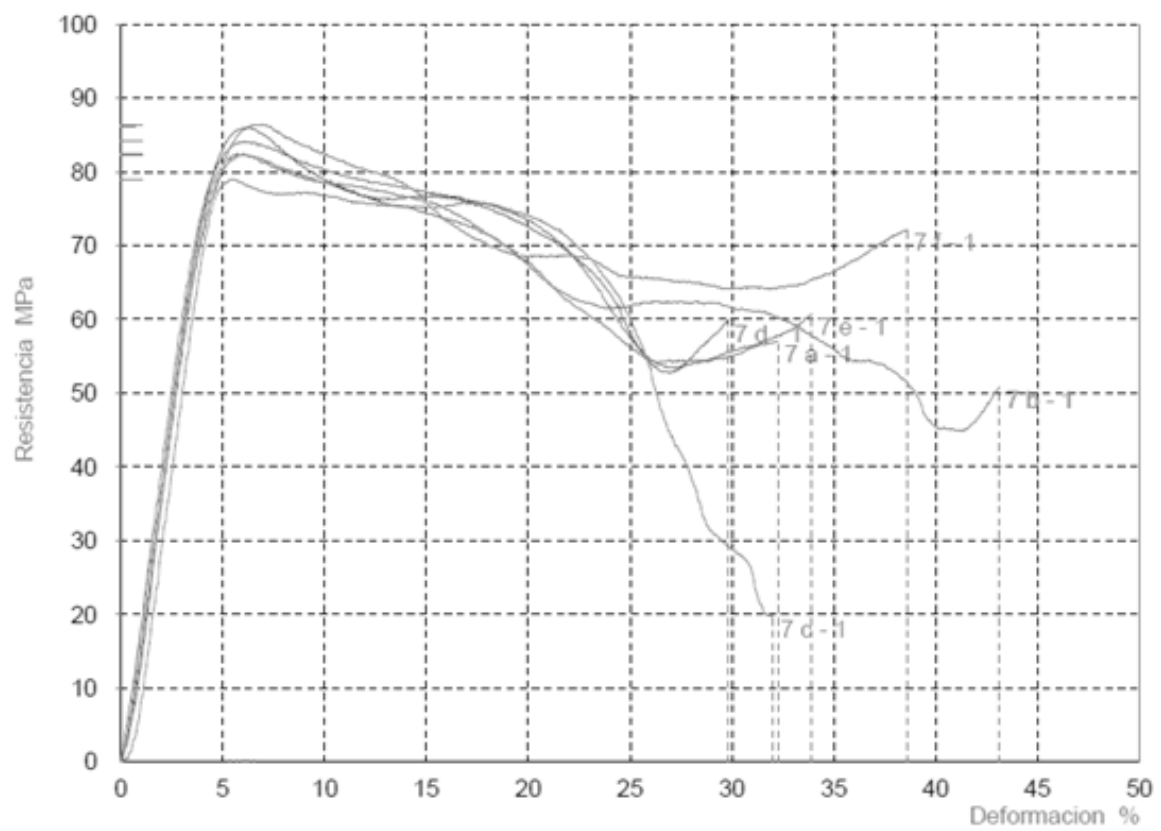


FIGURA 2

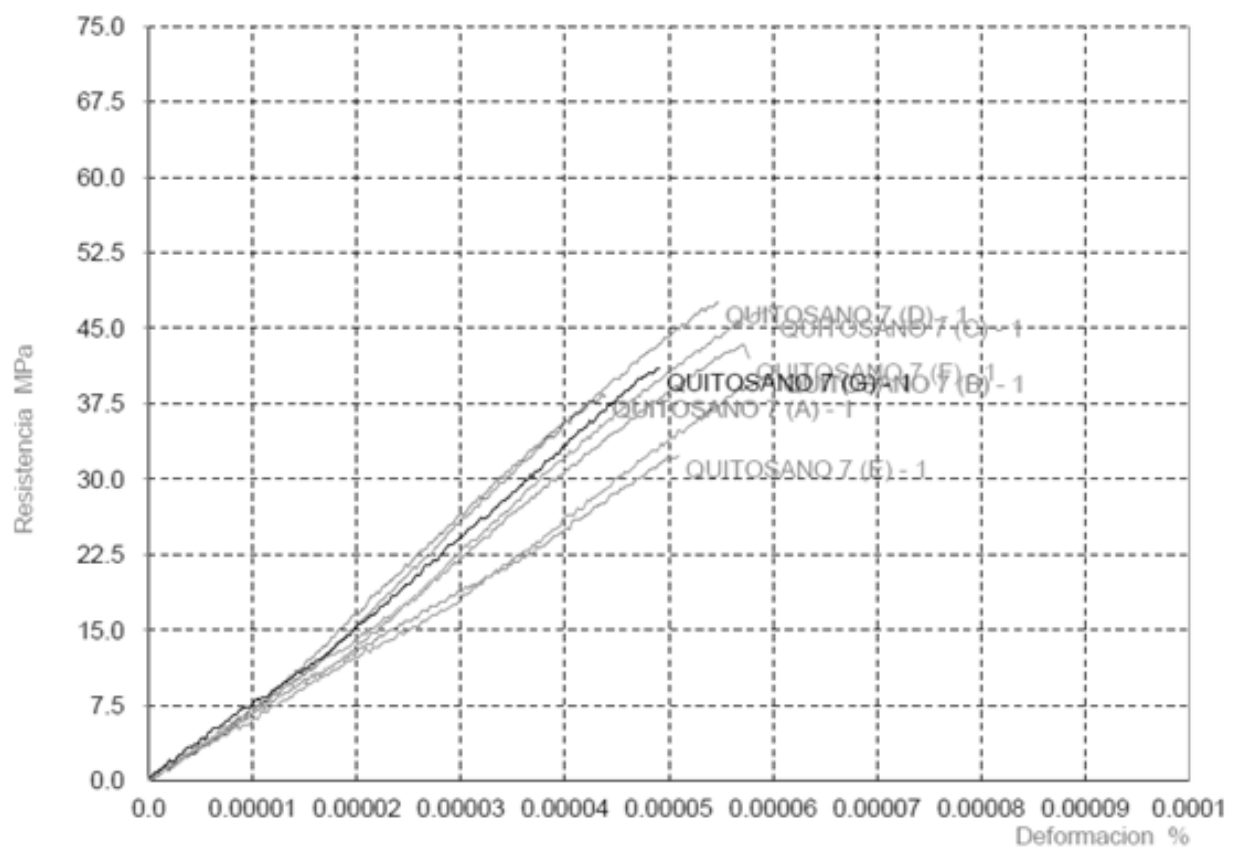


FIGURA 3

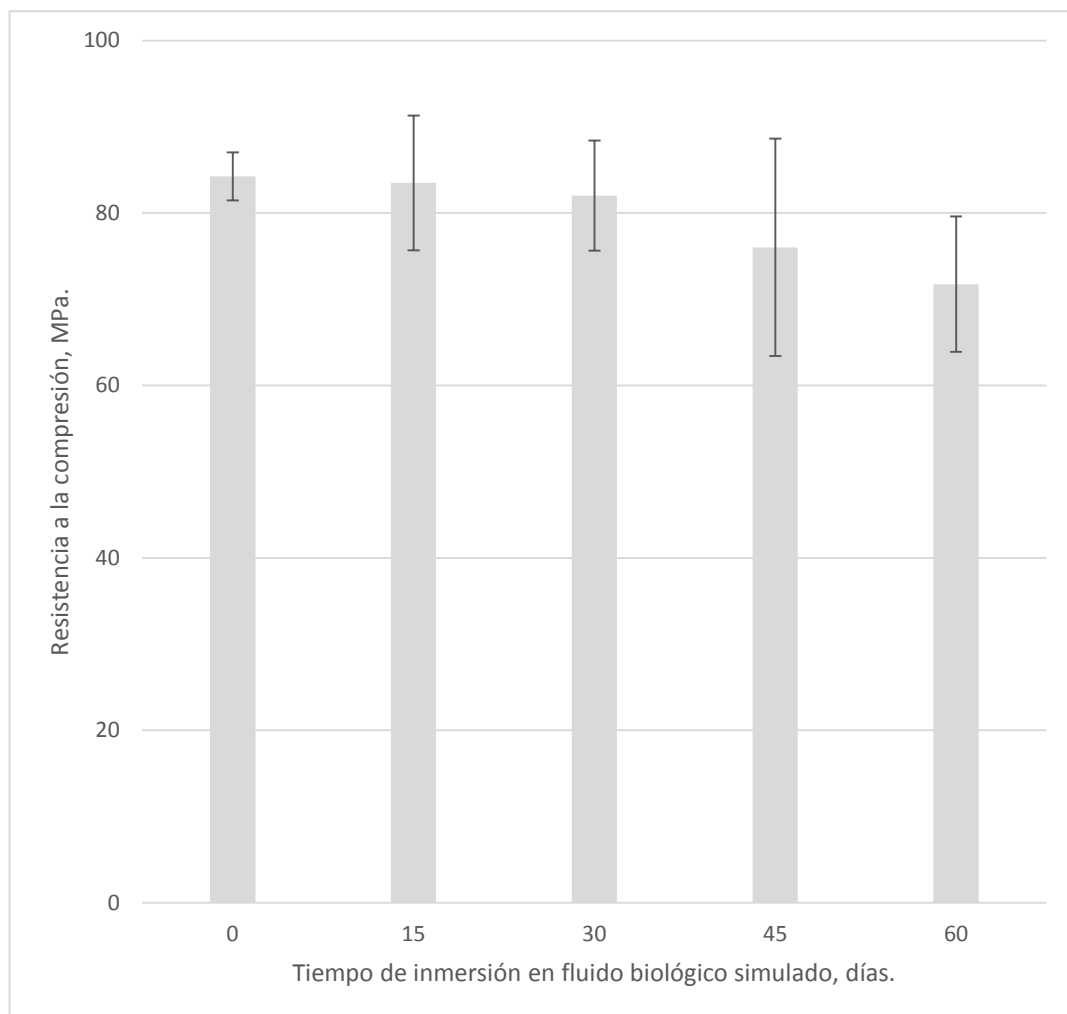


FIGURA 4

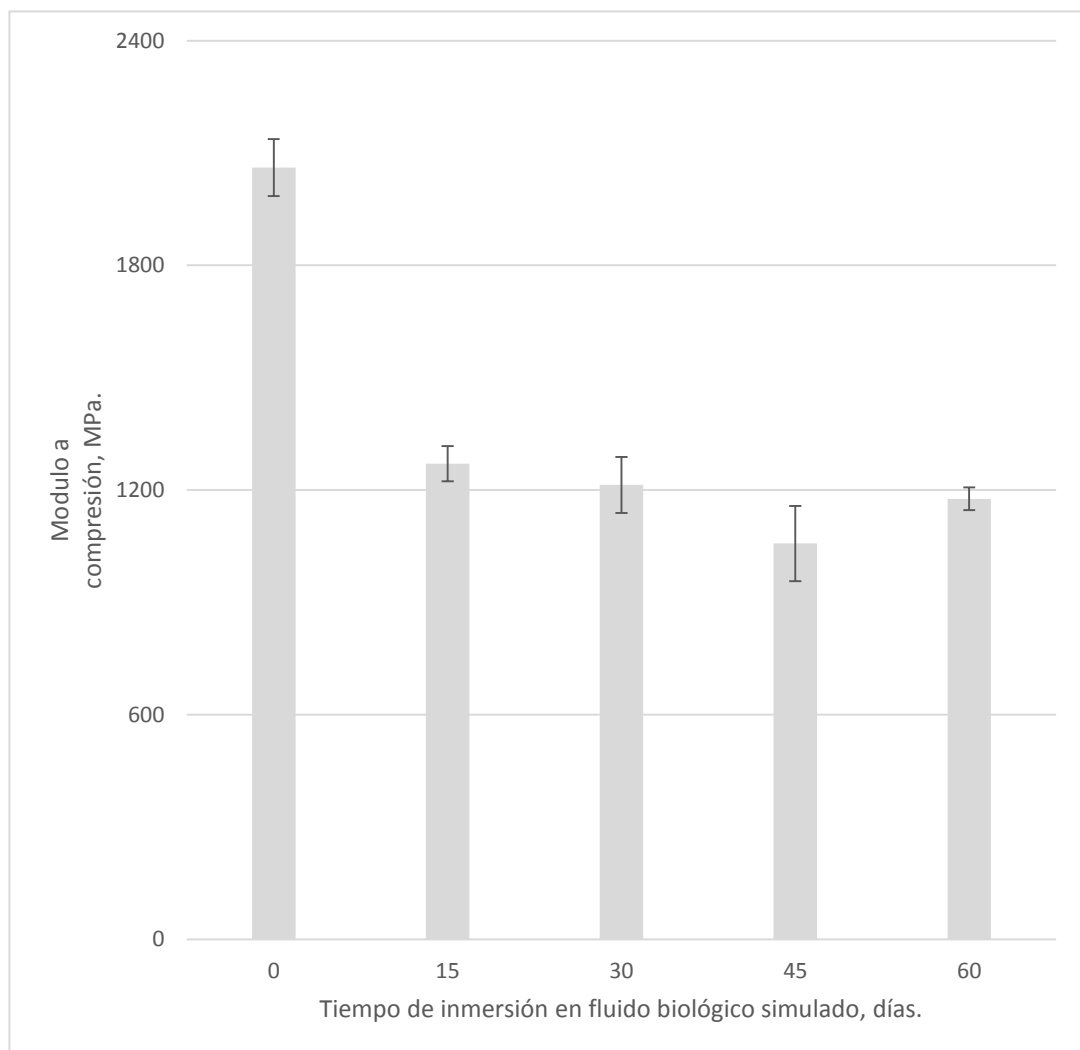


FIGURA 5

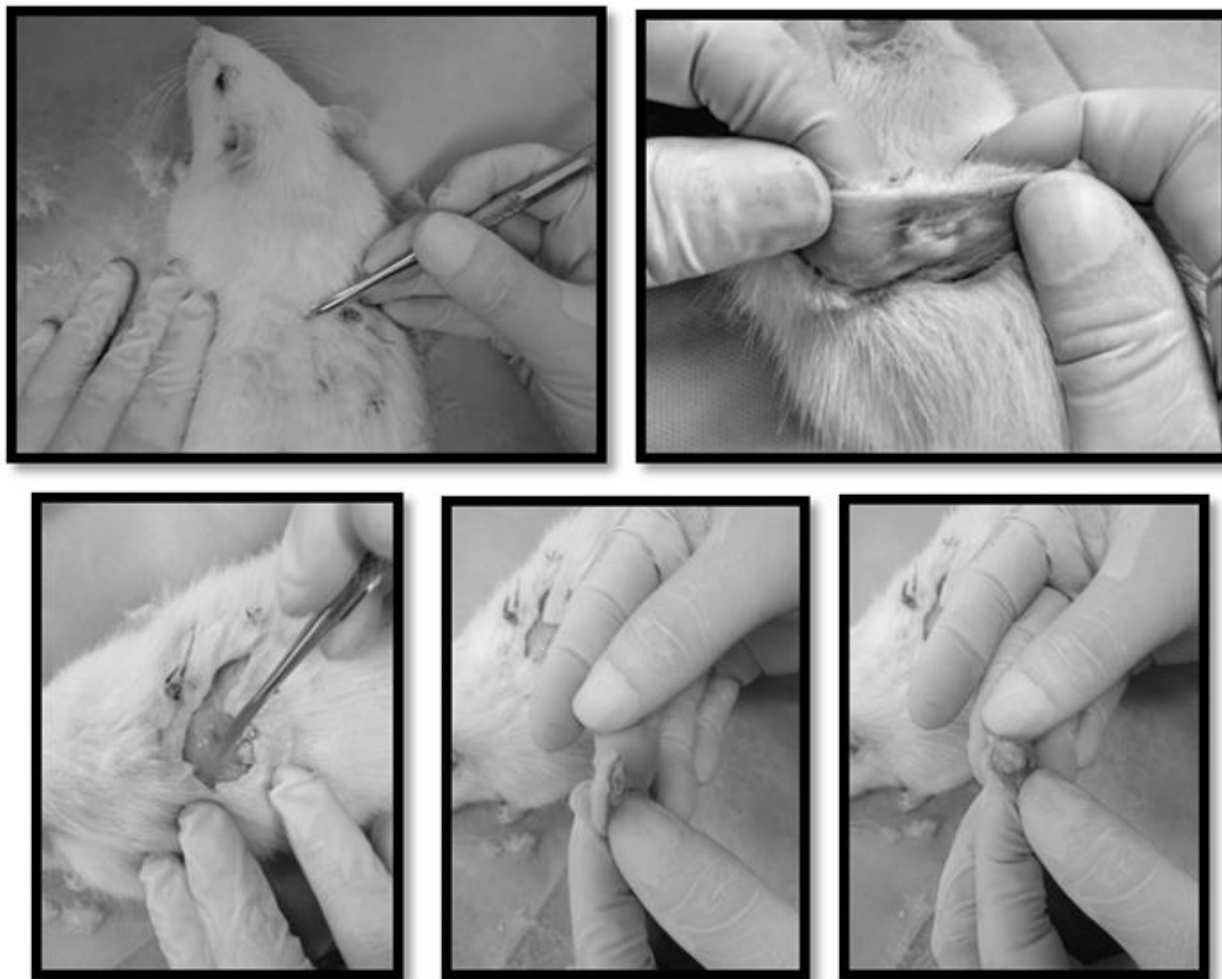


FIGURA 6

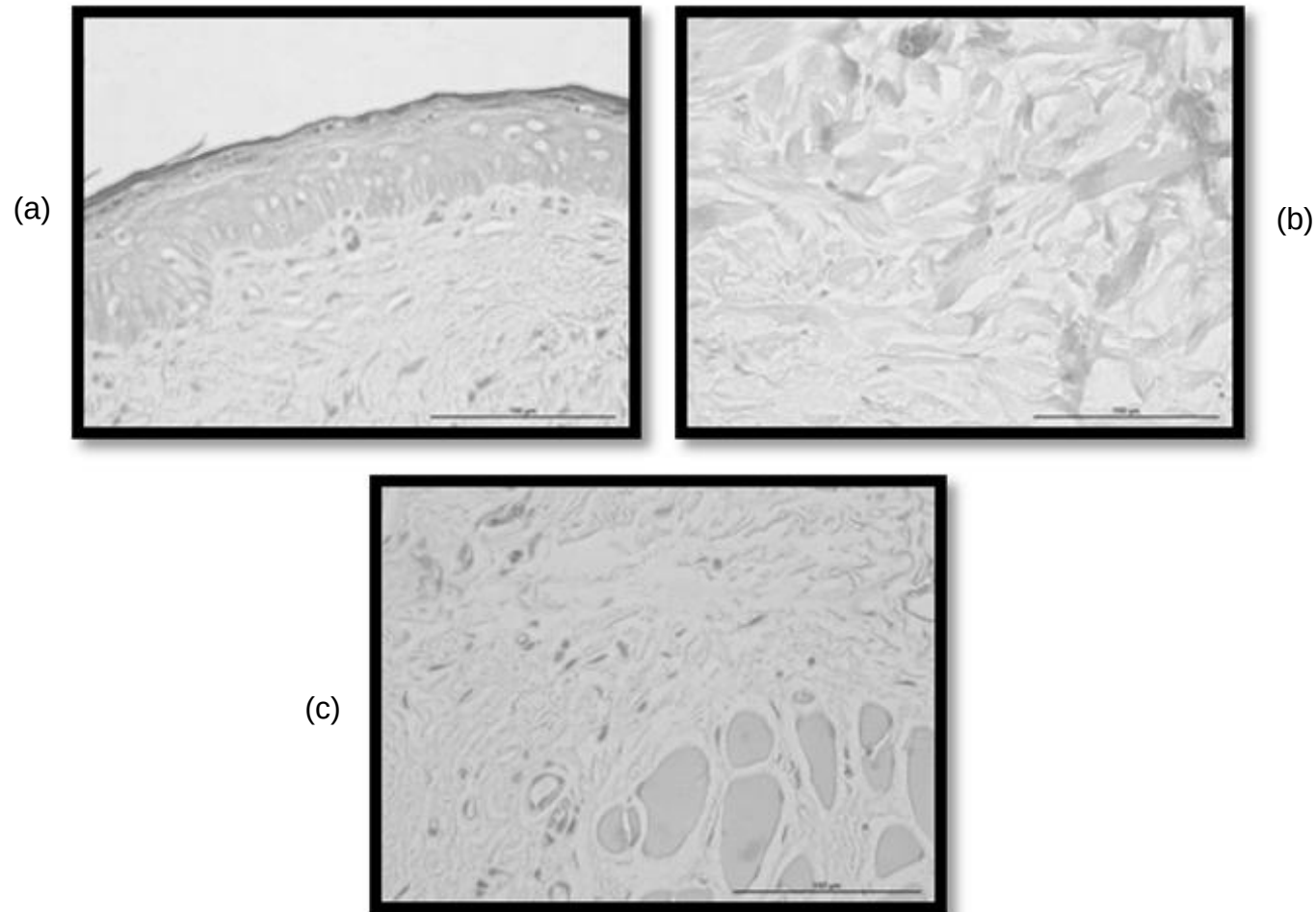
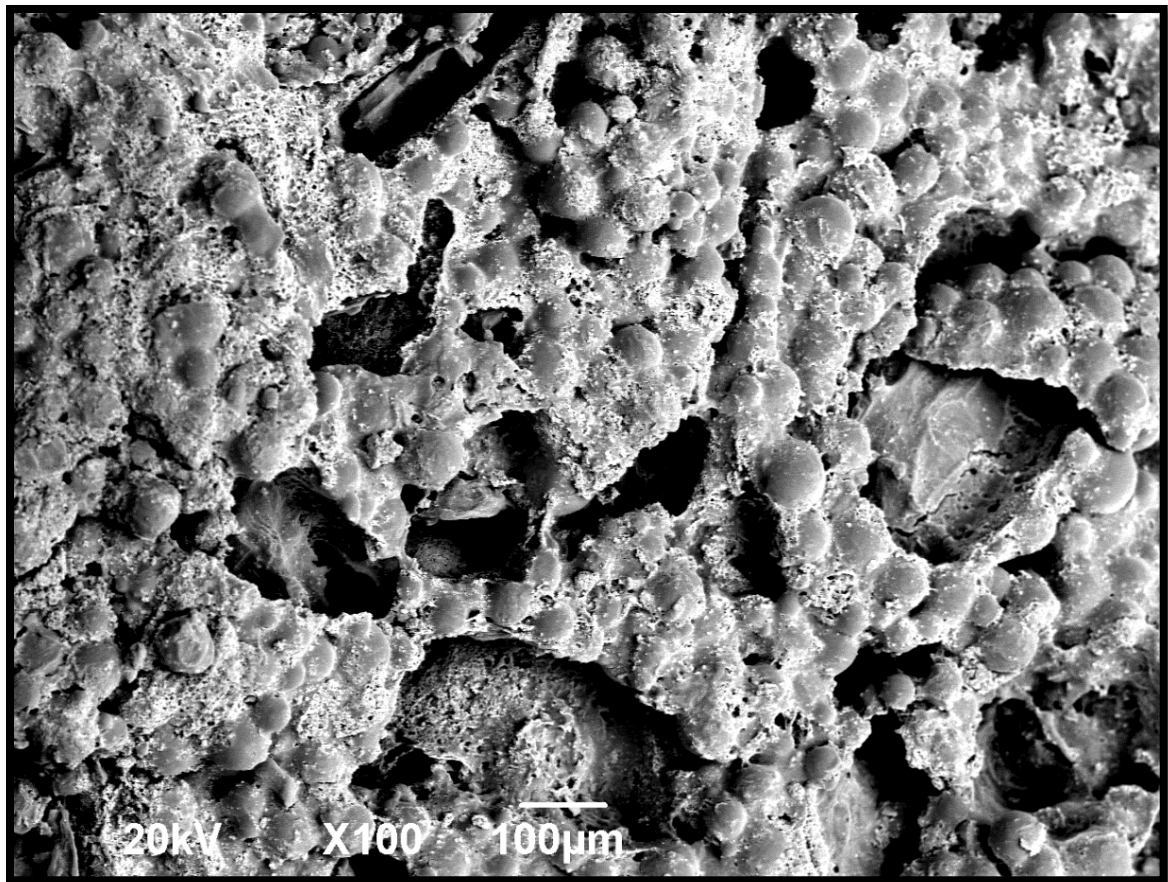


FIGURA 7



4.5.2 Participación en Eventos de Divulgación Científica

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**ESTUDIANTES DE
DOCTORADO EN
INGENIERÍA**
COLOMBIA INVESTIGA 2020

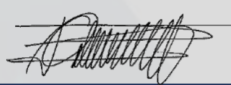
16 al 18
de septiembre | **2020**
www.acofi.edu.co

CERTIFICA QUE:

Mayra Eliana Valencia Zapata
38683378
Universidad del Valle, Colombia

Presentó la ponencia titulada Cemento óseo acrílico bioactivo y antibacterial modificado con óxido de grafeno y quitosano en el Segundo Encuentro Nacional de Estudiantes de Doctorado en Ingeniería.

En testimonio de lo anterior se firma a los dieciocho (18) días del mes de septiembre del año dos mil veinte (2020).


Roberto Carlos Hincapié Reyes
Presidente ACOFI

Organiza:
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1975-2020


Luis Alberto González Araujo
Director Ejecutivo

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REPÚBLICA DE COLOMBIA

El Servicio Nacional de Aprendizaje SENA

En cumplimiento de la Ley 119 de 1994

Hace constar que

MAYRA ELIANA VALENCIA ZAPATA

Con Cédula de Ciudadanía No. 38683378

Participó en la acción de Formación

V SIMPOSIO DE MATERIALES POLIMÉRICOS

con una duración de 16 horas

En testimonio de lo anterior, se firma el presente en Cali, a los veintinueve (29) días del mes de octubre de dos mil diecinueve (2019)

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Subdirector (E)
CENTRO NACIONAL DE ASISTENCIA TÉCNICA A LA INDUSTRIA - ASTIN
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La organización del X Congreso Internacional de Materiales

CIM 2019

Certifica que:

El trabajo titulado **“Evaluación de las propiedades mecánicas de cementos óseo acrílicos dopados con óxido de grafeno”**, de los Autor(es) **Lina Marcela Ruiz Rojas, M. Valencia Zapata, J. Hernadez**, fue presentado en calidad de **Presentación Poster** en el **X Congreso Internacional de Materiales** realizado los días **23, 24 y 25 de octubre de 2019**, en la ciudad de **Bucaramanga, Santander.**

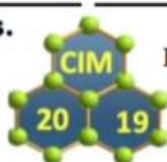
Se expide el 29 de octubre de 2019.

Darío Peña Ballesteros.

Director X CIM 2019

Ximena Acevedo.

Directora ACIEM Santander





La organización del X Congreso Internacional de Materiales

CIM 2019

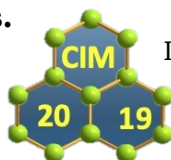
Certifica que:

El trabajo titulado **“QUITOSANO Y NANO-LÁMINAS DE ÓXIDO DE GRAFENO EN CEMENTO ÓSEO”**, de los Autor(es) **Andrea Muñoz Martinez, Mayra Valencia, José Mina.**, fue presentado en calidad de **Presentación Oral** en el **X Congreso Internacional de Materiales** realizado los días **23, 24 y 25 de octubre de 2019**, en la ciudad de **Bucaramanga, Santander**.

Se expide el 29 de octubre de 2019.

Darío Peña Ballesteros.
Director X CIM 2019

Ximena Acevedo.
Directora ACIEM Santander





La organización del X Congreso Internacional de Materiales

CIM 2019

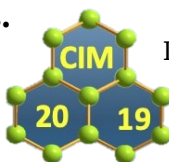
Certifica que:

El trabajo titulado **“SÍNTESIS DE ÓXIDO DE GRAFENO POR EL MÉTODO DE HUMMERS MODIFICADO”**, de los Autor(es) **Kelly J. Obando H., Mayra Valencia Zapata y José Mina Hernández.**, fue presentado en calidad de **Presentación Oral** en el **X Congreso Internacional de Materiales** realizado los días **23, 24 y 25 de octubre de 2019**, en la ciudad de **Bucaramanga, Santander.**

Se expide el 29 de octubre de 2019.

Darío Peña Ballesteros.
Director X CIM 2019

Ximena Acevedo.
Directora ACIEM Santander





Certifican que:

Mayra Eliana Valencia Zapata,

Asistió al **Segundo Simposio de Innovación en Excelencia Clínica**, realizado en la ciudad de Cali el pasado 14 de septiembre de 2018 en Valle del Pacífico Centro de Eventos.


Carlos Andrés Pérez
Director Económico y de competitividad
Cámara de Comercio de Cali



Certifica que:

Mayra Eliana Valencia Zapata, José Hermínsul Mina Hernández y Carlos David Grande Tovar

Participaron en el 10° Simposio de Investigaciones de la Facultad de Ingeniería
con la ponencia titulada:

**CEMENTOS ÓSEOS ACRÍLICOS MODIFICADOS CON NANOPARTÍCULAS DE ÓXIDO
DE GRAFENO**

Firmado en Cali, a los 7 días del mes de Mayo de 2018

Carlos Arturo Lozano Moncada
Decano
Facultad de Ingeniería – Universidad del Valle

Diego Fernando Manotas Duque
Vicedecano de Investigaciones y Posgrados
Facultad de Ingeniería – Universidad del Valle



Certifica que:

Jefferson Murillo Salas, Sara Zamora, Mayra Valencia y José Mina

Participaron en el 10° Simposio de Investigaciones de la Facultad de Ingeniería
con el poster titulado:

**EFFECTO DE LA MORFOLOGÍA DEL QUITOSANO SOBRE LAS PROPIEDADES
MECÁNICAS Y BIOLÓGICAS DE CEMENTOS ÓSEOS ACRÍLICOS**

Firmado en Cali, a los 7 días del mes de Mayo de 2018

Carlos Arturo Lozano Moncada
Decano
Facultad de Ingeniería – Universidad del Valle

Diego Fernando Manotas Duque
Vicedecano de Investigaciones y Posgrados
Facultad de Ingeniería – Universidad del Valle



Certifica que:

Sara Isabel Zamora Lagos, Jefferson Murillo Salas, Mayra Valencia y José Mina

Participaron en el 10° Simposio de Investigaciones de la Facultad de Ingeniería
con la ponencia titulada:

MICROESFERAS DE QUITOSANO PARA APLICACIONES BIOMÉDICAS

Firmado en Cali, a los 7 días del mes de Mayo de 2018

Carlos Arturo Lozano Moncada
Decano
Facultad de Ingeniería – Universidad del Valle

Diego Fernando Manotas Duque
Vicedecano de Investigaciones y Posgrados
Facultad de Ingeniería – Universidad del Valle

CERTIFICATE OF THE ATTENDANCE

I certificate that **Mayra Eliana Valencia Zapata, Jose Hermínsul Mina, Hector Fabio Zuluaga , Carlos David Grande Tovar, Blanca Vázquez Lasa, Julio San Roman e Luis Rojo** presented the work **Bioactive acrylic bone cements nanocomposites modified with graphene oxide and chitosan** in the 1st Pan American Congress of Nanotechnology - Fundamentals and Applications to Shape the Future, which was held on Casa Grande Hotel - Guarujá - São Paulo - Brazil from 27th to 30th November, 2017.

Solange Kazumi Sakata

Solange Sakata
Co-Chair

Debora Ingrid Rodrigues

Debora F. Rodrigues
Co-Chair







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El Servicio Nacional de Aprendizaje SENA

En cumplimiento de la Ley 119 de 1994

Hace constar que

MAYRA ELIANA VALENCIA ZAPATA

Con Cedula de Ciudadania No. 38.683.378

Participó en el evento de Divulgación Tecnológica

III SIMPOSIO INTERNACIONAL DE MATERIALES POLIMÉRICOS

con una duración de 16 horas

En testimonio de lo anterior, se firma el presente en Cali, a los treinta y un (31) días del mes de agosto de dos mil diecisiete (2017)

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SERVICIO NACIONAL DE APRENDIZAJE - SENA
Autenticidad del Documento
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AURA ELVIRA NARVAEZ AGUDELO
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4.5.3 Certificado Pasantía de Investigación



Instituto Ciencia y Tecnología de Polímeros

Isabel Quijada Garrido

Vice Director of the Institute of Polymer Science and Technology (ICTP-CSIC)

Juan de la Cierva 3, 28006 – Madrid, Spain

To whom it may concern

Isabel Quijada as Vice Director of ICTP-CSIC

INFORMS:

That **MAYRA VALENCIA** (passport 38683378), PhD student in Engineering at Valle University of Colombia, has performed a stay in the Institute of Polymer Science and Technology (ICTP-CSIC) during the period **3rd May 2017 to 30rd July 2017** within the Biomaterials Group of the Polymeric Nanomaterials and Biomaterials Department whose head is Prof Julio San Román.

3rd August 2017

A handwritten signature in blue ink that reads 'Isabel Quijada'.

Isabel Quijada



C/JUAN DE LA CIERVA 3
28006 MADRID ESPAÑA
TEL.: 91 56229 00
FAX: 91 564 48 53

Capítulo 5. ANEXOS

ANEXO A: Constancia de sometimiento del artículo “*The Role of Chitosan and Graphene Oxide in Bioactive and Antibacterial Properties of Acrylic Bone Cements*”

[Biomolecules] Manuscript ID: biomolecules-997561 -
Submission Received Recibidos x

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para Carlos, mí, and ▼

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Thank you very much for uploading the following manuscript to the MDPI submission system. One of our editors will be in touch with you soon.

Journal name: Biomolecules

Manuscript ID: biomolecules-997561

Type of manuscript: Review

Title: The Role of Chitosan and Graphene Oxide in Bioactive and Antibacterial Properties of Acrylic Bone Cements

Authors: Mayra Eliana Valencia Zapata, Carlos David Grande Tovar *, and José Hermínsul Mina Hernández *

Received: 27 October 2020

E-mails: valencia.mayra@correounivalle.edu.co,

carlosgrande@mail.uniatlantico.edu.co, jose.mina@correounivalle.edu.co

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ANEXO B: Constancia de sometimiento del artículo “*Optimization of mechanical and setting properties in acrylic bone cements added with graphene oxide by using a completely randomized factorial design*”

RSC Advances



RSC Advances

Optimization of mechanical and setting properties in acrylic bone cements added with graphene oxide by using a completely randomized factorial design

Journal:	RSC Advances
Manuscript ID	RA-ART-10-2020-009062
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ANEXO D: Lozano, K.; Mina, J.; Valencia, C.; **Valencia, M.**; Ospina, C. *Cementos Óseos Acrílicos preparados con un Co-monómero Alcalino e Hidroxiapatita: Estudios In Vitro e In Vivo*. *Revista Latinoamericana de Metalurgia y Materiales*. **2019**, 39, 131–145.

CEMENTOS ÓSEOS ACRÍLICOS PREPARADOS CON UN CO-MONÓMERO ALCALINO E HIDROXIAPATITA: ESTUDIOS *IN VITRO* E *IN VIVO*

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RESUMEN

A partir del análisis estadístico de los resultados de las pruebas mecánicas y de curado de formulaciones de cementos óseos adicionadas con Hidroxiapatita en porcentajes entre 0 – 20 p/p y Metacrilato de dimetil amino etilo (DMAEM) en porcentajes entre 0 – 10 p/p, reportadas en estudios previos [1], se seleccionaron cuatro formulaciones de cementos óseos con el fin de evaluar la formación de capas apatíticas sobre la superficie del material después de su inmersión en un fluido biológico simulado (FBS) durante 15 y 30 días. De igual manera, se estudió la reacción tisular mediante pruebas histológicas en tibias de conejos implantadas con los cementos. De acuerdo con el análisis por Microscopía Electrónica de Barrido y Espectroscopia de Energía Dispersiva (SEM – EDS), la presencia de la hidroxiapatita y el co-monómero favoreció la fijación de una capa similar a la apatita biológica en la superficie del material después de su inmersión durante 15 y 30 días en el FBS; mientras que, las formulaciones de cementos óseos sin modificar no evidenciaron la adhesión de la capa de apatita después del mismo tiempo de inmersión en el FBS. Por otra parte, las pruebas histológicas mostraron de manera cualitativa y cuantitativa que no se generó reacción inflamatoria en el tejido al estar en contacto con el cemento óseo con o sin modificación.

Palabras Claves: Cemento óseo, regeneración ósea, co-monómeros alcalinos, Hidroxiapatita, prueba *in vitro* e *in vivo*.

ACRYLIC BONE CEMENTS PREPARED WITH AN ALKALINE CO-MONOMER AND HYDROXYAPATITE: STUDIES *IN VITRO* AND *IN VIVO*

ABSTRACT

Based on the statistical analysis results of the mechanical and curing tests of bone cements formulations added with Hydroxyapatite in percentages between 0 – 20 w/w and Dimethyl amino ethyl methacrylate (DMAEM) in percentages between 0 - 10 w/w reported in previous studies [1], four bone cements formulations were selected to evaluate the fixation of an apatite layer on its surface after immersion in a simulated biological fluid (FBS) for 15 and 30 days, and the tissue reaction through histological tests in rabbits tibias implanted with the material. According to the analysis by Scanning Electron Microscopy and Dispersive Energy Spectroscopy (SEM - EDS), the presence of hydroxyapatite and the co-monomer favored the fixation of a layer similar to biological apatite on the surface of the material after immersion for 15 and 30 days in the FBS; whereas, the formulations of unmodified bone cements showed no adhesion of the apatite layer after the same time of the immersion in the FBS. In general, the histological tests showed qualitatively and quantitatively that there was no inflammation reaction in the tissue when in contact with the modified or unmodified bone cement.

Keywords: Bone cement, bone regeneration, alkaline co-monomers, Hydroxyapatite, *in vitro* and *in vivo* tests.

1. INTRODUCCIÓN

El cemento óseo acrílico es un compuesto no bioactivo y altamente hidrofóbico debido a la naturaleza del monómero (metil metacrilato). Lo anterior genera que al ser implantado, este sea encapsulado mediante la formación de un tejido fibroso como respuesta de las células encargadas de la regeneración ósea [2, 3]. Los cementos óseos acrílicos también presentan reacciones de polimerización altamente exotérmicas, monómero residual, contracción después del curado, propiedades mecánicas inadecuadas, y aflojamiento a largo plazo de la prótesis como consecuencia de la falta de fijación secundaria entre el cemento y el hueso [4, 5].

Con el fin de contrarrestar estas desventajas y promover la bioactividad, nuevas formulaciones de cementos óseos acrílicos con componentes que difieren ligera o notablemente de las formulaciones comerciales, han sido desarrolladas. Tal como la incorporación de partículas biocerámicas en la matriz de PMMA, que han mostrado un rendimiento óptimo en cuanto a propiedades mecánicas y tribológicas, con la capacidad de iniciar reparación de tejido óseo, al igual que los nanomateriales de carbono, bisfosfonatos y algunas proteínas; también, se ha indicado que la incorporación de algunos co-monómeros, activadores de baja toxicidad y/o polímeros biodegradables ayudan a disminuir la temperatura máxima de polimerización, además de generar superficies con zonas hidrófilas que permiten el reemplazo de hueso nuevo [6]-[12]. No obstante, estos materiales deberán proporcionar inicialmente la resistencia mecánica necesaria a la zona reconstruida y permitir o inducir la regeneración ósea, permitiendo de esta manera el proceso usual de remodelación para optimizar las propiedades mecánicas del hueso neoformado, convirtiéndolo en una estructura de soporte mecánicamente competente [13].

De acuerdo a lo anterior, en la presente investigación se estudió una alternativa que permite aumentar la bioactividad y mejorar la biocompatibilidad del cemento, promoviendo el crecimiento de tejido óseo sobre la superficie del material con la incorporación de Hidroxiapatita (HA), puesto que se ha logrado evidenciar en distintos modelos animales la formación de un enlace directo entre el implante y el tejido óseo, confirmándose el carácter bioactivo de estas

cerámicas [14]-[17]. Adicionalmente, se logró modificar la naturaleza de la carga superficial del cemento con la incorporación de un co- monómero con grupos amino (DMAEM), puesto que las células generadoras de hueso (osteoblastos) se depositan y proliferan mejor en superficies parcialmente hidrofílicas cargadas positivamente, como las generadas por la protonación de los grupos amino del co-monómero [18]-[20].

Hasta el momento es escasa la bibliografía asociada al estudio del comportamiento in vivo e in vitro de cementos óseos modificados con un fosfato de calcio y un co-monómero alcalino [21], enfocándose los estudios principalmente en la evaluación fisicoquímica, térmica y mecánica del material. En este sentido, se espera que los resultados obtenidos asociados al efecto mixto de la Hidroxiapatita y el DMAEM contribuyan a un mejor entendimiento del comportamiento biológico de la interface Hueso-Cemento, y que algunas de las formulaciones de cementos desarrolladas sean susceptibles de ser utilizadas en implantes de regeneración ósea tanto en ortopedia como en odontología.

2. PARTE EXPERIMENTAL

2.1 Reactivos

La fase sólida de los cementos estuvo compuesta por perlas de poli(metacrilato de metilo) (PMMA) con tamaño de partícula promedio de 63,6 μm adquiridas en Veracril® (New Stetic), peróxido de benzoilo (BPO) con 25 % de humedad y 97 % de pureza, marca Alfa Aesar® como iniciador de la reacción de polimerización por radicales libres del monómero, sulfato de bario (BaSO_4) marca Alfa Aesar® para facilitar el seguimiento radiográfico de los cementos durante y después de la implantación, e Hidroxiapatita comercial grado reactivo de referencia sigma-aldrich® que presentó un diámetro medio de partícula de 25,82 μm y una distribución de tamaño entre 14,77 μm y 44,32 μm . La fase líquida se preparó con metil metacrilato (MMA) y metacrilato del 2(dimetilamino etilo) (DMAEM) adquiridos en Merck®, además contiene una amina aromática, N. N – dimetil- p-toluidina (DMPT) que actúa como activador para que la iniciación de la reacción de polimerización se dé a temperaturas y tiempos acordes a los requerimientos clínicos. Tanto los componentes de la fase sólida, como los de la fase líquida se usaron tal como se recibieron del

proveedor, sin ninguna purificación previa.

2.2 Diseño experimental

Basado en el análisis estadístico arrojado por el software Minitab 16® para los resultados de la caracterización mecánica, térmica y de curado de los cementos óseos acrílicos modificados con HA y DMAEM reportados por Lozano et al. [1] y con el fin de evaluar la influencia de la HA y del DMAEM en los cementos óseos bajo condiciones *in vitro* e *in vivo*, se definió estudiar las formulaciones que se presentan en la Tabla 1. Para fines comparativos se utiliza como referencia una muestra patrón que corresponde al cemento sin carga y sin comonomero.

Tabla 1. Formulaciones seleccionadas para la preparación de cementos óseos modificados con HA y/o DMAEM.

Formulación	Composición (% p/p)						
	Fase líquida			Fase sólida			
	DMAEM	MMA	DMPT	BaSO ₄	PMMA	BPO	HA
Patrón	0	97,5	2,5	10	88	2	0
1	5	92,5	2,5	8,9	78,6	2	10,5
2	0	97,5	2,5	8,9	78,6	2	10,5
3	5	92,5	2,5	10	88	2	0

2.3 Preparación de los cementos óseos

De acuerdo con las proporciones de cada uno de los componentes, establecidas en la Tabla 1, se prepararon los cementos óseos acrílicos. Primero se dosificaron los componentes líquidos (MMA + comonomero + DMPT) y sólidos (PMMA + BPO + BaSO₄ + carga) en recipientes separados, y luego se mezclaron manualmente ambas fases a temperatura ambiente, manteniendo siempre una relación sólido/líquido de 2. Una vez el líquido entra en contacto con el sólido se inicia la reacción de polimerización por radicales libres del MMA que conlleva al endurecimiento del cemento.

2.3.1 Curado de los cementos óseos

Al entrar en contacto el peróxido presente en la fase sólida y la amina presente en la fase líquida tiene lugar la reacción redox que da lugar a la generación de los radicales libres. (Figura 1(a)). Los primeros radicales libres generados son consumidos por los inhibidores, y una vez consumidos los inhibidores, estos se unen a los monómeros insaturados, formando centros activos (figura 1b). Esta unión del monómero con el radical libre da lugar a una especie reactiva que es capaz de unirse a otro monómero dando lugar a la cadena polimérica creciente (figura 1c). La reacción de terminación ocurre cuando se dejan de producir radicales libres y, en el caso del PMMA se da principalmente por la unión entre cadenas activas [22].

2.4 Prueba In Vitro

Para estudiar la interacción del cemento óseo con el medio fisiológico, se sumergieron 6 probetas de cada una de las cuatro formulaciones estudiadas, en un Fluido Biológico Simulado (FBS) y se extrajeron 3 muestras en cada período de ensayo (15 y 30 días). Las probetas consistieron en cilindros de 6 mm de diámetro y 12 mm de altura, a los cuales se les calculó el área superficial para determinar el volumen del FBS a emplear, mediante el modelo presentado en la Ecuación 1. El FBS fue preparado según la formulación planteada por Miyazaki et al. [23] y posee una composición iónica similar a la del plasma sanguíneo humano. Este se preparó disolviendo en agua Tipo I (libre de contaminantes disueltos o iónicos coloidales y orgánicos) [24] cada uno de los reactivos especificados en la formulación, manteniendo agitación constante y teniendo en cuenta que cada reactivo fue añadido después de la completa disolución del anterior. Una vez calculado el volumen adecuado de la solución del FBS, éste se colocó en un recipiente de plástico y el espécimen se sumergió completamente en la solución a una temperatura de $23 \pm 2^\circ\text{C}$. Transcurridos 15 y 30 días, respectivamente, se retiraron las probetas de la solución, se lavaron con agua destilada y se secaron moderadamente con aire caliente.

$$V_s = S_a/10 \quad (1)$$

Donde V_s es el volumen de FBS (ml) y S_a es la superficie aparente de la muestra (mm^2).

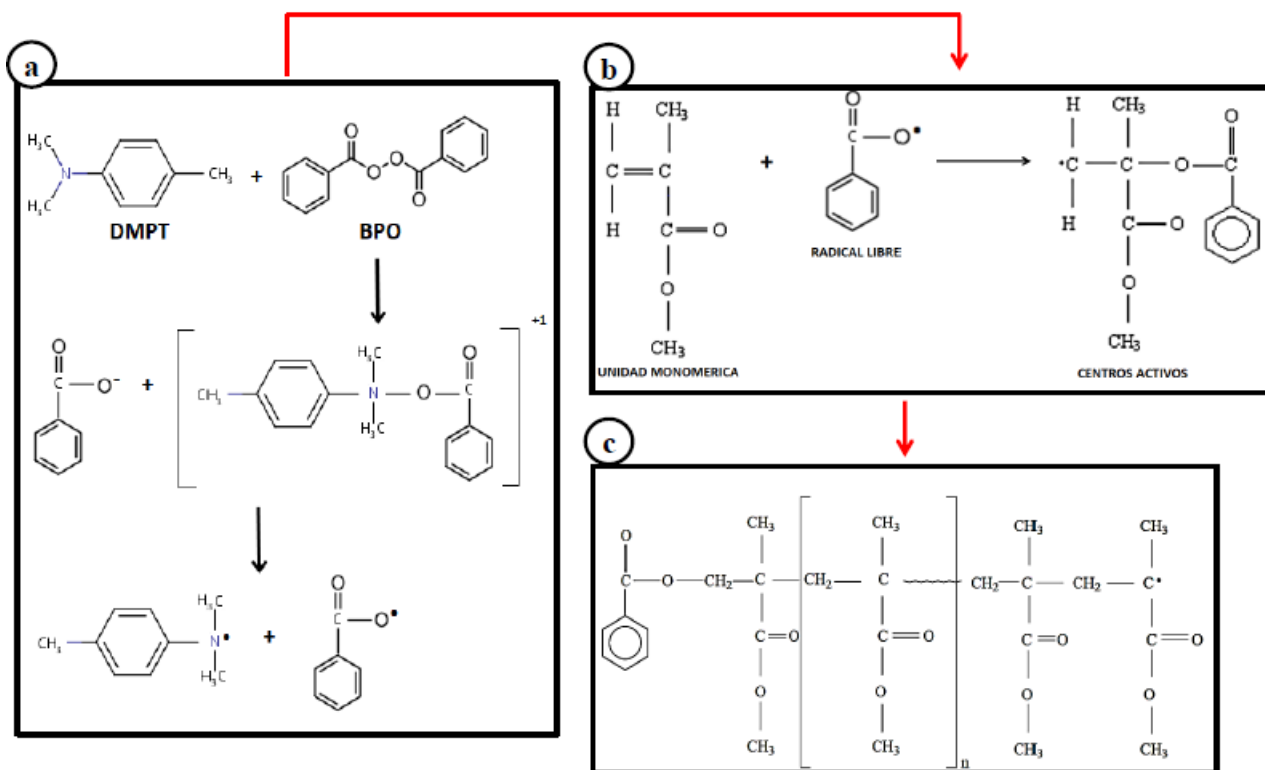


Figura 1. Polimerización vía radicales libres del PMMA de los cementos óseos: (a) Formación radicales libres, (b) Reacción de iniciación, (c) Reacción de propagación.

Se empleó un Microscopio Electrónico de Barrido marca JEOL SEM modelo JSM – 6490LV con imágenes de electrones secundarios para observar la formación del crecimiento de capas apatíticas en la superficie, posterior a la inmersión en el fluido biológico simulado. El equipo SEM se acopló a un sistema de micro-análisis químico por espectroscopia de energía dispersiva (EDS) para el análisis cualitativo y semicuantitativo de la composición química de las muestras. Las muestras fueron montadas en porta muestras de aluminio y pegadas con cinta de carbono, posteriormente se recubrieron con una película de oro, por un tiempo de aproximadamente dos minutos, mediante la técnica de espolvoreado catódico.

2.5 Prueba *In Vivo*: Estudio de reacción tisular por medio de pruebas histológicas en tibias de conejos.

La prueba de reacción tisular fue de tipo

experimental. Como biomodelos se utilizaron conejos, machos, de raza *New Zealand*, de 3 meses de edad y un peso entre 2600 – 3800 gramos. El criterio para determinar el número de sujetos se basó en la norma ISO 10993-6, el cual arrojó un total de 20 conejos (5 por cada formulación) [25].

Previamente el componente líquido se filtró por membranas en una cabina de flujo laminar y el componente sólido se sometió a proceso de esterilización con óxido de etileno; luego se mezclaron ambas fases y posteriormente el cemento óseo fue implantado en tibias de conejo en un defecto de 2,5 mm de diámetro. Después de 28 días de implantado el material, se realizaron eutanasias y se enviaron los especímenes a patología con instrucción de realizar tres cortes longitudinales (cresta, medio y medular) y reportar el tipo y contenido de células encontradas.

Los protocolos asociados con la adecuación del bioterio, selección de los animales, así como el manejo y ética de la investigación, fueron aprobados

por el Comité de Ética Animal de la Facultad de Salud de la Universidad del Valle mediante el Acta No. 001 de 2010.

Siguiendo la norma ISO 10993-6:2016 [25], se determinó el número de animales (cinco para cada formulación). Se evaluaron las condiciones de salud de los animales, mediante toma de registros de temperatura corporal, peso, consumo de alimentos; y se realizaron observaciones acerca del comportamiento del animal, tales como: acicalamiento, mantenimiento del hábitat, transporte de materiales, posiciones en reposo y alerta. Al llegar al bioterio todos los animales fueron vacunados con 0,2 miligramos de Ivermectina al 1%, subcutáneo.

Esta prueba se realizó en varias fases:

2.4.1. Primera fase (etapa quirúrgica).

Se preparó un defecto óseo circular de 2,5 mm en la tibia derecha de cada biomodelo, utilizando fresas de titanio para hueso de diámetro secuencial iniciando con la de 1,8 mm.

Para el procedimiento quirúrgico se siguió el protocolo utilizado en la Fundación CIEO (Centro de Investigación en Estudios Odontológicos). Así como los instructivos establecidos para la fase quirúrgica de enjertación de hueso y/o substitutos óseos, de la siguiente forma [26]:

- Anestesia y sedación

A cada biomodelo se le suministró vía parenteral 0,3 mg por kilogramo de Tranquilán (Maleato de Acepromicina) y 0,4 mg por kilogramo de Xilasyn (Oxilacina al 2%).

- Preparación del campo quirúrgico

Se realizó tricotomía del área a operar, técnica de asepsia y antisepsia en la zona con Isodine en espuma, colocación de campo quirúrgico, Aplicación Lidocaína al 2% con Epinefrina 1:80,000, técnica infiltrativa para anestesia local para vasoconstricción.

- Procedimiento quirúrgico

Se elaboró la incisión y la disección para preparar un defecto óseo circular, en el cual posteriormente se implantó el cemento óseo. Luego, se realizó confrontación de tejidos y cierre, y finalmente se

aplicaron fármacos post-quirúrgicos (analgésico y antibiótico) y se protegió el área con antibiótico tópico localmente.

- Eutanasia

Después de 28 días de la implantación del material, se efectuó la eutanasia mediante aplicación intravenosa de una dosis de 5 ml de pentobarbital sodico en la vena marginal de la oreja; el procedimiento fue realizado siguiendo recomendaciones internacionales y bajo la supervisión directa de un médico veterinario, quien se encargó del desarrollo del aspecto ético.

2.4.2. Segunda fase.

Se recuperaron las muestras del tejido óseo injertado con los diferentes cementos, se decalcificaron los tejidos con solución de ácido etilendiaminotetraacético (EDTA) al 10% en búfer fosfato (PBS) por 2 meses, se retiraron las partículas de cemento óseo, se realizó la preparación de la muestra mediante deshidratación, aclaramiento e inclusión en parafina; se realizaron tres cortes a cada espécimen, uno a nivel crestal, otro a nivel medio, y el tercero en proximidad al canal medular; y se estudió el tipo de células y cantidades presentes en porcentaje por campo.

2.4.3. Tercera fase.

Con los informes de la histología se realizó un análisis estadístico descriptivo basado en los valores promedios de los resultados relacionados con las células encontradas en el material.

3. RESULTADOS Y DISCUSIÓN

3.1 Prueba in vitro por inmersión en fluido biológico simulado.

A pesar de los cuestionamientos ya reportados en algunas investigaciones respecto a la "capacidad de formación de apatita" sobre la superficie de un material al ser sumergido en un FBS [27], es apropiado examinar la posible formación de éstos precipitados sobre los compuestos de cemento óseo a base de PMMA modificados con mineral bioactivo, antes de la experimentación con biomodelos, puesto que el número y la duración de los experimentos pueden reducirse notablemente con la aplicación preliminar de esta caracterización.

Los posibles mecanismos de formación de apatita sobre la superficie de las formulaciones de cemento óseo, PMMA-HA y PMMA-DMAEM-HA, se presentan de manera esquemática en la Figura 2. En principio el material presenta una carga superficial negativa, conferida por los iones fosfato (PO_3^{4-}) e hidroxilo (OH^-) de la HA presente en el cemento (Figura 2(a)); lo anterior permite que después de la inmersión en el FBS se dé la interacción superficial con los iones calcio (Ca^{2+}) de la solución, formando

una capa inicial de fosfato de calcio ($\text{Ca}_3(\text{PO}_4)_2$) amorfo “rico en calcio” con una carga superficial positiva (Figura 2(b)). Posteriormente, esta capa interactúa con los iones PO_3^{4-} de la solución, precipitando dichos iones sobre la superficie del cemento y transformando el recubrimiento anterior en una capa de $\text{Ca}_3(\text{PO}_4)_2$ amorfo “pobre en calcio” (Figura 2(c)), la cual cristaliza finalmente en una capa de apatita neoformada cuyos principales componentes químicos son calcio y fósforo (Figura 2(d)) [28] [29].

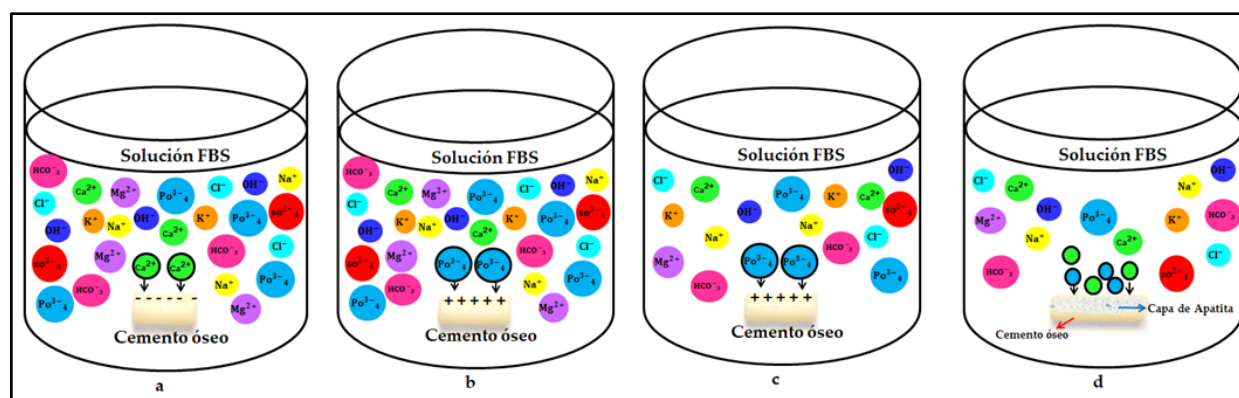


Figura 2. Mecanismos de formación de apatita: (a) cemento con carga negativa, (b) cemento con capa de fosfato de calcio amorfo rico en calcio, (c) cemento con capa de fosfato de calcio amorfo pobre en calcio, (d) cemento con capa de apatita neoformada.

En la Figura 3, se muestra la imagen SEM de la superficie del cemento óseo sin cargas bioactivas (muestra patrón), inmerso durante 3 semanas en el fluido biológico simulado. Se pueden observar las perlas de PMMA (zona A) sin disolver, rodeadas de una matriz de monómero polimerizado (zona B). Poco se sabe acerca de las características de inclusión de las perlas de polímero dentro de la matriz de monómero. Sin embargo, algunas investigaciones mediante Microscopía Electrónica de Barrido (SEM) demuestran que el proceso de incorporación es muy sensible a las condiciones químicas y ambientales durante el mezclado [30]. Las imágenes obtenidas en la presente investigación son comparables con muestras de cementos óseos recuperados en cirugías de revisión [31], donde es evidenciado que efectivamente existe una interfaz entre las perlas de polímero y el monómero

polimerizado. De igual manera, en este trabajo plantean que el proceso de incorporación resulta a menudo incompleto y las perlas de polímero se liberan y/o aflojan desde la matriz de monómero que las rodea; afectando a su vez la resistencia a la tensión como consecuencia de la pérdida de adherencia interfacial entre dichas perlas y la matriz [31].

Por otro lado, con los resultados del micro-análisis hecho por Espectroscopia de Energía Dispersiva (EDS), para determinar la composición química de la muestra patrón (Tabla 2), No se identificó la presencia de P o Ca, lo que sin lugar a duda demuestra que el cemento óseo acrílico es un polímero no bioactivo, su matriz polimérica (MMA polimerizado) es altamente hidrofóbica por lo que debilita el contacto carga – solución y se dificulta el crecimiento de apatita sobre su superficie.

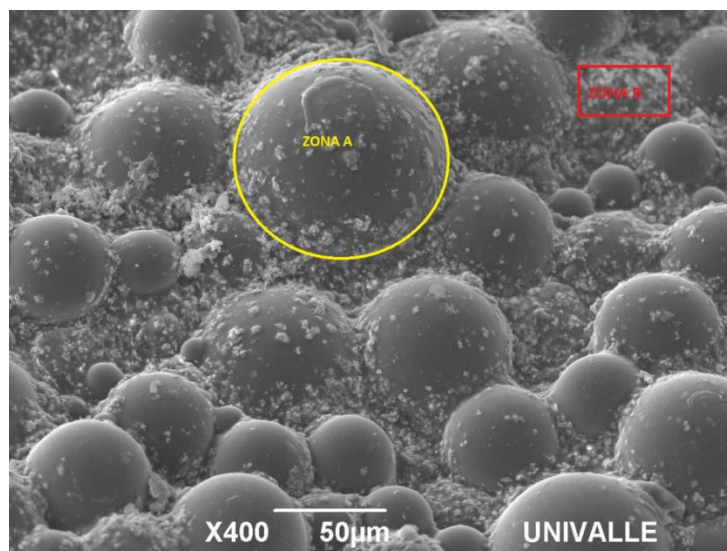


Figura 3. Imagen SEM a 800X del cemento óseo acrílico patrón (0% HA – 0% DMAEM).

Tabla 2. Análisis EDS de formulaciones de cementos óseos modificadas con DMAEM y/o HA (1, 2, 3) y sin modificar (patrón), después de 15 y 30 días de inmersión en un FBS.

formulación	C (%atom)	O (%atom)	S (%atom)	Ba (%atom)	P (%atom)	Ca (%atom)	Ca/P	Na (%atom)	K (%atom)	Cl (%atom)
Patrón (15 días)	75,2	21,91	1,04	0,85	-	-	-	-	-	-
Patrón (30 días)	76,83	22,44	0,3	0,43	-	-	-	-	-	-
1 (15 días)	50,79	41,57		0,23	2,46	3,79	1,54	0,7	0,11	0,35
1 (30 días)	65,43	23,39	0,74	1,27	3,25	5,09	1,57	-	-	0,83
2 (15 días)	41,4	40,61	-	-	8,86	9,13	1,03	-	-	-
2 (30 días)	63,46	16,63	-	1,38	6,4	12,13	1,90	-	-	-
3 (15 días)	46,82	49,05	-	0,16	0,16	-	-	3,43	0,38	-
3 (30 días)	62,61	31,19	0,74	0,47	0,78	1,16	1,49	2,53	0,2	0,32

Ha sido reportado que la incorporación de comonómeros con grupos amino a formulaciones de cemento óseo, conduce a una mejora en la biocompatibilidad del material debido a que las células generadoras de hueso (osteoblastos) se depositan y proliferan mejor en superficies parcialmente hidrófilas cargadas positivamente, como las generadas por la protonación de los grupos

amino del comonómero [18]-[20]. Lo cual se corrobora en el presente estudio a partir de los resultados obtenidos después de la inmersión de la muestra que contenía 5 % p/p de DMAEM (formulación 3), en un fluido biológico simulado durante cuatro semanas. En las Figuras 4(a) y 4(b) se muestra la imagen SEM de este material transcurridos 15 y 30 días de inmersión,

respectivamente. En estas se identifican pequeños precipitados, depositados desde las primeras semanas, lo que demuestra que es factible que el comonomero empleado aumente la bioactividad del cemento óseo. El análisis por EDS sintetizado en la Tabla 2 arrojó bajos porcentajes de fósforo en la superficie del cemento óseo durante las primeras semanas (0,16 % átomos), lo que puede ser atribuido a que en un principio la amina terciaria es insoluble en agua; pero la presencia de un ácido diluido en el FBS (disolución acuosa) hace que la misma forme sales de amonio y aumente su solubilidad. Lo anterior da como resultado la protonación de la amina y la atracción de iones PO_4^{3-} hacia la superficie del cemento aumentando la cantidad de fósforo depositada sobre la superficie a 0,78 % átomos y alcanzándose una relación entre iones Ca y P de 1,49.

En la Figura 4(c) y 4(d), se muestran las imágenes SEM de las superficies del cemento óseo de la formulación 2, que contenía 10,5 % de HA, en estas se observa la presencia de pequeños núcleos dispersos tanto en la matriz de monómero polimerizado como en las perlas del polímero, con tamaño y forma irregular que se han formado después de 15 días de inmersión en FBS (Figura 4(c)), los cuales después de 30 días de inmersión muestran un aumento en cantidad que los lleva a unirse entre sí formando estructuras de precipitados que cubren casi en su totalidad las perlas de PMMA y la matriz de monómero. Con el análisis EDS se logró identificar la presencia de calcio y fósforo respectivamente en proporciones de 9,13 % átomos y 8,86 % átomos, a los 15 días y de 12,13 % átomos y 6,36 % átomos a los 30 días, de estar sumergidas las muestras (Tabla 2).

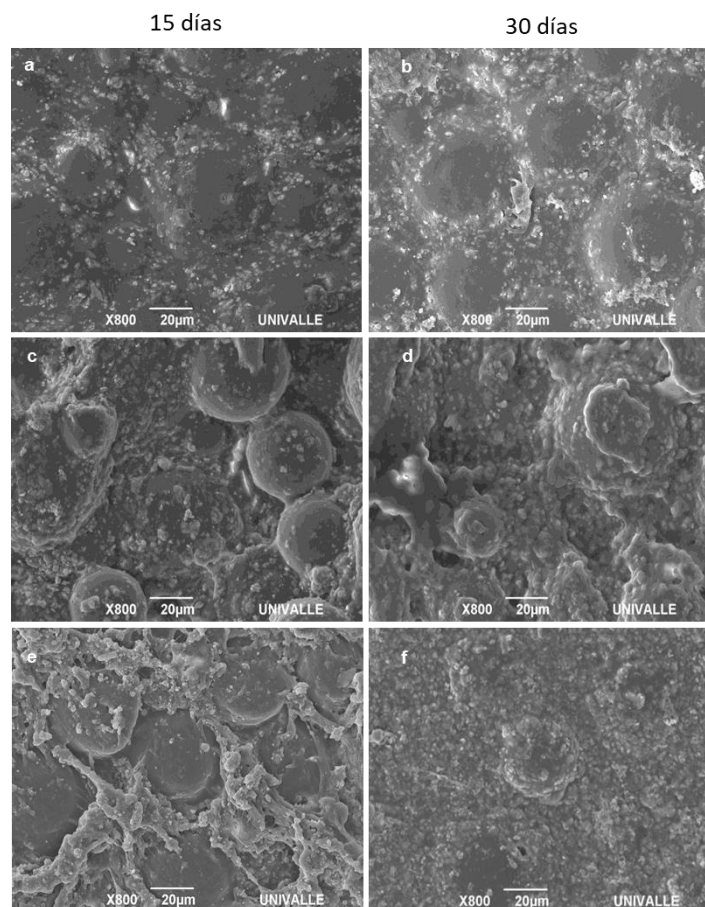


Figura 4. Imagen SEM a 800X de: a) y b) formulación 3 de cemento (5% DMAEM); c) y d) formulación 2 de cemento (10,5% HA); e) y f) formulación 1 de cemento (5% DMAEM y 10,5% HA). a), c) y e) corresponden a muestras después de 15 días de inmersión y b), d) y f) corresponden a muestras después de 30 días de inmersión.

En las Figura 4(e) y 4(f), se presentan las imágenes SEM de la superficie de las muestras de cemento óseo con 10,5 y 5 % p/p de HA y DMAEM, respectivamente, después de estar inmersas por 15 y 30 días en el FBS. La Figura 4(e) evidencia que después de 15 días de inmersión hay una leve aparición de aglomerados de partículas con apariencia densa, producto de la formación de nuevas estructuras sobre las partículas iniciales, algunas zonas de estas capas densas conservan una morfología porosa, la cual tiende a desaparecer con el tiempo de inmersión. También se aprecia la presencia de partículas de apariencia esférica formando pequeños cúmulos débilmente condensados; esta nueva capa es atribuida a la formación de apatita, semejante a la apatita biológica, formada por el material bioactivo al estar en contacto con el FBS. Por otra parte, después de 30 días de inmersión (Figura 4(f)) la apariencia de la apatita neoformada está mejor distribuida sobre la superficie del material, generándose capas ligeramente más densas y conservando una apariencia porosa. La evidencia de la apatita neoformada está en la variación de la relación de los iones Ca y P de la capa generada en la superficie del cemento reportada en la tabla 2, con respecto a la relación de la apatita estequiométrica incorporada en las diferentes formulaciones de cemento que corresponde a 1.67 [32].

Es importante resaltar que se observa desde los primeros días de inmersión en el FBS la formación de fosfato de calcio amorfo rico en calcio, siendo esta afirmación sustentada en el resultado del

microanálisis por EDS que se llevó a cabo en diferentes regiones asociadas a la superficie del cemento óseo (Tabla 2). En este sentido, se logró comprobar que la capa depositada estaba constituida por 3,79 % átomos de Ca y 2,46 % átomos de P, para los primeros 15 días de acondicionamiento. Por otro lado, como era de esperarse, al aumentar el tiempo de inmersión los consumos de calcio y fósforo de la capa depositada a partir de la solución fueron mayores, lo que se logra apreciar en el EDS después de haber estado las muestras 30 días en el FBS, (5,09 y 3,25 % átomos Ca y P, respectivamente). Con base a lo anterior, se puede afirmar que el tratamiento estudiado mostró tener potencialidad para la formación de una capa de apatita sobre la superficie del cemento al ponerse en contacto con un FBS.

3.2 Prueba in vivo: Estudio de reacción tisular por medio de pruebas histológicas en tibias de conejos.

En el análisis microscópico se logró identificar los diferentes tipos de células presentes en los alrededores de la interfaz hueso-cemento, después de transcurridos 28 días de haberse implantado el material en las tibias de los conejos, éstas fueron: células óseas (osteoblastos, osteoclastos y osteocitos), así como estructuras biológicas importantes para la regeneración ósea como los vasos sanguíneos y la médula ósea (roja o amarilla) [33]. En la Tabla 3 se resumen los resultados.

Tabla 3. Porcentajes de células, vasos sanguíneos y médula ósea encontrados en las histologías de las tibias de los conejos, que contenían cemento óseo acrílico. Los porcentajes reportados corresponden a los promedios de los tipos celulares de interés (Ob, OC, Oc), obtenidos en 10 campos de microscopio óptico diferentes, observados aleatoriamente.

Células	Patrón	Formulación 1	Formulación 3
Osteoblastos (Ob)	24%	17%	25%
Osteoclastos (OC)	3%	13%	10%
Osteocitos (Oc)	73%	70%	65%
Vasos sanguíneos	40%	40%	40%
Inflamatorias	Negativo	Negativo	Negativo
	Amarilla	Amarilla	Rojas
Médula ósea	Roja	Roja	Arteriolas
	Arteriolas	Arteriolas	Adipocito

Los valores de osteoblastos en las muestras control y con DMAEM son muy similares, sin embargo en la muestra con DMAEM e hidroxiapatita se observa una disminución importante en esta población, pero a la vez un aumento en el número de osteocitos, lo que podría estar indicando un proceso más rápido de diferenciación celular y por ende de neoformación ósea, lo que también se observa en el aumento de osteoclastos, las células encargadas del recambio óseo en el remodelado de la matriz ósea formada inicialmente.

La diferencia entre medula roja y amarilla es debida a la presencia de vasos sanguíneos en este tejido, en general se considera que la roja es joven o recién formada y tiene muchos vasos sanguíneos y menos células grasas (adipocitos), mientras que la amarilla es una medula más madura, más vieja y tiene menos vasos sanguíneos y más células grasas.

Es de esperarse que el procedimiento quirúrgico que se realizó para llegar al canal haya ocasionado un estrés quirúrgico que estimuló la formación de medula nueva [34]; en general después del procedimiento quirúrgico se encuentra la presencia de medula roja (tejido neoformado), responsable a su vez de la formación de nuevas líneas celulares tanto óseas como hematopoyéticas, pero también se debe encontrar medula amarilla (tejido preexistente); una vez superada la etapa aguda la medula roja se transformara progresivamente en medula amarilla. En la tabla 3, Se puede observar que hubo presencia de los dos tipos, solo que en la muestra de cemento sin modificar y en la muestra con DMAEM e hidroxiapatita predominó la medula amarilla mientras que en la muestra con solo DMAEM predominó la roja.

Los resultados mostrados en la tabla 3 corresponden al día 28 después de la implantación, para este momento ya se superó la etapa de inflamación aguda, situación que se confirma por la ausencia de células inflamatorias; la presencia de osteoblastos y osteoclastos indican que la lesión quirúrgica que se creó todavía está en proceso de reparación lo que explica la presencia tan alta de vasos sanguíneos (40 % en todas las muestras), ya que estos son necesarios para garantizar el proceso debido a su papel en el transporte de células, nutrientes y oxígeno.

De igual manera, vale la pena resaltar que la ausencia de células inflamatorias (macrófagos y linfocitos) y la presencia de células responsables de la reparación y remodelado óseo como osteoblastos, osteocitos, y osteoclastos indican la biocompatibilidad del material implantado. Teniendo en cuenta los resultados anteriores, se puede inferir que el cemento óseo no produjo una respuesta citotóxica ya que se encuentra rodeado de hueso neoformado y células óseas.

3.2.1 Formulación Patrón

En la Figura 5, se aprecian dos cortes histológicos que muestran el proceso de neoformación ósea de una cavidad a la cual se le implantó la formulación patrón. En la figura 5(a), se observa el canal medular (1) rodeado por hueso cicatrizal periférico (2) y conteniendo la medula ósea (3). Además, se pueden identificar algunas células óseas, posiblemente osteoblastos (4) rodeando el canal medular; identificado con el número 5 se encuentra la zona de la preparación (zona del defecto óseo), donde se implantó el material; en el canal medular también se identifica medula ósea amarilla (6) y algunos vasos sanguíneos, marcados con estrellas.

En la figura 5(b) el corte corresponde al hueso cicatrizal correspondiente a la zona donde se realizó la preparación para implantar el material, se aprecia cicatrización completa del defecto y se diferencian dos zonas, una marcada con el numero 8 correspondiente a una zona más antigua y otra marcada con el número 7 que parece corresponder a un tejido en proceso de remodelado.

3.2.2 Formulación 1 (5 % DMAEM – 10,5 % HA)

En la Figura 6, se aprecia el corte histológico que muestra el proceso de neoformación ósea de una cavidad a la cual se le implantó cemento óseo acrílico modificado con carga bioactiva y comonomero alcalin.

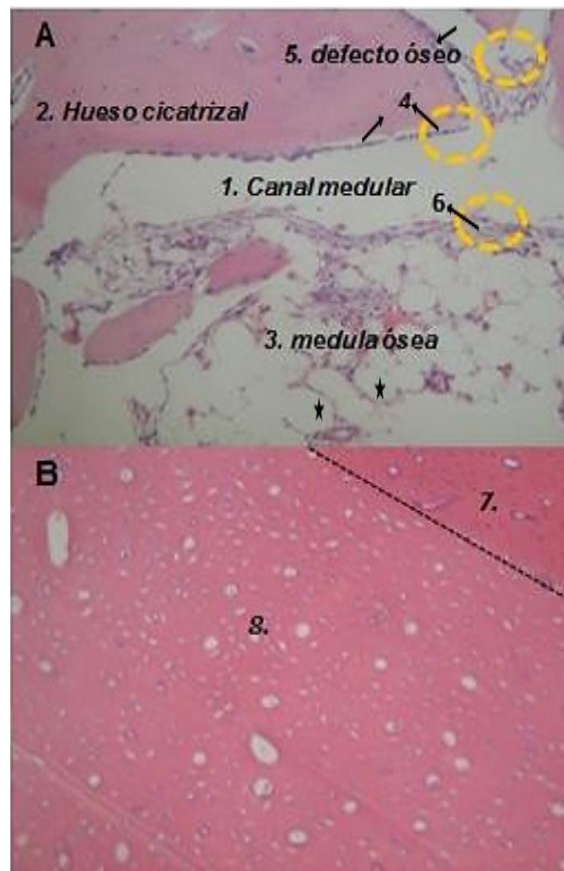


Figura 5. Histología de la interfaz hueso-cemento óseo acrílico sin carga y sin comonomero (0% DMAEM-0% HA). Que muestra el corte del espécimen en la proximidad con el canal medular (A) y a nivel medio (B).

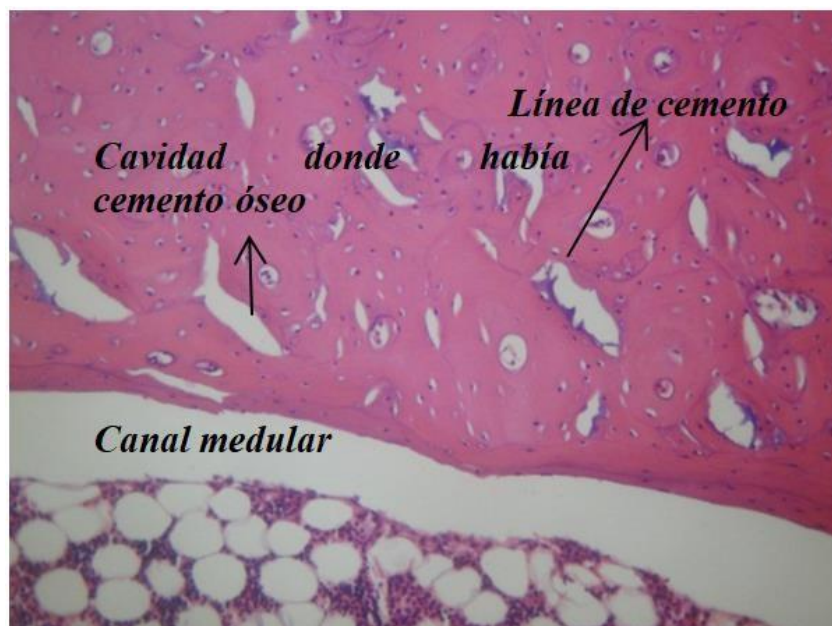


Figura 6. Histología de la interfaz hueso-cemento óseo acrílico modificado con 10,5% de HA y 5% DMAEM.

El porcentaje de medula ósea roja del defecto óseo fue alto (70 %), lo cual es bueno puesto que el aumento del porcentaje de medula roja ayuda a incrementar la cantidad de células óseas (Osteoblastos y Osteocitos). En la proximidad con el canal medular se identificaron líneas de cemento que tienen la función de separar las diferentes etapas de formación de tejido óseo.

El porcentaje de osteocitos, es mayor para esta formulación que contiene HA que para la que contiene solo comonomero, lo cual unido a la observación de un número menor de osteoblastos (17% vs 25%), podrían estar indicando un proceso de remodelado más rápido para las muestras a las que se les incorporó tanto DMAEM como HA.

Con los resultados anteriores y en vista de que no se encontraron células inflamatorias en la interfaz hueso-cemento se puede inferir que el cemento óseo modificado con HA y DMAEM no afectó la cicatrización normal del hueso. Además, la masiva presencia de osteocitos en su cavidad osteocitaria y de líneas de cemento, dan a entender que el proceso de cicatrización del cemento pudo haber ocurrido más rápido en comparación con los casos anteriores.

3.2.3 Formulación 3 (5 % DMAEM)

En la Figura 7, se muestra el proceso de

regeneración ósea de una cavidad en la cual se implantó cemento óseo acrílico con 5 % de DMAEM. En esta imagen se observa que la interfaz donde se ubicó el cemento óseo (1) se rodeó completamente de células óseas (osteoblastos y/o osteoclastos); además, ocurrió el proceso de cicatrización normal del hueso, diferenciándose al igual que en el caso de la formulación patrón, el hueso periférico del viejo.

La cantidad de osteoblastos en la periferia de la muestra con DMAEM fue un poco superior a la de la muestra control, lo que está de acuerdo con los reportes acerca de que la protonación del grupo amina en el co-monómero promueve una mayor adhesión y proliferación de las células formadoras de hueso [18]-[20]. La razón por la que el aumento en la cantidad de osteoblastos es tan baja, se puede atribuir a que los osteoblastos son células transitorias cuyo valor debe estar elevado en los momentos iniciales de la cicatrización pero disminuye a medida que el proceso regenerativo madura debido a su diferenciación terminal a osteocitos. En la formulación no se encontraron células inflamatorias, razón por la cual se puede deducir que el cemento óseo acrílico con comonomero alcalino es biocompatible al ser implantado en tejidos óseos.

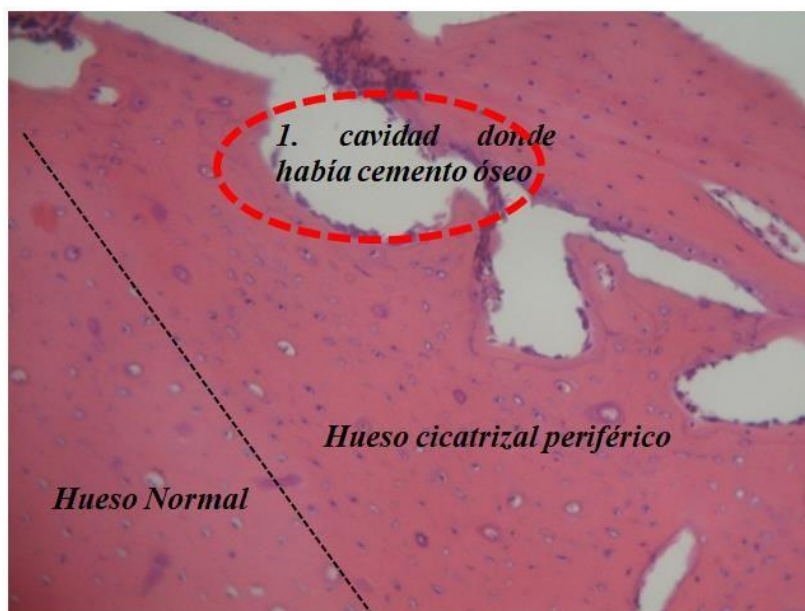


Figura 7. Histología de la interfaz hueso-cemento óseo acrílico modificada con 5% de DMAEM y sin Hidroxiapatita.

Finalmente, a los 28 días la presencia de osteoclastos en las tres formulaciones (3 %, 13 %, y 10 %) es normal porque se está dando la remodelación después del proceso inicial de cicatrización ósea, y la proporción con relación al número de osteoblastos y osteocitos es adecuada porque indica que hay más aposición que resorción.

La presencia de osteoclastos es mayor en las 2 formulaciones modificadas que en la patrón, lo cual sumado a valores adecuados para osteoblastos (17 y 25 %) podrían estar indicando un mayor estímulo a la neoformación ósea. Se observa también una presencia de osteoclastos ligeramente mayor en la formulación de cemento 1 lo cual se explica por ser estas las células las encargadas de digerir las partículas fosfocálcicas [35].

En general para las tres formulaciones estudiadas el porcentaje de osteoblastos es inferior al de osteocitos puesto que el proceso de cicatrización y formación de hueso iniciales ya ha pasado; lo normal es que en este periodo halla un porcentaje alto de osteocitos porque son células maduras derivadas de osteoblastos que quedan atrapados en sus lagunas dentro de la matriz ósea calcificada.

4. CONCLUSIONES

Las formulaciones modificadas en la fase líquida o sólida, mostraron propiedades bioactivas en condiciones *in vitro* e *in vivo* a diferencia de la formulación sin modificación (patrón), en la cual no se evidenció presencia de formación de capa apatítica sobre su superficie en condiciones *in vitro*, y se presentó una menor actividad de las células formadoras de hueso (osteoblastos, osteocitos y osteoclastos).

Los resultados en condiciones *in vitro*, muestran que la adición de DMAEM aumentó en menor grado la bioactividad de los cementos óseos en comparación con la adición de HA.

Los resultados *in vivo* demuestran que el cemento óseo modificado con 5 % de DMAEM en la fase líquida y 10,5 % de HA en la fase sólida presenta mayor bioactividad comparada con las otras formulaciones, ya que acelera el proceso de cicatrización del defecto óseo generado en la tibia de los biomodelos.

Por su buen comportamiento bioactivo tanto *in vitro*

como *in vivo*, las formulaciones estudiadas en este trabajo son susceptibles de ser empleadas en la fijación de prótesis ortopédicas y/o dentales.

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ANEXO E: Tamayo Marín, A.J.; Londoño, R.S.; Delgado, J.; Navia Porras, P.D.; **Valencia Zapata, M.E.**; Mina Hernandez, H.J.; Valencia, H.C.; Grande Tovar, D.C. *Biocompatible and Antimicrobial Electrospun Membranes Based on Nanocomposites of Chitosan/Poly (Vinyl Alcohol)/Graphene Oxide. International Journal of Molecular Sciences.* **2019**, 20.



Article

Biocompatible and Antimicrobial Electrospun Membranes Based on Nanocomposites of Chitosan/Poly (Vinyl Alcohol)/Graphene Oxide

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Abstract: Tissue engineering is gaining attention rapidly to replace and repair defective tissues in the human body after illnesses and accidents in different organs. Electrospun nanofiber scaffolds have emerged as a potential alternative for cell regeneration and organ replacement. In this paper, porous membranes, based on nanofibrous chitosan (CS), polyvinyl alcohol (PVA), and graphene oxide (GO), were obtained via electrospinning methodology. Three different formulations were obtained varying GO content, being characterized by Fourier Transform Infrared spectroscopy (FTIR), scanning electron microscopy (SEM), and energy dispersive spectroscopy (EDS). In vitro tests were carried out, consisting of hydrolytic degradation inside simulated biological fluid (SBF), and in vivo tests were carried out, where the material was implanted in Wistar rats' subcutaneous tissue to determine its biocompatibility. The antibacterial activity was tested against Gram-positive bacteria *Bacillus cereus* and *Staphylococcus aureus*, and against Gram-negative *Salmonella enterica* and *Escherichia coli*, by contact of the electrospun nanofiber scaffolds above inoculum bacterial in Mueller Hinton agar with good inhibition only for scaffolds with the higher GO content (1.0%). The results confirmed good biocompatibility of the nanofibrous scaffolds after in vivo tests in Wistar rats, which evidences its high potential in applications of tissue regeneration.

Keywords: antibacterial nanofibrous membranes; chitosan; electrospinning; graphene oxide; polyvinyl alcohol

1. Introduction

Currently, in the world, millions of people are affected by bone defects due to accidents, traumas, tumors, natural aging, bone fractures, obesity, and physical activity [1]. Due to this problem, the search for biomaterials with application in tissue engineering for the development of three-dimensional porous structural materials that imitate bone behavior has increased. This porous framework must

possess the properties of a natural bone, such as biocompatibility, biodegradability, support for cell adhesion, proliferation, and cell growth, to overcome accidents, traumas, tumors, natural aging, bone fractures, obesity, and physical activity [1]. The primary function of the scaffolding is to mimic the natural extracellular matrix temporarily, while the new bone is growing, which serves as a biological substitute for adhesion, allows cell migration, and, finally, guides the development of the new tissue with the appropriate functions [2].

Typically, various materials have been used for the manufacture of scaffolds, especially of the polymeric type, due to its excellent biocompatibility, adjustable chemical composition, suitable biological rearrangement, and acceptable degradation. However, the main drawback of natural polymers is their low mechanical resistance, which makes them unsuitable for a bone scaffold [3]. For this reason, it is necessary to reinforce the mechanical and thermal properties of the materials used as scaffolds with nanocomposites, which can increase mechanical and thermal resistance, as well as the barrier and antimicrobial properties. Therefore, the development of nanocomposites based on biopolymers, such as chitosan (CS), thanks to its biocompatibility, biodegradability, physiological inertness, remarkable affinity to proteins, and antibacterial, hemostatic, fungistatic, and antitumor properties, becomes an excellent alternative for the design of biomaterials for cell regeneration [4].

Graphene is a solid material composed of a thin single atomic sheet of sp^2 carbon atoms [5–7]. Functionalization of graphene for several uses in biomedicine (e.g., biosensors, nanocarriers, and devices for cell imaging and phototherapy for cancer) is an active area nowadays [8–12]. Usually, it is synthesized from the allotropic form graphite, one of the most abundant chemical elements in nature [13]. Several nanocomposites based on graphene and other materials have been used for stimulating neural stem cell adhesion, proliferation, differentiation, and neural regeneration [14].

Graphene oxide (GO), one of graphene's derivatives, has become a highly used nanofiller thanks to its high mechanical resistance, chemical stability, large surface area, and low toxicity [15]. Also, due to its antibacterial properties and a high surface/volume ratio, GO is a promising material for the development of antimicrobial surfaces [16]. Graphene oxide nanocomposites have been prepared to improve surface roughness and promote neural growth [14,17–20].

Recently, our group prepared antimicrobial films based on nanocomposites of chitosan/polyvinyl alcohol/graphene oxide by the drop-casting method, which showed excellent biocompatibility in the subcutaneous tissue of Wistar rats, antimicrobial activity against Gram-positive and Gram-negative bacteria, and better thermal and mechanical stability with the increase of graphene oxide [21]. We also prepared scaffolds by the freeze-drying method based on CS–GO nanocomposites demonstrating excellent biocompatibility after 30 days of implantation in Wistar rats' subcutaneous tissue. Reabsorption of the material by phagocytic activity and new bone formation in experiments on critical size defects has also been demonstrated [22,23]. However, there is a growing need to obtain materials with a tunable size of porosity to better stimulate cell adhesion and regeneration through the use of these devices.

Micro/nanofibrous scaffolds have been investigated extensively for tissue engineering and drug delivery [24]. Since micro/nanofibrous scaffolds mimic the natural extracellular matrix (ECM), they stimulate cell adhesion, proliferation, migration, and differentiation better than particulate structures. By the other side, due to its higher surface-area to volume ratio and higher interconnected porosity, cell adhesion and proliferation occurs quickly, which makes it a promising material for tissue engineering [25].

Electrospinning technique has been used to develop these controlled nanostructures with high porosity ratio for the formation of polymer fibers. This technique has advantages because, by using electric forces, fibers with diameters ranging from 2 nm to several micrometers can be obtained using solutions of both natural and synthetic polymers [26]. This process offers unique capabilities for the production of new nanofibers and natural tissues with controllable pore structure [27]. Electrospun nanofibrous membranes can be easily tuned to match irregular bone defects to promote osteointegration [28]. For all of the above reasons, electrospinning of polymeric micro/nanofibrous

scaffolds has the potential for application in traumatic or disease states, such as in skin regeneration or the treatment of cancer [29].

Even though several studies on CS/ polyvinyl alcohol (PVA)/GO films and electrospun polymeric micro/nanofibrous scaffolds have been investigated extensively in the literature, there is a lack of information about the physical, chemical, and mechanical characterization of the nanofibrous membranes, and their biocompatibility and antimicrobial performance. Therefore, this research proposed the study of biodegradable nanofibrous membranes based on PVA/CS/GO with potential applications for tissue regeneration and antimicrobial devices.

2. Results and Discussion

2.1. GO Synthesis and Characterization

GO synthesis followed the methodology used by Mangadlao [30]. GO synthesis and characterization were previously reported by our group [21].

2.2. Electrospun CS/PVA/GO Composite Nanofibrous Membranes Characterization

2.2.1. Fourier-Transform Infrared Spectroscopy (FTIR)

In Figure 1, the ATR-FTIR spectrum of the scaffolds are shown. It was observed that a band appears at 1701 cm^{-1} due to the stretching vibration of carbonyl groups $\text{C}=\text{O}$ ($-\text{COOH}$) at the edges of GO. The vibration band coupled in the OH plane (1328 cm^{-1}) becomes more pronounced due to the destruction of the original hydrogen bonds of CS/PVA compounds and the formation of a strong interaction between CS, PVA, and GO, with the increase in the amount of GO. The $\text{C}-\text{OH}$ bond observed at 1382 cm^{-1} is weakened with the increase in GO due to the strong hydrogen bond. On the other hand, no changes were noted in the functional groups in the compound system, although it is known that generally between the CS and the GO there will be hydrogen bonds that stabilize their compounds. The bands of the secondary amino groups and amide groups formed would be overlapping with the bands of amino groups and amide groups of the CS [31]. Furthermore, the band at 3251 cm^{-1} , due to the stretching vibration of the OH group, was displaced to higher wavenumbers (3288 cm^{-1}) and was further expanded, which can be attributed to the hydrogen interaction of GO with the mixture CS-PVA [32].

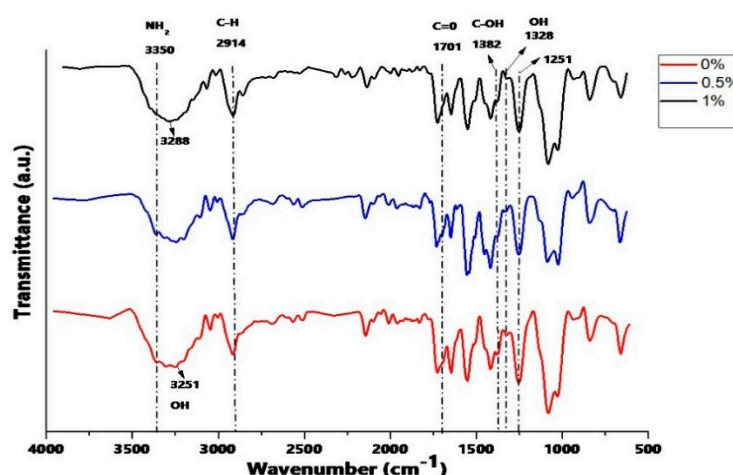


Figure 1. Attenuated total reflectance Fourier-transform infrared spectroscopy (ATR-FTIR) of electrospun chitosan (CS)/polyvinyl alcohol (PVA)/graphene oxide (GO) composite nanofibrous membranes with different GO amounts (0%, 0.5%, and 1.0%).

2.2.2. Scanning Electron Microscopy (SEM)

The morphology of the electrospun fibers is controlled by various parameters, such as the applied voltage, the flow velocity of the solution, the distance between the nozzle and the collector, and, especially, the concentration and surface tension of the solution [33].

As the Figure 2 shows, the composite nanofibers exhibit a random fibrous morphology and an interconnected porous structure, where the effect of the addition of GO is reflected in the increase of the average diameters of the fibers, due to the rise in the viscosity of the mixture with the addition of the GO [34,35]. The diameter of the fibers increased in average 144.45, 152.94, and 202.79 nm for GO percentages of 0%, 0.5%, and 1% by weight, respectively (Table S1).

Viscosity refers to the resistance exerted by a fluid against a tangential deformation. This resistance to flow is generated from the friction between the molecules. The increase in the viscosity can be justified because, when increasing the concentration of GO, the quantity of electrospun CS/PVA/GO composite nanofibrous membranes that interacted with each other, both with the polymeric molecules, increased. In addition, the formation of agglomerates in suspension was induced and they act as a resistance to the flow. When the jet is generated, the solution exerts strength to being stretched by the electric field, creating larger diameter fibers [36,37]. However, the defects presented can be attributed to an agglomeration of the GO sheets due to the high concentration that was used. On the other hand, it can be attributed to the effect of the residual solvent that was not evaporated during the process. Therefore, the flat fibrillar structure was not obtained in some regions of the material [36,38].

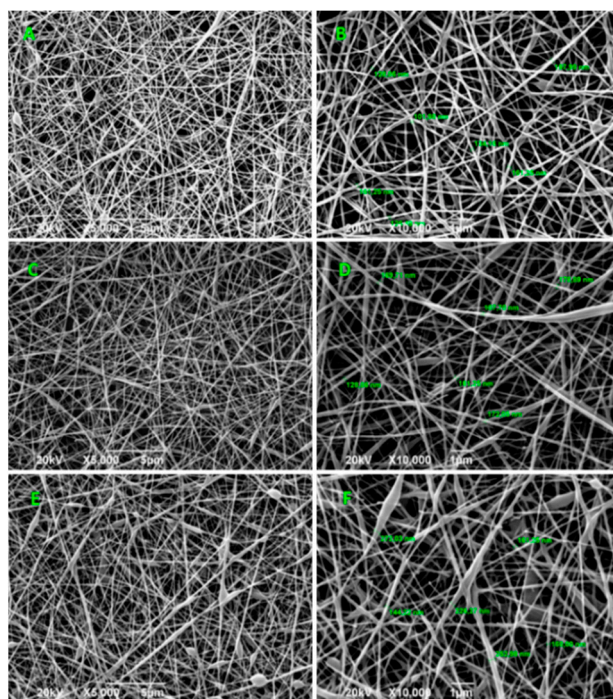


Figure 2. SEM images of electrospun CS/PVA/GO composite nanofibrous membranes with different GO amounts (0%, 0.5%, and 1.0%). Images (A,C,E) at 500 $\times$, and images (B,D,F) at 10,000 $\times$. For all the experiments, the voltage used was 20 kV.

2.2.3. Degradation in a Simulated Biological Fluid (SBF)

Figure 3 shows the results of the degradation percentage (weight loss) of the scaffolds with different proportions of GO after 14 days of being subjected to the process of hydrolytic degradation in a simulated biological fluid (SBF). The rate (%) of weight loss was calculated by employing Equation (1). It can be observed that the incorporation of GO in the polymer matrix improved the stability of the scaffolds against the SBF. The weight loss was lower for the samples with higher GO content, the

samples with 0% GO presented a final degradation of 75.4%, which is higher in comparison with the samples that contained some percentage of GO: the scaffolds with 0.5% GO exhibited an ultimate degradation of 72.33% and the frameworks with 1% GO showed the lowest deterioration of 71.90%. Therefore, it was observed that with a higher content of GO there is a lower weight loss, attributed to the higher content of GO in the CS/PVA binary mixture due to hydrogen bonds with the CS. Since many secondary bonds are present, these forces will multiply, and it will have high energy along with these links, evidenced in more excellent stability in the polymer chains [39].

The degradation behavior is explained by the interaction of the SBF with the CS and PVA. This interaction generates random excisions of the polymer chains since the introduction of water inside the polymer matrix causes the hydration of the molecules, rupture of hydrogen bonds, swelling, and, finally, the hydrolysis of unstable bonds [40]. Ultimately, resulting in a decrease in molecular weight and, therefore, higher susceptibility to a weight loss of the samples [41].

$$W_l (\%) = \frac{W_0 - W_d}{W_0} \times 100 \quad (1)$$

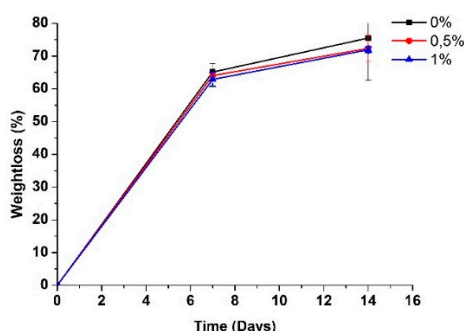


Figure 3. Weight loss of electrospun CS/PVA/GO composite nanofibrous membranes with different GO amounts (0%, 0.5%, and 1.0%), after several periods of immersion in a simulated biological fluid SBF.

In Figure 4, the average variation in pH of the SBF solution is shown. The drop in pH has been associated with the attack on amorphous zones because they retain more acidic species and because their blocks are more susceptible to degradation since they do not have segments of crystalline zones that hold them together, according to Figueira Maldonado [42]. Another possible cause could be the remaining acetic acid present in the scaffolds due to the production process used and the degradation of byproducts typical in CS, such as glucosamine or N-acetylglucosamine, which can also be found in the extracellular tissue matrix of humans, making them harmless when released into the human body [43].

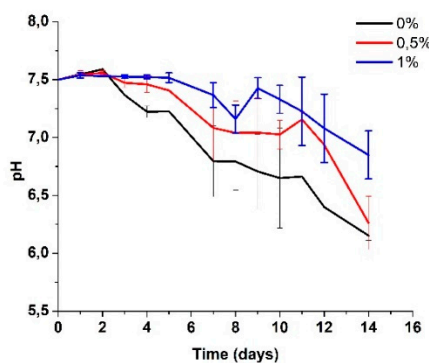


Figure 4. Change in pH in the SBF after several days of immersion of the electrospun CS/PVA/GO composite nanofibrous membranes with different GO amounts (0%, 0.5%, and 1.0%).

The pH of the biological media is a fundamental constant for the maintenance of vital processes. The enzymatic action and the chemical transformations of the cells are carried out within strict pH ranges. In the human body, the admissible scales that promote life and maintenance of vital functions oscillate between 6 and 8, as determined by the ASTM F1635 standard and confirmed in various investigations [44,45], which indicates that the values obtained during the degradation of the scaffolds are within the known ranges for biological processes.

2.2.4. SEM of the Electrospun CS/PVA/GO Composite Nanofibrous Membranes after Immersion in SBF

It was expected that the characteristic fibrillar structure of these materials would resist dissolution during the incubation in SBF. However, it could be seen by the SEM that there are notable differences between the morphologies before and after the degradation process in SBF (Figures 2 and 5), respectively, giving evidence of a rough surface and with fibers fused together. The fibrillar and porous structure disappeared, possibly due to the swelling of the fibers, which was attributed to the hydrophilic character of CS and PVA in a high degree of hydrolysis and medium molecular weight [44].

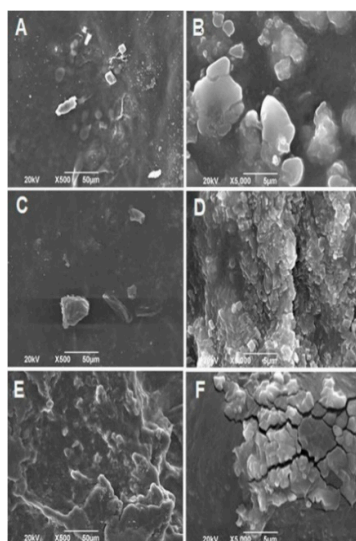


Figure 5. SEM images of the electrospun CS/PVA/GO composite nanofibrous membranes after fourteen days in the degradation process in SBF. (A,B) 0% GO, (C,D) 0.5% GO, and (E,F) 1.0 % GO. Images (A,C,E) at 500 $\times$, and images (B,D,F) at 10,000 $\times$. For all the experiments, the voltage used was 20 kV.

The immersion of the scaffolds in SBF was also carried out to evaluate the capacity of apatite formation on the surface of the material. The SEM images showed that, in some places, the scaffolds were covered by hydroxyapatite (HA) crystals, as evidenced for the scaffolding with 0.5% GO (Figure 5D) after 14 days of immersion, confirming the capacity of the material to stimulate the formation of new bone. This was verified through dispersive energy spectroscopy (EDS, data not shown) where it showed that the peak signals of Ca and P were present in the scaffolds, with proportions of calcium of 7.09% and phosphorus of 3.11%, which confirms the formation of apatite. In addition to these main elements, the presence of small amounts of Na, Cl, K, and Mg was detected. The above components were derived from SBF.

Figure 5 shows the SEM images of the surface of the CS/PVA/GO scaffolds that were successfully made by the electrospinning method, showing a fibrillar morphology, as expected. With a volume ratio of PVA/CS of 7:3 and different concentrations of GO (0%, 0.5%, and 1% by weight of CS) the nanofibers were uniform, smooth, continuous and without defects in almost the entire sample.

The results showed the formation of hydroxyapatite compounds. Therefore, CS/PVA/GO scaffolds could provide a promising construction for the application of bone tissue engineering, as long as the chemical stability in SBF is improved. However, it is advisable to perform tests in subdermal

implantation in the longer term to verify if there is bone ectopic formation or to perform tests on critical size intraosseous defects to study the osteogenic capacity of the biomaterial.

2.2.5. Antibacterial Activity

As shown in Table 1, no inhibition of pathogens was observed in the treatments with 0% and 0.5% of GO. Although inhibition against Gram-positive and Gram-negative bacteria can occur due to GO and chitosan [46,47], the fact that there was no inhibition when there is only chitosan (0% GO) shows the strong interaction of hydrogen bonds between chitosan and PVA, which decreases the solubility of chitosan in the medium [45]. This interaction prevents its interaction with the cell membrane of the microorganisms, causing no inhibition to occur.

This explanation suggests that the inhibition found in the treatment with 1.0% GO is mainly due to the GO effect. The sharp atomic edges of the GO penetrate the cell's membrane and destabilize its integrity. Chemically, they can promote lipid peroxidation induced by the natural oxidative nature of GO depending directly on their concentration in the medium [46,47]. This inhibition was presented against all the bacteria evaluated, which agrees with that found by other authors against Gram-positive bacteria [48,49] and Gram-negative bacteria [48–55].

Table 1. Inhibition of electrospun CS/PVA/GO composite nanofibrous membranes against bacterial strains.

Strain	0% GO	0.5% GO	1.0% GO
<i>Bacillus cereus</i>	---	--	+
<i>Staphylococcus aureus</i>	---	--	+
<i>Salmonella spp</i>	---	--	+
<i>Escherichia coli</i>	---	--	+

(+) Weak inhibition of the pathogen; (--) Weak pathogen growth; (---) Complete growth of the pathogen.

2.2.6. Biomodel Tests In Vivo

After 30 days of implantation in the biomodels, the samples were recovered. In all cases, repair of the created surgical defect was observed. Furthermore, all the biomodels showed a hair recovery and absence of injuries and infections in the intervened areas with healthy healing and restoration of the tissue architecture (Figure 6). The material, although quite compatible, generated an inflammatory response to a foreign body where the cells surrounded the fragments with a fibrous capsule and the rest of the soft tissues with healthy appearance, for the case of higher GO content (1.0%).

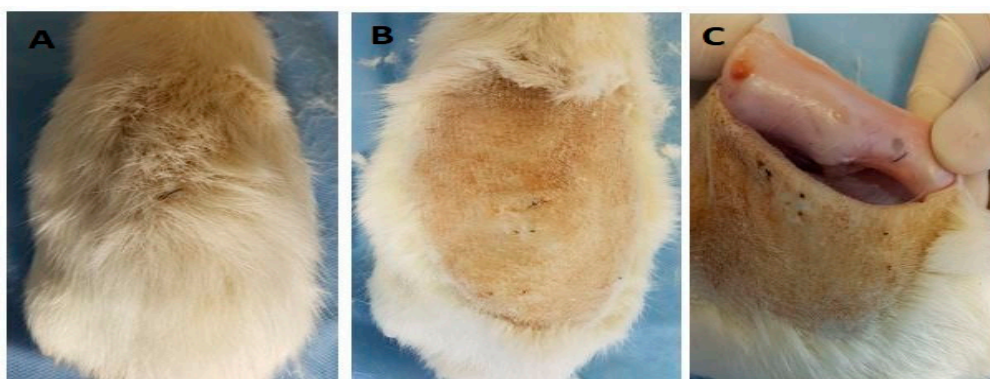


Figure 6. The dorsal area of the rat Wistar after 30 days of the implantation: (A) hair recovery, (B) absence of injuries and infections, and (C) internal surface of the skin where the implanted samples are encapsulated by scar tissue.

Figure 7 shows the images of the histological study of the subcutaneous tissue after 30 days of implanting the scaffolds in the biomodels (Wistar rats). In Figure 7, we have the histological image of the control sample (porcine collagen), where it was observed that there was resorption of the material with the recovery of the anatomical architecture of the tissue.

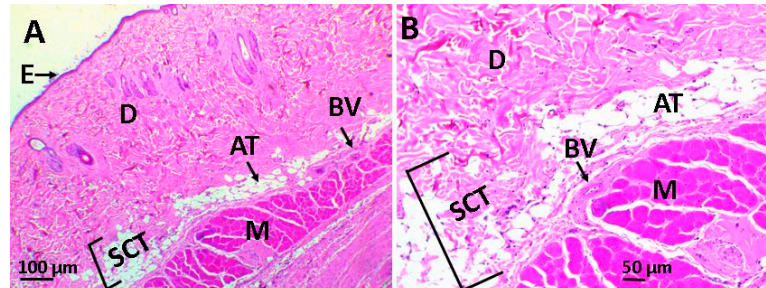


Figure 7. Image of the control sample using the hematoxylin and eosin technique. Image (A) at 4× and image (B) at 10×. E: epidermis, D: dermis, AT: adipose tissue, BV: blood vessel, SCT: subcutaneous cellular tissue, and M: muscle.

Figure 8 corresponds to scaffolds with 0% GO, recovery of tissue architecture, and fragments of the material in the process of resorption with the presence of inflammatory infiltrate are noted. It was also possible to observe the presence of a fibrous capsule (FC), corresponding to a series of collagen fibers that surrounded the biomaterial and that is a common finding in the experiments that include the implantation of materials, as part of the healing process within the chronic inflammation phase.

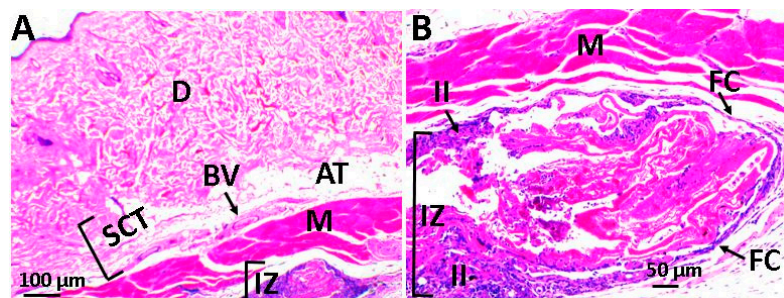


Figure 8. Scaffold with 0% GO using the hematoxylin and eosin technique. Image (A) at 4× and image (B) at 10×. D: dermis, AT: adipose tissue, BV: blood vessel, SCT: subcutaneous cellular tissue, II: inflammatory infiltrate, IZ: implantation area, M: muscle, and FC: fibrous capsule.

Figures 9 and 10 correspond to the implanted material with graphene oxide content. In both of them, the recovery of tissue architecture is observed. Figure 9 corresponds to the material with 0.5% GO, which shows a normal healing process, with the presence of traces of material in the process of resorption and are surrounded by fibrous tissue with the presence of inflammatory cells, although the inflammatory response seems to be lower in scaffolds with 0.5% GO. In the case of structures with 1% GO (Figure 10), a continuity solution was observed in the tissue, healing was delayed, and a greater inflammatory process was apparent when compared to the two previous cases.

In all cases, a normal healing process was observed with the recovery of the tissue architecture. In scaffolds with 0.5% graphene oxide, traces of the material being phagocytized by an inflammatory infiltrate were observed, whereas in frameworks with 1% graphene oxide, resorption was minimal after 30 days of implantation and the situation has been solved as a reaction to a foreign body that has encapsulated the scaffold (FC).

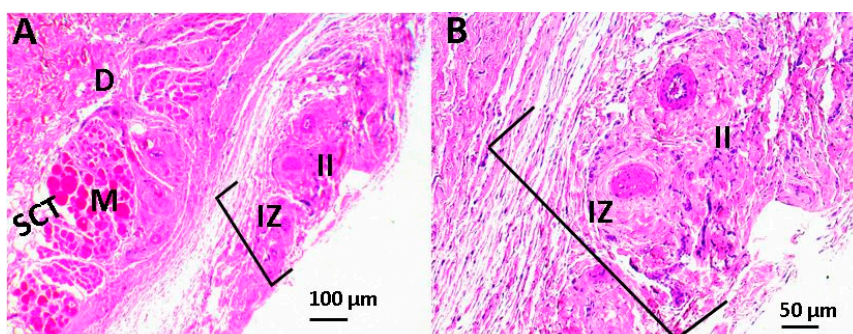


Figure 9. Scaffold with 0.5% GO using the hematoxylin and eosin technique. Image (A) at 4× and image (B) at 10×. D: dermis, AT: adipose tissue, SCT: subcutaneous cellular tissue, II: inflammatory infiltrate, IZ: implantation area, and M: Muscle.

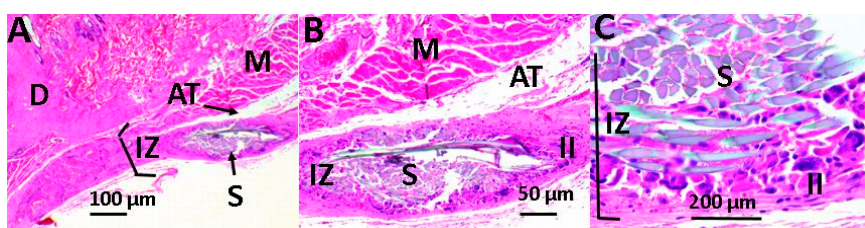


Figure 10. Scaffold with 1% GO using the hematoxylin and eosin technique. Image (A) at 4× and image (B) at 10×, Image (C) at 40×. D: dermis, AT: adipose tissue, II: inflammatory infiltrate, S: scaffold, IZ: implantation area, and M: muscle.

3. Materials and Methods

3.1. Materials

For graphene oxide synthesis, graphite flakes (99.8%) were used (Alfa Aesar, Tewksbury, MA, USA). Concentrated sulfuric acid (H_2SO_4), potassium permanganate (KMnO_4), hydrogen peroxide (H_2O_2), and isopropanol were supplied by Merck (Burlington, MA, USA). For the production of the films, chitosan of low molecular weight (M_v 144,000 g/mol) and a deacetylation degree between 89–90%, polyvinyl alcohol (PVA), with hydrolysis between 87–89% and viscous molecular weight of 93,000 g/mol was used (Sigma-Aldrich, Palo Alto, CA, USA). Glacial acetic acid comes from Merck (Burlington, MA, USA). For the elaboration of the simulated biological fluid, NaCl , $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, CaCl_2 , Na_2SO_4 , and tris-(hydroxymethyl aminomethane) $[(\text{CH}_2\text{OH})_3\text{CNH}_2]$ were acquired from Sigma Aldrich (Palo Alto, CA, USA); NaHCO_3 , KCl , and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ from Fisher Chemical (Pittsburgh, PA, USA); and hydrochloric acid (HCl) from Merck (Burlington, MA, USA). All reagents used were analytical degree.

3.2. Methods

3.2.1. GO Synthesis

GO synthesis followed the methodology used by Mangadlao [30]. GO synthesis and characterization were previously reported by our group [21].

3.2.2. Preparation of Electrospun CS/PVA/GO Composite Nanofibrous Membranes

Scaffolds were manufactured by the electrospinning method using a vertical configuration. A high voltage source (Gamma High Voltage Research INC., Model E30) was used. It was connected to the nozzle and the collector, then the polymer solution was introduced in a 10 mL syringe, and, by means of a polypropylene hose, it was coupled to the electrospinning nozzle in stainless steel. Finally,

a voltage of 22 kV was applied and a pump (Braintree Scientific INC) was used to control the flow of the solution, maintaining it at 1.0 mL/h. As a result of the positive polarization of the solution and the electric field, the solution was attracted by the negatively charged collector, thus forming the fibers, which were collected on a slotted Teflon mold, which is arranged on an aluminum sheet. The whole process was carried out at a temperature (21–30 °C) and relative humidity (45–60%), average to the environmental conditions of the city of Santiago de Cali, Colombia.

The solution for electrospinning was carried out with individual solutions of CS at 5% (*w/v*) dissolved in 2% acetic acid (*v/v*), 8% PVA solution (*w/v*) dissolved in distilled water at 80 °C, and 300 rpm. The GO was dispersed in the ultrasonic medium. A volume ratio of PVA:CS of 7:3 was used, and the GO was added concerning 1% by weight of the CS.

3.3. Characterization

3.3.1. Fourier Transform Infrared Spectroscopy (FTIR)

The chemical identification of the films was carried out using FTIR in the ATR mode (attenuated total reflectance) (Shimadzu, Kyoto, Japan).

3.3.2. Scanning Electron Microscopy (SEM)

The morphological inspection of the surfaces of the film was carried out through a scanning electron microscope (SEM) (JEOL JSM-6490LA, Musashino, Tokyo, Japan). The working conditions were 20 kV and mode of secondary backscattered electrons. All the samples were coated with gold to create an electronic density in the material since the polymers lack it.

3.3.3. Degradation in Simulated Biological Fluid

The hydrolytic degradation was carried out following the procedure outlined in the American Society for Testing and Materials (ASTM) F1635-16 standard. The scaffolds were immersed in a simulated biological fluid (SBF) at 37 °C for 14 days in a Memmert IN 110 incubator (Memmert, Schwabach, Germany). The SBF was prepared according to the method proposed by Kokubo and Takadama [56], and the degradation was evaluated by examining the weight of the films before and after immersion for different periods (1, 3, 5, 7, and 14 d).

The initial weight of the samples before immersion was recorded as W_0 and the weight after drying for 48 h in the incubator at 37 °C was recorded as W_d . The weight loss (% W_l) was calculated according to Equation (1).

Each sample was immersed in 15 mL of SBF, and, in each period, three samples were evaluated per formulation. The pH of the SBF was measured every day until the total test time was completed using an Accumet AB150 pH meter (Fisherbrand, Ottawa, ON, Canada). The morphology of the films after drying was studied using SEM.

3.3.4. Antimicrobial of Electrospun CS/PVA/GO Composite Nanofibrous Membranes Assay

The antimicrobial activity of the electrospun CS/PVA/GO composite nanofibrous membranes was evaluated against Gram-positive bacteria, *Bacillus cereus* (ATCC 13061) and *Staphylococcus aureus* (ATCC 55804), and Gram-negative bacteria, *Salmonella enterica* (ATCC 13311) and *Escherichia coli* (ATCC 11775), by contact of the electrospun CS/PVA/GO composite nanofibrous membranes above inoculum bacterial in agar. The methodology used by Ruiz et al. (2018) was followed without modifications [21]. The test was repeated three times for each of the treatments.

3.3.5. Biomodels Test In Vivo

The inflammatory response to the implantation of films in the subcutaneous tissue was measured. Samples of CS–PVA films with different percentages of GO (three replicates per formulation) with 10 mm in diameter and 2 mm in thickness were implanted in the subdermal tissue of three adult Wistar

rats, in preparations made on the dorsal surface, according to the recommendation of ISO 10993-6. As a control sample, commercial porcine collagen with the same dimensions was used. All the biomodels were supplied by the Bioterium of the Faculty of Medical Sciences of the Universidad del Valle. The procedures carried out were approved by the Animal Ethics Committee of the Universidad del Valle, by the CEAS 001-016 certificate of May 20th of 2016.

After 30 days of implantation, the samples were recovered, fixed in buffered formalin, dehydrated in alcohol solutions of ascending concentration (70%, 80%, 95%, and 100%), diaphanized with xylol and infiltrated with paraffin for later cutting to 4 μ m. The samples were processed for histological analysis by hematoxylin and eosin (H&E) and masson trichromacy (MT) techniques.

4. Conclusions

In this research, we demonstrated a simple protocol for obtaining electrospun scaffolds based on CS/PVA/GO nanocomposites, which showed adequate chemical and biological properties for their application in tissue engineering. The addition of GO in the electrospun scaffolds did not interfere with the correct formation of the fibrillar structure. Rather, it gave a more excellent stability against the degradation in SBF. It was also evidenced through EDS that in some places the scaffolds were covered by a mild apatite layer.

Finally, the material that presented the best tissue biocompatibility was the one that had a GO content of 0.5%. Interestingly, there was a better antibacterial response when the GO was increased to 1%, being active both for Gram-positive bacteria and Gram-negative bacteria. However, the inflammatory response also increased and the degradability was reduced when it was implanted. According to this observation, the use of biomaterial the most suitable in subdermal applications is the scaffold with GO at 0.5%, and scaffolds with 1% GO should be recommended in areas where an antibacterial effect is needed, such as in wounds exposed and infected skin, since the inflammatory response seems to be higher as the percentage increases. It is also necessary to perform additional studies to evaluate the osteogenic effect in applications for bone tissue engineering.

Supplementary Materials: Supplementary material Table S1 can be found at <http://www.mdpi.com/1422-0067/20/12/2987/s1>.

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Supplementary Materials: Biocompatible and antimicrobial electrospun membranes based on nanocomposites of chitosan/poly (vinyl alcohol)/graphene oxide

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Table S1. Measurements of fiber diameters in scaffolding

Fiber Number	Diameters (nm)		
	0 % GO	0.5% GO	1% GO
1	128.06	169.71	272.03
2	101.98	128.06	144.22
3	161.25	107.7	226.27
4	126.49	161.25	252.98
5	134.16	172.05	160
6	161.25	178.89	161.25
7	197.99		
Mean	144.45	152.94	202.79
Desv. stand	31.44	28.48	54.50

ANEXO F: Diego, L.; Valencia, C.H.; Valencia, C.; Zuluaga, F.; **Valencia, M.E.**; Mina, J.H.; David, C.; Tovar, G. *Evaluation of the Biocompatibility of CS-Graphene Oxide Compounds In Vivo. International Journal of Molecular Sciences.* **2019**, co, doi:10.3390/ijms20071572.



Article

Evaluation of the Biocompatibility of CS-Graphene Oxide Compounds *In Vivo*

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Abstract: In the last few years, graphene oxide (GO) has gained considerable importance in scaffold preparation for tissue engineering due to the presence of functional groups that allow the interaction between the extracellular matrix and the components of the cellular membrane. The interaction between GO and chitosan (CS) can not only improve the biomechanical properties of the scaffold but also generate a synergistic effect, facilitating tissue recovery. *In vivo* studies on GO are scarce; therefore, biocompatibility tests on CS-GO scaffolds and bone regeneration experiments on critical size defects were carried out on *Wistar* rats. Scaffolds made of CS, CS-GO 0.5%, and CS-GO 1% were prepared and implanted on *Wistar* rats cranial bones for three months. Scaffold samples were analyzed through histochemistry and scanning electron microscopy. The analysis performed showed reabsorption of the material by phagocytic activity and new bone formation. The CS-GO 0.5% formulation gave the best performance in bone regeneration, with excellent biocompatibility. These results show the potential of this compound for tissue regeneration opening and medical applications.

Keywords: bone-regeneration; chitosan; graphene oxide; scaffolds

1. Introduction

In recent years, the use of graphene oxide (GO) to reinforce scaffolds for tissue engineering has gained importance. Besides improving biomechanical properties, it can produce a synergistic effect that would improve biological properties when combined with other materials such as CS [1].

However, some doubts remain about the biocompatibility of GO in tissue engineering as there is not enough *in vivo* experimental evidence, and *in vitro* cytotoxicity results indicate that some side effects may occur.

It is clear that the systemic effects of a biomaterial on human beings' health depend on some factors such as particle size, concentration, physicochemical properties, time of exposure, and route of entry into the organism. According to Peruzynska et al. [2], the biocompatibility of structures elaborated with graphene depend not only on dose and time but also on some chemical parameters such as layer number, shape, size, chemical functionalization, and surface characteristics.

In the case of GO, different investigations have been carried out to evaluate its effects on different types of cells and white organs, as well as to mitigate its cytotoxic effect.

In a review by Pinto et al. [3], it is reported that bacteria and mammalian cells decrease their viability when directly exposed to GO materials. *In vivo* studies showed high toxicity (approximately 50% of deaths), with accumulation in lung and liver and inflammation depending on dose and concentration. After a few hours of oral ingestion, there was an increase in these effects in the stomach and intestine but not inside other essential organs. After one day, some amounts were found in organs that disappear within a few days. Intratracheal introduction produced pulmonary inflammation and the subdermal route generating a slight inflammatory response. However, they also found that GO toxic effects decreased when it was incorporated into biomaterials [3,4].

Qu et al. [5] intravenously injected solutions of GO in buffer phosphate (PBS) in rats, confirming a toxic effect with accumulation mainly in the lungs. Dose-dependent cytotoxicity has been reported in human epithelial cells cultures [6].

In specialized literature, there is a consensus that GO cytotoxic effects occurred when there was direct contact with cells by physically damaging the lipid layer of the cell membrane [4] and increasing production of reactive oxygen species (ROS) [7], possibly with the initiation of apoptotic processes. However, some authors also found that this cytotoxic effect can be controlled by functionalization and binding to other compounds, which, in addition to improving biocompatibility, allow increased solubility and stability [4].

Awaja et al. [8] found low *in vitro* toxicity when coating polymers like polyether ether ketone (PEEK) and polyethylene terephthalate (PET) with GO. Wang et al. [9] demonstrated cytocompatibility and *in vivo* degradation of CS-GO scaffolds implanted in rat subdermal tissues. Xie et al. [10] found that incorporating GO into calcium phosphate (CaP) frameworks did not affect biocompatibility but instead improved surface characteristics that allowed a bone-inductive effect.

Our group previously reported the synthesis and characterization of CS-GO scaffolds by the freeze-drying method, having good results in the biocompatibility analysis in subdermal tissue regeneration after 30 days of implantation. It was clear that the material was biocompatible and that it did not generate an inflammatory response. However, more *in vivo* assays for critical size defects are necessary in order to obtain more information about the biocompatibility of the materials and their influence on the tissue regeneration processes, due to the fact that in this kind of defect, the tissue will not regenerate by itself, it will need some stimulation from the implanted materials.

For the above reasons, the objectives of this research were to evaluate the *in vivo* biocompatibility of Chitosan-graphene oxide (CS-GO) scaffolds and their potential in stimulating bone regeneration of critical size defects after three months of implantation in *Wistar's* parietal bone.

2. Results

2.1. Characterization of CS, GO, and CS-GO Scaffolds

All this characterization was reported by our group elsewhere [11]. Briefly, chitosan was characterized using Fourier Transform Infrared Spectroscopy (FTIR) and Proton Nuclear Magnetic resonance spectroscopy ($^1\text{H-NMR}$), in order to elucidate the structure of the molecule and its deacetylation degree (66.7%). Gel permeation chromatography was used to determine the average molecular weight of the polymer (6481.87 g/mol) [11].

Graphene oxide was characterized by FTIR, Raman, and X-ray diffraction spectroscopy. Finally, to study GO tridimensional and nanometric structure, Atomic Force Microscopy (AFM) and Dynamic Light Scattering (DLS) were used. CS-GO scaffolds were prepared and characterized by FTIR, TGA, and scanning electron microscopy (SEM).

2.2. Histological Observations on Control Samples

In the experimental designs with negative defect control, the objective of the empty defect or control defect is to validate the investigation. In this work, three samples used as control showed non-binding in the defect, i.e., the surgical preparation performed behaved as critical size, and the

organism was not able to complete the healing by bone regeneration presenting a fibrous type of scarring.

Figures 1 and 2 correspond to a sample of this group in preparations with hematoxylin-eosin stain (HE). It is apparent how the fibrous material (fibrous interface (If)) occupies the preparation area, and old bone areas (peripheral bone (B)) are in the process of remodeling.

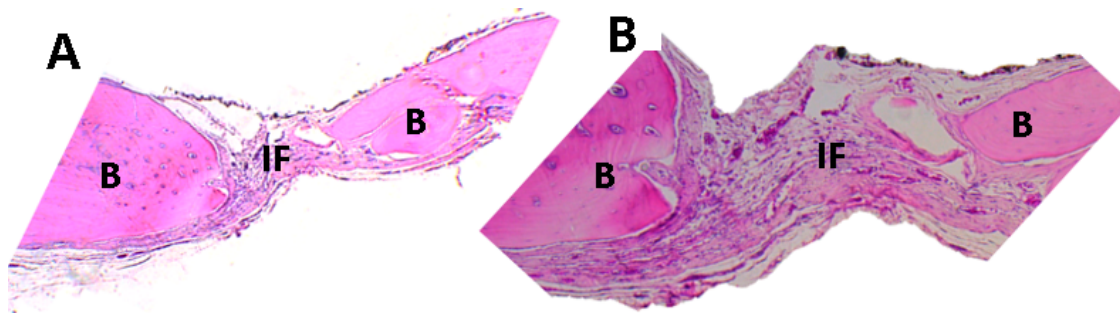


Figure 1. Control site (empty defect). Image (A) at 5× image (B) at 10× preparation zone. (B): peripheral bone, FI: a fibrous interface. Hematoxylin-eosin (HE) stain was used for the analysis of the preparation area.

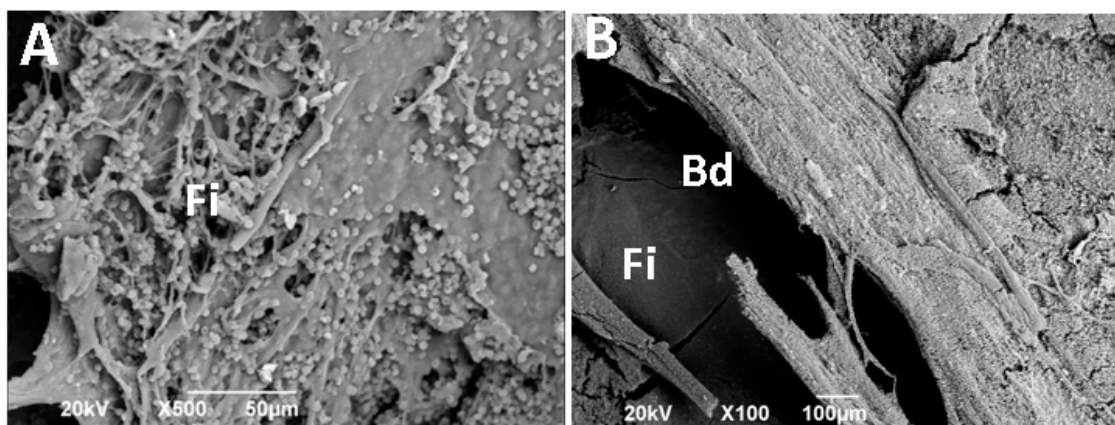


Figure 2. Scanning electron microscopy (SEM) image of the control site. Image (A) corresponds to the periosteal surface, and image (B) corresponds to the view from the meningeal surface. FI: Fibrous interface, BD: Bone defect.

Figure 2 corresponds to an image obtained by scanning electron microscopy in the preparation area. Here, it is notorious how the defect is covered by a material of fibrous appearance (If). Image A corresponds to the surface in contact with the periosteum. At the left end of the image, there is a preparation area corresponding to the defect. Image B corresponds to the same surface seen from the meningeal side, where it is clear that the deficiency remains without regeneration.

The previous observations are expected for this type of design, similar to reports in the literature. Some reports mention scarring of fibrous nature with parallel collagen fibers that seem to continue with the periosteum, with a thickness lower than the original bone and with evidence of regeneration in the edges of the preparation area [12–15].

2.3. Histological Observations in Experimental Samples

In general, all samples showed a similar behavior with partial degradation/resorption of the materials and replacement by newly formed bone tissue. The staining techniques used corresponded to hematoxylin-eosin (HE), Masson trichromacy (TM), and Gomori trichromacy (TG).

Figure 3 corresponds to 10× images of samples implanted with the three types of material, newly formed areas plus areas with remaining material, and regions in degradation/reabsorption processes are observed.

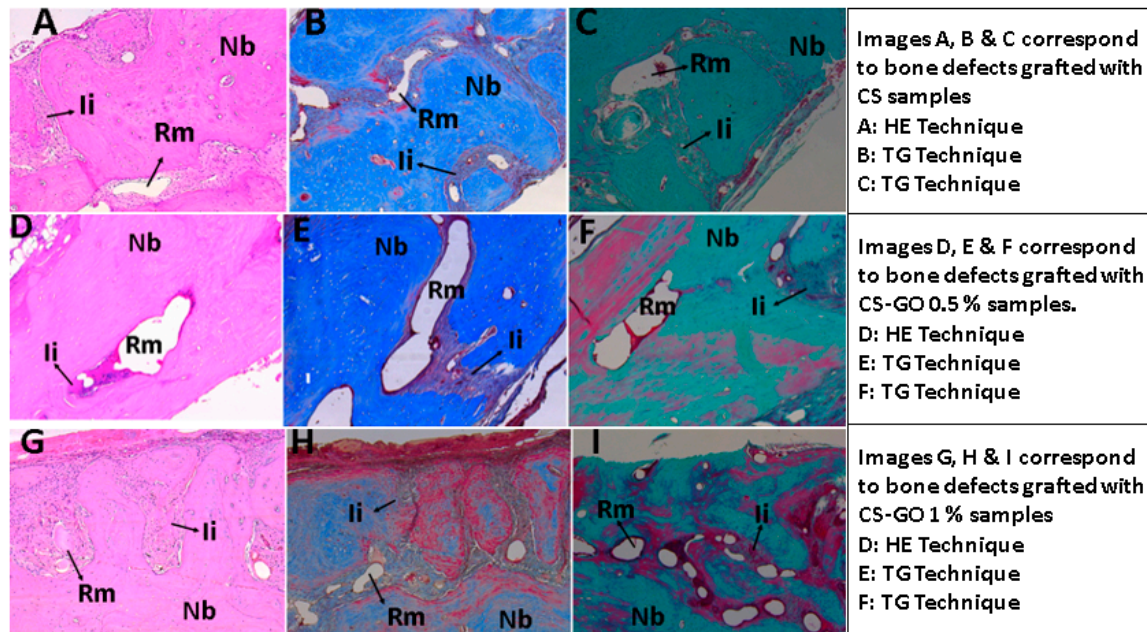


Figure 3. Experimental samples with images at 10×. Nb: Newly formed bone, Rm: Remnant material, li: Inflammatory infiltrate.

At higher magnification, it is possible to observe particles' remaining material surrounded by a fibrous capsule which is phagocytized by cells of a mixed inflammatory infiltrate, with the presence of abundant blood vessels (Figure 4).

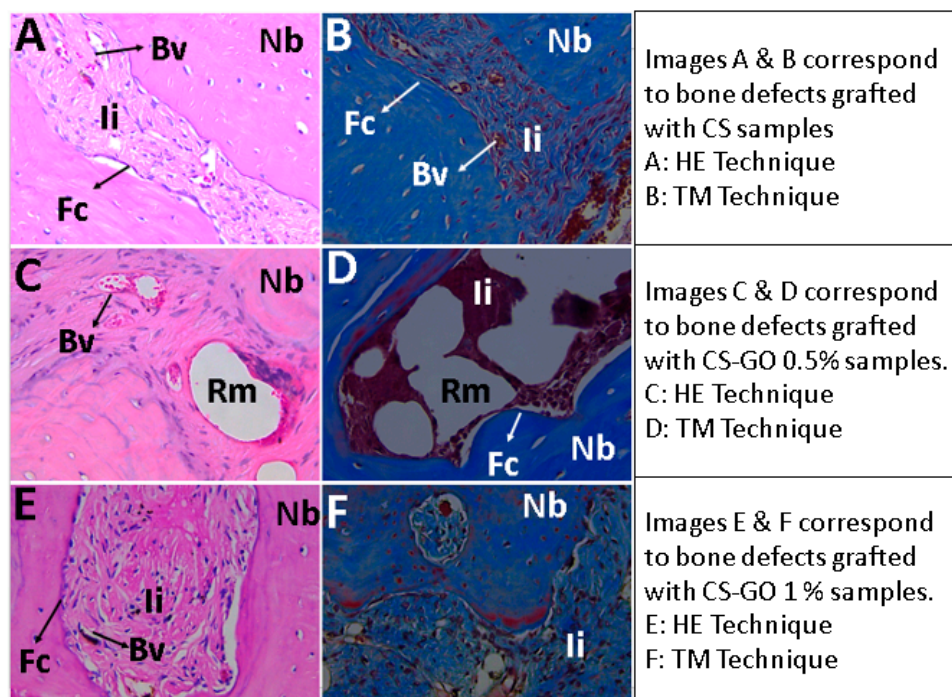


Figure 4. Experimental samples with images at 40×. Nb: Newly born bone, li: Inflammatory infiltrate, Rm: Remnant material, Fc: Fibrous capsule, Bv: Blood vessel.

From histological images taken at 4 $\times$, a histomorphometrically analysis was performed to determine the area of newly formed bone and the areas occupied with collagen I and collagen type III. The three groups were made up of three specimens each. The area of bone formation was greater in the group of specimens treated with CS-GO 0.5% with an average area formed of $95.287 \pm 6.126\%$, as compared to 52.79% for those implanted with CS-GO at 1%. This previous result is statistically significant at the significance level of 0.1 ($p = 0.0509$). The post-hoc test showed differences between CS vs. CS-GO 0.5% ($p = 0.005$) and between CS-GO 0.5% vs. CS-GO 0.1% ($p = 0.028$).

SEM and dispersive energy spectroscopy (EDS) analyses showed differences in the surface appearance of the implanted area and the contents of calcium and phosphorus.

Figures 5–7 show the images of the three samples implanted with the different materials, the presence of structures compatible with collagen fibers, cells, and extracellular matrix in different proportions are observed.

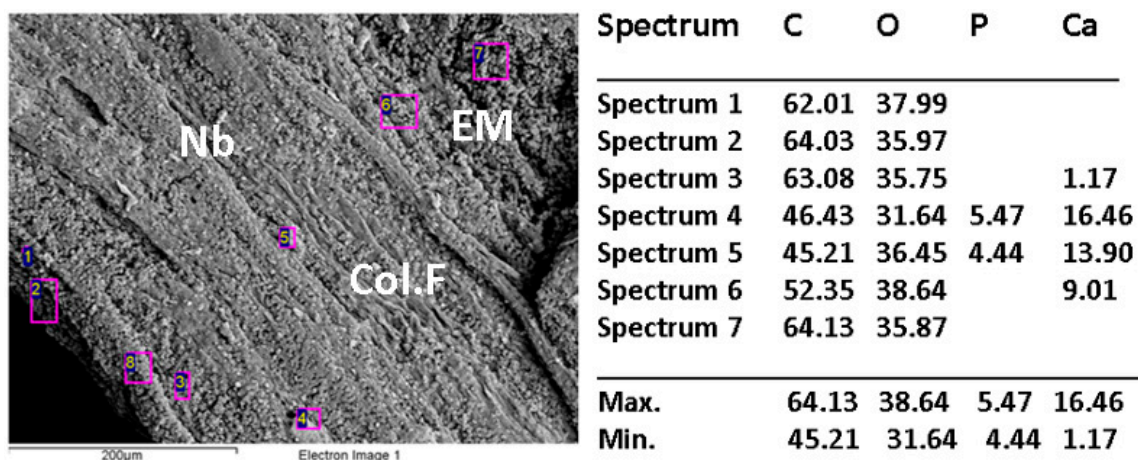


Figure 5. Scanning electron microscopy (SEM)/dispersive energy spectroscopy (EDS) analysis of control sample implanted in critical size defects in rat parietal bone *Wistar*. Nb: Newly born bone. EM: Extracellular matrix. Col-F: collagen fiber.

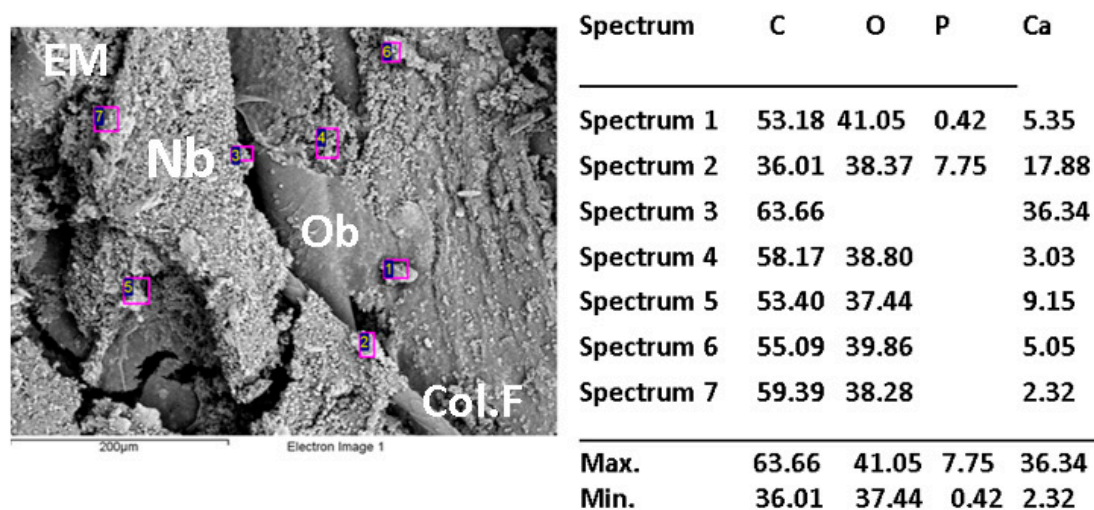


Figure 6. Scanning electron microscopy (SEM)/dispersive energy spectroscopy (EDS) analysis of CS-GO 0.5% sample implanted in critical size defects in rat parietal bone *Wistar*. Nb: Newly born bone. EM: Extracellular matrix. Col-F: collagen fiber. Ob: Osteoblast.

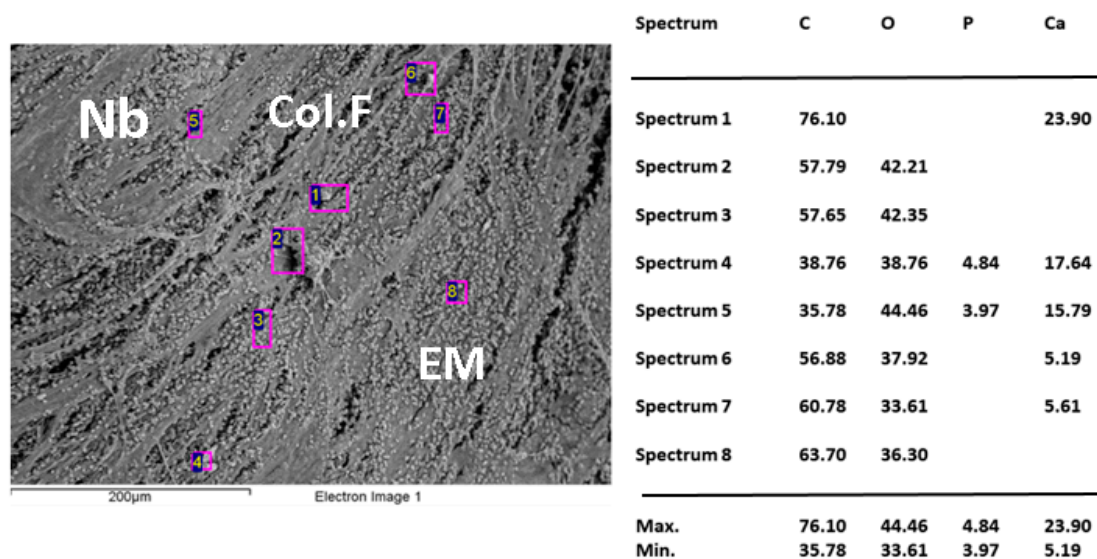


Figure 7. Scanning electron microscopy (SEM)/dispersive energy spectroscopy (EDS) analysis of CS-GO 1% sample implanted in critical size defects in rat parietal bone *Wistar*. Nb: Newly born bone. EM: Extracellular matrix. Col-F: collagen fiber.

The elemental analysis by EDS indicates that the sample CS-GO 0.5% presented the highest percentages of calcium and phosphorus.

3. Discussion

In this investigation, the differences in the results obtained are a consequence of the design of the experiment itself (critical defect with negative defect control), i.e., differences in defects (regeneration of the preparation) are due to the influence that the three formulations had on the repairing cells.

If we review the overall percentages of bone-formation in the three defects, the next observations arise: 34.19% bone formation for those implanted with CS, 95.28% for those embedded with CS-GO 0.5%, and 52.79% for those implanted with CS-GO 1%. A considerable difference arises between the scaffolds with GO and the ones that were made of CS alone. It is also notorious that CS with 0.5% GO was more effective in stimulating bone regeneration than the other two.

When comparing the results of the three groups in relation to the area of newly formed bone, statistically significant differences were found at 0.1, with a value very close to 0.05 ($p = 0.05090$) and with statistically significant differences in the post-analysis with comparison between CS vs. CS-GO 0.5% ($p = 0.005$) and between CS-GO 0.5% vs. CS-GO 0.1% ($p = 0.028$).

We explain these results between defects grafted with CS versus those grafted with GO through a chitosan-GO synergistic effect. Regarding the difference between GO 0.5 and GO 1%, the higher concentration of the GO seems to encourage a greater inflammatory response, which delays the healing process.

The statistical results that show the 0.5% CS-GO formulation as the one with the highest new bone-formation area agree with the SEM images that present the samples of this formulation with a better level of development of the extracellular matrix, and with the elemental analyses (EDS) that show higher percentages of calcium and phosphorus in the sample (Figures 5–7).

The lack of homogeneity in the analysis of the samples is due to bone mineralization, which is not uniform and varies in each specific site. Besides that, the fact that the values obtained by EDS correspond to particular locations means a high number of areas of analysis was selected across the same sample.

When reviewing the values, it is observed that the highest amounts of Ca and P were obtained for the sample of CS-GO 0.5%, indicating a more advanced mineralization process (bone formation).

Currently, the combination of CS and GO is considered one of the most promising nanocomposites in tissue engineering, due to the synergistic effect between CS (a material with recognized biocompatibility) and the excellent mechanical properties of GO [16], which would explain the superiority in the biocompatibility, mechanical, and thermal stability of the CS-GO nanocomposites compared to scaffolds of CS only.

The hydrogen bonding interaction between the two compounds has allowed a better resistance material with different applications today, with the ability to positively modulate cell activity and cell regeneration [1].

It is proposed that GO beside promoting the adhesion and proliferation of osteoblasts, thanks to its high conductivity, can electrically stimulate osteoblasts during matrix formation [16,17]. For example, in a previous study conducted by Lee et al. (2011) [18], it was found that GO promoted stem cell differentiation. This observation was also pointed by Ming et al. in 2014 [19] and Dubey et al. (2015), highlighting graphene's ability to stimulate osteoblastic differentiation [20].

Concerning the presence of collagen type I and III, there are no statistical differences between the three formulations, with the 0.5% CS-GO formulation having the highest percentages. Also, there are no differences in the portions of the two collagens. The presence of the two collagens in such similar rates indicates the presence of a young extracellular matrix, in a mineralization process.

In all cases of implantation, both CS and CS-GO are degraded by a mixed inflammatory infiltrate, surrounded by a fibrous capsule compatible with a chronic inflammatory response strong evidence of biocompatibility.

4. Experimental

4.1. Materials

Aspergillus niger mycelium was obtained from Sucroal (Cali, Colombia). All other reagents were obtained from Sigma-Aldrich (Palo Alto, CA, USA). CS was extracted and characterized previously (yield: 11%) [11]. GO was synthesized following Marciano. D. et al. methodology, obtaining 5 g of GO [21]. All the characterization was reported previously [11]. CS-GO scaffolds were prepared and characterized according to our previous report [11].

4.2. In Vivo Biodegradation

Scaffolds made of CS, CS with GO at 0.5% (CS-GO 0.5%), and CS with 1% GO (CS-GO 1%), were reduced to particles of irregular shape and size in a range of 200 to 600 μm and implanted in cranial defects of critical size in 16 Wistar adult male rats (5 mm diameter and 0.8 mm thickness). Biomodels were obtained from the Bioterio of the Universidad del Valle. As a control, four biomodels were left without implant (control by empty defect).

A group of twelve biomodels underwent histological analysis with hematoxylin-eosin (HE) and histochemistry (Masson trichromacy and Gomori trichromacy); the second group consisted of four biomodels which were analyzed by SEM and elemental analysis by dispersive energy spectroscopy (EDS).

After three months of implantation, euthanasia was performed with excess isoflurane®, and samples were recovered. The samples for immunohistochemistry technique were fixed in buffered formalin, dehydrated in alcohol solutions of ascending concentration (70%, 80%, 95%, and 100%), diaffinized with xylol and infiltrated with paraffin for later cutting at 5 μm using a Thermo Scientific™ Histoplast Paraffin™ and a Autotechnicon Tissue Processor™ (Leica Microsystems, Mannheim, Germany). The samples were processed for histological analysis by hematoxylin and eosin and Masson trichromacy techniques. For the analysis of the images, a Leica DM 750 microscope with a Leica DFC 295 camera and Leica Application Suite version 4.12.0 (Leica Microsystems, Mannheim, Germany) imaging software was used.

The samples for SEM/EDS were dehydrated in ascending alcohols and analyzed through scanning electron microscope (JEOL JSM-6490LA, Musashino, Tokyo, Japan). Each sample was coated with a copper bath.

This research was reviewed, endorsed, and supervised by the Institutional Ethics Review Committee with experimental animals of the Universidad del Valle (CEAS 001-016).

4.3. Statistical Design

4× histological images were used to perform a histomorphometrical study to determine the formation of bone tissue in the defects created. The Image J program 1.3, NIH, was used to analyze the images.

The statistical analysis consisted of calculating measures of central tendency and dispersion for the variables of area of bone formation in percentage and presence of collagen type I and type III, for each of the intervention groups: CS (CS), CS plus 0.5% GO (CS-GO 0.5%), and CS plus 0.1% GO (CS-GO 0.1%).

Assumptions of normality were verified by the Shapiro–Wilk test and equality of variances with the Levene test. The contrast between groups to determine differences between distributions was performed using the nonparametric Kruskal Wallis test, and Bonferroni's post-test was used in case of statistical significance. The level of confidence was set at 95% and of significance at 0.1 and 0.05. All estimates were conducted in STATA IC 15 (StataCorp LLC).

5. Conclusions

This study shows how materials based on chitosan and chitosan/graphene oxide are biocompatible, and its elimination in a living organism occurs by a foreign body reaction process where the content is fragmented and phagocytosed by the cells. Bone formation was simultaneous with the absorption of the scaffold material.

The CS-GO 0.5% formulation showed significant differences in terms of the ability to stimulate bone regeneration in critical size defects, maintaining adequate levels of biocompatibility, which allows to propose it as an election material in tissue engineering.

Implanted samples of Chitosan and graphene oxide 0.5% presented an advanced extracellular matrix development and with higher percentages of phosphorus and calcium, essential components of the extracellular bone matrix in the mineralization process.

The collagen distribution was similar for type I and II collagen, which indicated the presence of new bone tissue, (Woven bone). All these results show that the elaboration of scaffolds of chitosan reinforced with 0.5% graphene oxide is a good alternative in the regeneration of bone defects in critical size defects.

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ANEXO G: Ruiz, S.; Tamayo, A.J.; Delgado Ospina, J.; Navia Porras, P.D.; **Valencia Zapata, M.E.**; Mina Hernandez, H.J.; Valencia, H.C.; Zuluaga, F.; Grande Tovar, D.C. *Antimicrobial Films Based on Nanocomposites of Chitosan/Poly(vinyl alcohol)/Graphene Oxide for Biomedical Applications. Biomolecules.* **2019**, 9.

Article

Antimicrobial Films Based on Nanocomposites of Chitosan/Poly(vinyl alcohol)/Graphene Oxide for Biomedical Applications

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Abstract: Today, tissue regeneration is one of the greatest challenges in the field of medicine, since it represents hope after accidents or illnesses. Tissue engineering is the science based on improving or restoring tissues and organs. In this work, five formulations of chitosan/poly(vinyl alcohol)/graphene oxide (CS/PVA/GO) nanocomposites were studied for the development of biodegradable films with potential biomedical applications. The characterization of the films consisted of Fourier-transform infrared (FTIR) spectroscopy, scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS). The antibacterial activity was evaluated in vitro against Gram-positive bacteria *Bacillus cereus* and *Staphylococcus aureus* and Gram-negative *Salmonella* spp. and *Escherichia coli*, by contact of the film above inoculum bacterial in Müeller–Hinton agar. On the other hand, in vivo tests in which the material implanted in the subcutaneous tissue of Wistar rats demonstrated that the formulation CS/PVA/GO (14.25:85:0.75) was the best antibacterial film with adequate degradation in vivo. All together, these results indicate the potential of the films using nanocomposites of CS/PVA/GO in tissue engineering and cell regeneration.

Keywords: biodegradable films; chitosan; graphene oxide; poly(vinyl alcohol); tissue engineering

1. Introduction

Chitosan (CS) is a natural biopolymer derived from the deacetylation of chitin obtained from crustacean shells which can be processed to form films, fibers, beads, and dust [1,2]. Chitosan has become an attractive polymer for biomedical applications due to its antimicrobial properties and biocompatibility [3], with applications in tissue engineering of bone, cartilage, liver, tendons, ligaments and nerves, in wound dressings, separation membranes, blood anticoagulants, contact lenses, controlled release of drugs, as a fat-sequestering agent, in hydrogels and as food packaging material [4–7]. However, the high amount of hydrogen bonds of the amino and hydroxyl groups in

the CS has created some inherent disadvantages, such as low mechanical properties, poor solubility in conventional solvents as well as poor stability in physiological media [8–11].

Many studies are addressing the physicochemical attributes of hybrid polymers (natural and synthetic origin), thanks to their effectiveness in overcoming the disadvantages of the naturally occurring polymer [12]. Among the fillers used are ceramic nanoparticles [13], oxides [14], and cross-linking agents [15], among other materials. However, polyvinyl alcohol/chitosan (PVA/CS) compounds are traditionally used to prepare films with improved properties, because PVA is biocompatible, which allows its use in clinical applications, membrane fabrication and tissue repair [16].

Thanks to its high mechanical resistance, chemical stability, large surface area, and low toxicity [17,18], in recent years graphene oxide (GO) has had rapid growth in biomedical applications [19]. The presence of polar groups on the surface of this material improves compatibility with polymer matrices, such as CS [20]. Also, due to its antibacterial properties and its high surface/volume ratio, the GO is a promising material for the development of antimicrobial and antiviral surfaces [21].

Although several studies on CS/PVA/GO films are in the literature, there is a lack of works where the characterization includes the physical, chemical, and mechanical evaluation of the films, together with biological tests *in vitro* and *in vivo* conditions. Therefore, this research aimed to study biodegradable films from PVA/CS formulations reinforced with GO nanofilms grouping all this characterization.

2. Materials and Methods

2.1. Materials

For GO synthesis, graphite flakes (99.8%) were used (Alfa Aesar, Tewksbury, MA, USA). Concentrated sulfuric acid (H_2SO_4), potassium permanganate (KMnO_4), hydrogen peroxide (H_2O_2) and isopropanol were supplied from Merck (Burlington, MA, USA). For the production of the films, CS of low molecular weight (144,000 g/mol) and a deacetylation degree between 89% and 90%, PVA with hydrolysis between 87% and 89% and viscous molecular weight of 93,000 g/mol (Sigma-Aldrich, Palo Alto, CA, USA). Glacial acetic acid was purchased from Merck. For the elaboration of the simulated biological fluid, NaCl, $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, CaCl_2 , Na_2SO_4 , tris-(hydroxymethyl aminomethane) [$(\text{CH}_2\text{OH})_3\text{CNH}_2$] were acquired from Sigma-Aldrich; NaHCO_3 , KCl, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ from Fisher Chemical (Pittsburgh, PA, USA) and hydrochloric acid (HCl) from Merck. All reagents used were analytical grade.

2.2. Methods

2.2.1. Graphene Oxide Synthesis

Graphene oxide synthesis followed the methodology used by Mangadlao et al. [22]. In a round flask, 3 g of graphite were mixed with 400 mL of concentrated H_2SO_4 , after 10 min of stirring, 3 g of KMnO_4 were added. Subsequently, 3 g of KMnO_4 were added every 24 h for three days. The reaction was stopped at four days, for which a third of the contents of the balloon was mixed in 300 mL of a water/ice mixture and 2 mL of H_2O_2 were added. The solution was distributed in 50 mL flasks and washed in a UNIVERSAL 320R centrifuge (Hettich, Tuttlingen, Germany) for 10 min at 5000 rpm with Milli-Q water and isopropanol until reaching a neutral pH. Subsequently, it was purified by dialysis in 3.5K MWCO SnakeSkin tubes (Thermo Scientific, Waltham, MA, USA), followed by Milli-Q water washing and drying at -51°C and 0.12 mBar pressure in a Freezone 4.5 freeze dryer (LABCONCO, Kansas City, MO, USA) for 48 h. The graphite oxide was dispersed in water for 24 h in a Branson 5800 ultrasound equipment (Branson, Madrid, Spain) to finally obtain the GO.

2.2.2. Graphene Oxide Characterization

The GO and the starting graphite were chemically characterized by Fourier-transform infrared (FTIR) spectroscopy on an IR Affinity-1 infrared spectrophotometer (Shimadzu, Kyoto, Japan) in a

range of 500–4000 cm^{−1} in transmittance mode using the KBr pellet method. The X-ray spectra were taken on a Panalytical X'PERT PRO diffractometer (Malvern, Royston, UK) using Cu Kα1 radiation (1.540598 Å) and Kα2 (1.544426 Å), with an electron accelerator voltage of 45 kV, an electron-generating current of 40 mA, an optical grid of incident beam 1° and 1/2° and a diffracted beam grid of 9.1 mm, in a range 2θ between 5° and 40°. We used the Bragg law (Equation (1)) to determine the distance between two planes of the GO network:

$$d = \frac{\lambda}{2\sin\theta} \quad (1)$$

where d is the lattice spacing, λ is the X-ray wavelength and θ is the angle of incidence.

The characteristic bands of GO and graphite were acquired by Raman spectroscopy in a Thermo Scientific X-ray diffraction (XRD) equipment (Thermo Scientific), with an excitation source of 512 nm at room temperature.

2.2.3. Film Preparation

The films were prepared according to the film casting method described by Liu et al. [23], which consisted of clearing out the solutions described in Table 1, in acetate molds and subsequently, letting cure for 24 h in the environment, and then 24 h in the oven to a temperature between 38 and 40 °C, thus obtaining the solid film of CS/PVA/GO. Once collected, the test specimens were cut and conditioned for the tensile test according to ASTM D6287 and ASTM D618 standards with the dimensions specified in ASTM D882. The thickness of each specimen was determined using a Mitutoyo digital micrometer No. 293-330 (Kawasaki, Japan), from three average values along each sample. The samples were placed in a desiccator at 10% relative humidity (RH) until the time of the test.

For the preparation of the solutions, CS was dissolved in a 2% (w/v) acetic acid solution for 12 h at 80 rpm. The PVA was dissolved in distilled water for 3.5 h at 80 °C and 300 rpm, and finally, the GO was dispersed in distilled water using an ultrasonic bath (Branson) for 24 h. The formulations used in this research are in Table 1.

Table 1. Formulations (F1–5) used for the preparation of nanocomposite solutions.

Component	F1	F2	F3	F4	F5
CS (wt %)	20	14.75	19.5	14.25	29
PVA (wt%)	80	85	80	85	70
GO (wt %)	0	0.25	0.5	0.75	1

CS: Chitosan; GO: Graphene oxide; PVA: Poly(vinyl alcohol).

After the solutions were prepared separately, CS and PVA were mixed by stirring at 90 rpm for 2 h until a homogeneous solution was obtained which was filtered and finally, the GO dispersion was added with stirring until a homogeneous solution was obtained.

2.2.4. Film Characterization

Fourier-Transform Infrared Spectroscopy

The chemical identification of the films was carried out using FTIR in the attenuated total reflectance (ATR) mode (Shimadzu).

Scanning Electron Microscopy

The morphological inspection of the surfaces of the film was carried out by scanning electron microscopy (SEM) (JEOL JSM-6490LA, Musashino, Tokyo, Japan). The working conditions were 20 kV and mode of secondary backscattered electrons. All the samples were coated with gold, to create an electronic density in the material, since the polymers lack it.

The Tensile Strength of Films

For the tensile test, a universal SHIMADZU EZ-LZ test machine (Shimadzu) was used, following the ASTM D882 standard. Where at least five samples per formulation are required, the gap between jaws was 100 mm, the width of the film was 20 mm, and the test speed was 50 mm/min.

Degradation in Simulated Biological Fluid

The hydrolytic degradation was carried out following the procedure outlined in the ASTM F1635-16 standard. The films were immersed in a simulated biological fluid (SBF) at 37 °C for seven days in a Memmert IN 110 incubator (Memmert GmbH & Co. KG, Schwabach, Germany). The SBF was prepared according to the method proposed by Kokubo and Takadama [24] and the degradation was evaluated by examining the weight of the films before and after immersion for different periods (one, three, five, and seven days).

The initial weight of the samples before immersion was recorded as W_0 and the weight after drying for 48 h in the incubator at 37 °C was recorded as W_d . The weight loss (% W_l) was calculated according to Equation (2):

$$W_l (\%) = \frac{W_0 - W_d}{W_0} \times 100 \quad (2)$$

Each sample was immersed in 15 mL of SBF, and in each period three samples were evaluated per formulation. The pH of the SBF was measured every day until the total test time was completed using an Fisherbrand™ accumet™ AB150 pHmeter (Fischer Scientific, Inc., Ottawa, Canada). The morphology of the films after drying was studied using SEM.

Antimicrobial Film Assay

The antimicrobial activity of the films was evaluated against bacteria *Bacillus cereus*, *Staphylococcus aureus*, *Salmonella* spp., and *Escherichia coli*, by contact of the film above inoculum bacterial in agar. In short, the inoculum of each of the bacteria was carried out by adding 40 µL of dilution to 10⁻⁶ colony forming units (CFU) of each of the 24 h growth strains in 90 mm diameter Petri dishes containing 20 mL of Mueller–Hinton agar. Subsequently, a disc with 10 mm of diameter of each of the prepared films was placed in the center of the box on the inoculated agar. The plates with the strains were incubated at 37 °C, and the reading of the results was carried out 24 h after the inoculation. The inhibition was determined as the absence of growth observed between the transparent film and the previously inoculated agar. The test was repeated three times for each of the treatments.

Biomodel Tests In Vivo

The inflammatory response to the implantation of films in the subcutaneous tissue. Samples of CS/PVA films with different percentages of GO (three replicates per formulation) with 10 mm in diameter and 2 mm in thickness were implanted in subdermal tissue of three adult Wistar rats, in preparations made on the dorsal surface, according to the recommendation of ISO 10993-6 and as reported previously [25]. As a control sample, commercial porcine collagen BioMend® (ZIMMER BIOMET, Miami, FL, USA) with the same dimensions was used. All the biomodels were supplied by the Bioterio of the Faculty of Medical Sciences of the Universidad del Valle. The procedures carried out were approved by the Animal Ethics Committee of Universidad del Valle by the CEAS 001-016 certificate.

After 30 days of implantation, the samples were recovered, fixed in buffered formalin, dehydrated in alcohol solutions of ascending concentration (70%, 80%, 95%, and 100%), diaffinized with xylol and infiltrated with paraffin for later cutting at 4 µm using a Thermo Scientific™ Histoplast Paraffin™ and an Autotechnicon Tissue Processor™ (Leica Microsystems, Mannheim, Germany). The samples were processed for histological analysis by hematoxylin and eosin and Masson trichromacy

techniques. For the analysis of the images, a Leica DM 750 microscope with a Leica DFC 295 camera and Leica Application Suite version 4.12.0 (Leica Microsystems, Mannheim, Germany) imaging software was used. The macroscopic images were taken with a Samsung DualView DV150F 16 MP digital camera.

3. Results and Discussion

3.1. Graphene Oxide Characterization

3.1.1. Fourier-Transform Infrared Spectroscopy

Figure 1 shows the FTIR spectrum of graphite and graphene oxide. For the graphite, it exhibits few absorption signals, due to the difference in the state of charges between carbon atoms. This weak difference leads to a very small induced electric dipole, which provides a very clean spectrum [26]. It is clear that when an oxidation treatment is used on the material, bands corresponding to oxygenated functional groups appear, which indicates that the oxidative process was successful. The graphene oxide spectrum showed the band at 3389 cm^{-1} , which corresponds to the stretching vibration of the hydroxyl groups (O–H). The band at 2917 cm^{-1} corresponds to the vibration of the C–H bond that is in sp^3 hybridization. The band at 1726 cm^{-1} corresponds to the stretching vibration of the carbonyl group (C=O). The band at 1624 cm^{-1} corresponds to the stretch mode of the sp^2 carbon skeletal network (C=C). It is also evident, according to some results reported in the literature, that the GO obtained contains several oxygen functional groups, such as hydroxyl, carboxyl, and epoxy groups, which correspond to the bands presented at 1417 , 1226 , 1048 , 987 , and 671 cm^{-1} [25,27,28].

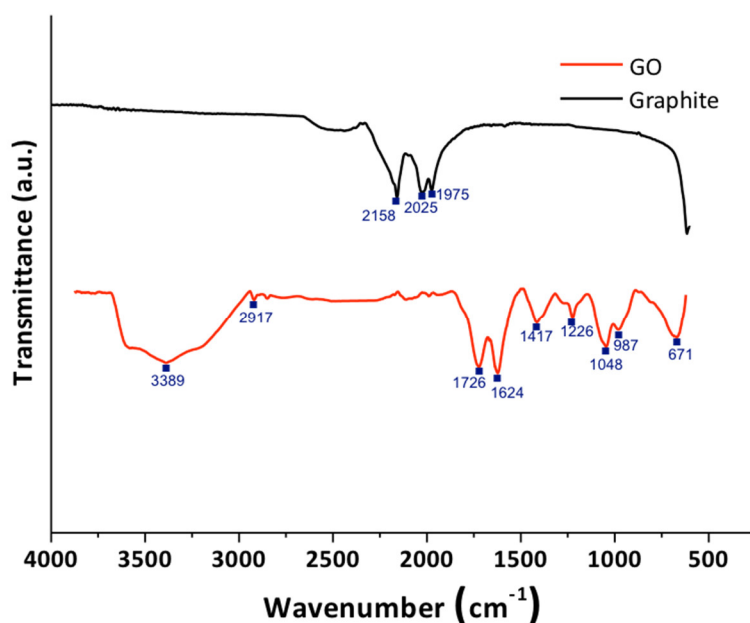


Figure 1. Fourier-transform infrared (FTIR) spectroscopy of graphite (black line) and graphene oxide (GO; red line). a.u.: Arbitrary units.

3.1.2. X-Ray Diffraction

Figure 2 shows the X-ray diffraction (XRD) pattern of graphite and GO. The graphite exhibits a peak at $2\theta = 26.46^\circ$ which corresponds to the reflection of the plane (002), while for the GO the peak appears at $2\theta = 10.77^\circ$ and corresponds to the reflection of the plane (001) [29–33]. The increase in the interplanar distance of the GO in comparison with the graphite is shown in Table 2 and due to the presence of functional oxygen groups introduced by the oxidation of the graphite, which facilitate

the exfoliation and hydration of the nanofilm of GO in an aqueous solution [30]. This increase in interplanar distance has been widely reported. The characteristic spacing of the GO generally ranges between 0.7 and 0.8 nm but may vary slightly at higher or lower values, depending on the degree of functionalization [30,34,35].

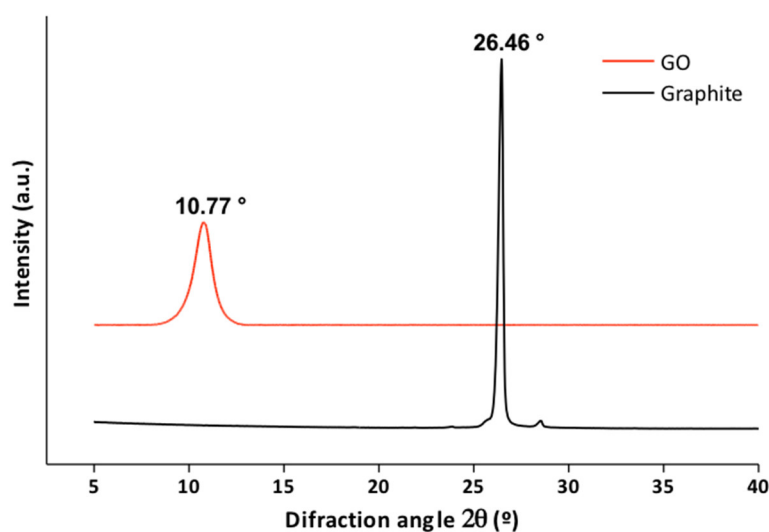


Figure 2. X-ray diffraction (XRD) of graphite (black line) and graphene oxide (GO; red line).

Table 2. Interplanar distance of graphite and GO.

Material	Diffraction Peak (°)	Interlayer Distance (nm)
Graphite	26.46	0.336
GO	10.77	0.82

GO: Graphene oxide.

3.1.3. Raman Spectroscopy

According to the Raman spectrum for the GO shown in Figure 3, the G peak attributed to the stretching of the bond of the carbon pairs in sp^2 hybridization, and the peak D associated with the breathing mode of the sp^2 carbon rings and activated by defects caused in the graphite network due to the presence of oxygen-rich functional groups [36]. The relationship between the intensities of bands D and G is directly related to the degree of oxidation of the GO sheets [37].

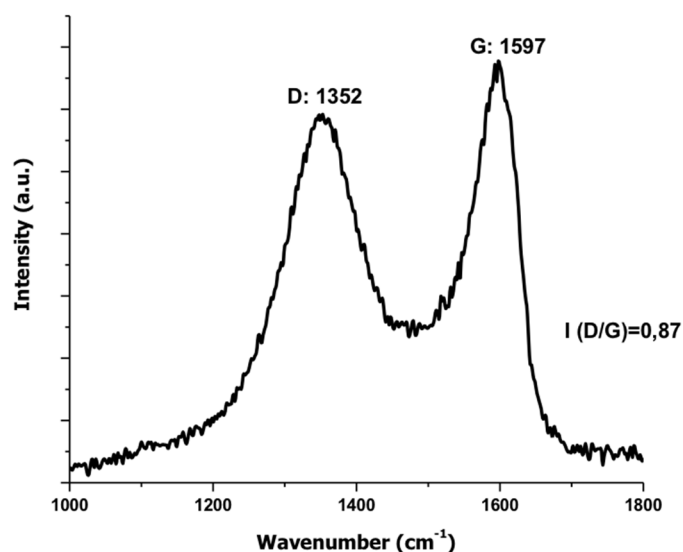


Figure 3. Raman spectrum of graphene oxide.

3.2. Film Characterization

3.2.1. Fourier-Transform Infrared Spectroscopy

In Figure 4 it is observed that an absorption band appears at 1701 cm^{-1} related to the stretching vibration of C=O carboxyl groups at the GO edges. The band of vibration associated with the –OH group (1328 cm^{-1}) becomes more pronounced with the increase of the GO content due to the destruction of the original hydrogen bonds of CS/PVA compounds and the formation of a strong interaction between CS, PVA, and GO. The C–OH bond, observed at 1382 cm^{-1} , is weakened due to the increase in GO due to the strong hydrogen bond. Besides, the band at 3251 cm^{-1} (stretching vibration of the –OH group) was displaced to higher values (3288 cm^{-1}) and was further extended, due to the interaction of the GO with the CS/PVA mixture by hydrogen bonds [35].

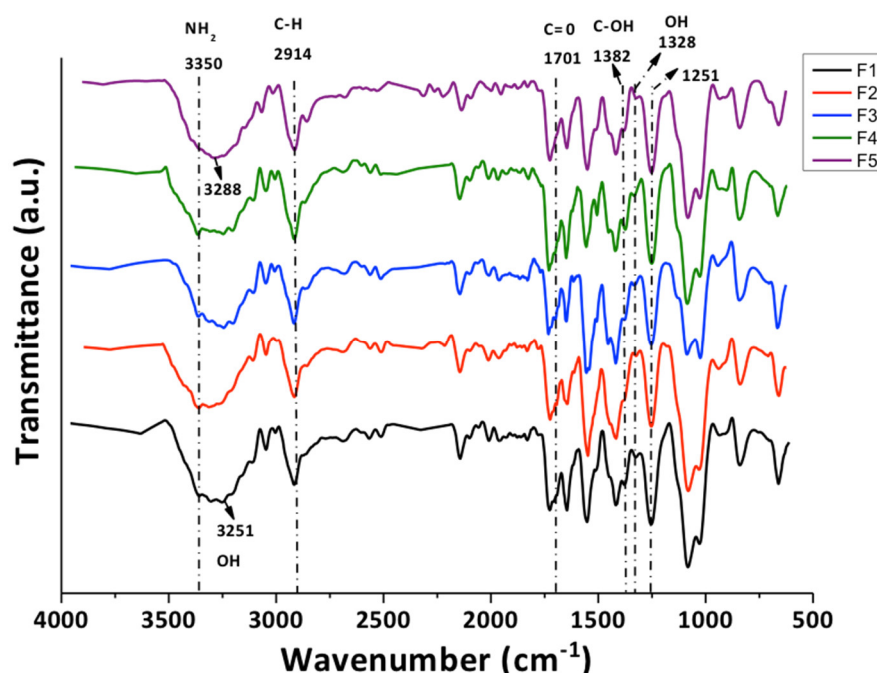


Figure 4. Attenuated total reflectance Fourier-transform infrared (ATR-FTIR) spectroscopy of chitosan/poly(vinyl alcohol)/graphene oxide (CS/PVA/GO) films for the five formulations. F1: CS/PVA/GO 20:80:0; F2: CS/PVA/GO 14.75:85:0.25; F3: CS/PVA/GO 19.5:80:0.5; F4: CS/PVA/GO 14.25:85:0.75 and F5: CS/PVA/GO 29:70:1.

3.2.2. Scanning Electron Microscopy

As shown in the SEM micrographs (Figure 5), an increase in the roughness of the film surface is evidenced in the different formulations, the morphology of the films changes from a smooth structure for formulation F1 (F1; Figure 5A), to an utterly rough morphology for formulation F5 (F5; Figure 5E). Previous studies have shown that the surface of pure CS and PVA films separately is smooth, continuous and compact [16,38], so it is expected that the mixture between them will also present a surface with the same conditions, which is consistent with the results found, because the PVA offers good compatibility when incorporated into polysaccharides such as CS. By adding GO to the CS/PVA mixture, the GO nanosheets generate an increase in the roughness of the films due to their texture [35]. There is also a specific porosity which is possibly associated with the rapid evaporation of the solvent during the curing of the films, air bubbles or separation of polysaccharide molecules during the curing process of the films [16].

In general, CS/PVA films were transparent, which can be explained by the inherent property of PVA and the amorphous nature of chitosan [35]. With GO content increasing, there was an increase in the intensity of the black tone and consequently an increase in the opacity of the film [39].

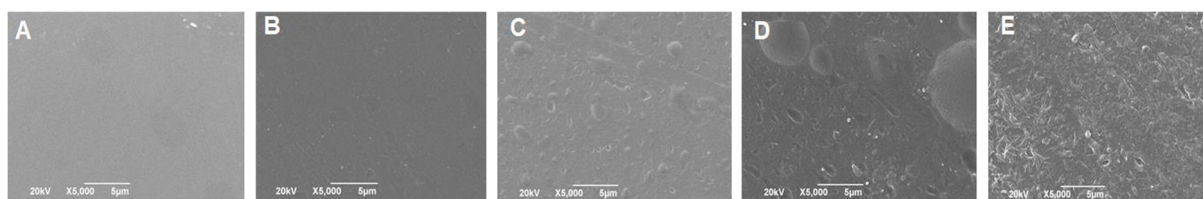


Figure 5. Morphology of CS/PVA/GO films. (A) F1: CS/PVA/GO 20:80:0; (B) F2: CS/PVA/GO 14.75:85:0.25; (C) F3: CS/PVA/GO 19.5:80:0.5; (D) F4: CS/PVA/GO 14.25:85:0.75; (E) F5: CS/PVA/GO 29:70:1.

3.2.3. The Tensile Strength of the Films

As shown in Figure 6, the tensile strength is affected by the fraction of each component. It is evident that the presence of GO in percentages lower than 1 wt % improves the traction properties of the films. It is evident that the incorporation of high fractions of PVA/CS, as well as small portions of GO, increases the tensile strength of the films. This reinforcing effect is attributed to the good GO sheets dispersion in the PVA/CS matrix, acting as a nanocharge that absorbs a good part of the force applied to the film. Also, it is due to the union between the amino groups of the CS and the carboxyl groups on the surface of the GO through covalent bonds and hydrogen bonds, thus contributing to the reinforcement of the films due to the formation of these molecular bonds [40].

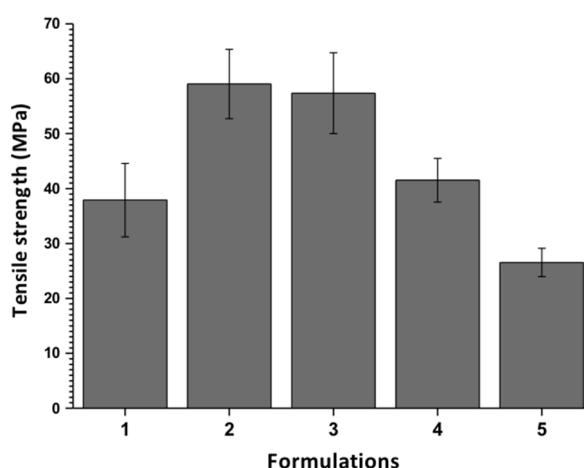


Figure 6. The tensile strength of CS/PVA/GO films. Data are shown as mean $\pm$ standard deviation of the mean ($n = 5$).

3.2.4. Degradation in a Simulated Biological Fluid

Weight Loss

Figure 7 shows the results of the degradation percentage (weight loss) of the films with different proportions of GO during the seven days of immersion in SBF. The incorporation of GO in the polymeric matrix increased the stability of the films against SBF since the percentage of weight loss after seven days of immersion decreased from 86.95% for formulation F1 that did not contain GO up to 33% for the formulation F5 comprising 1% GO. This behavior is because the GO in the CS/PVA binary mixture gives the material a higher number of hydrogen bonds between the CS and the GO, which provide the material with high chemical stability in the polymer chains of the CS [41].

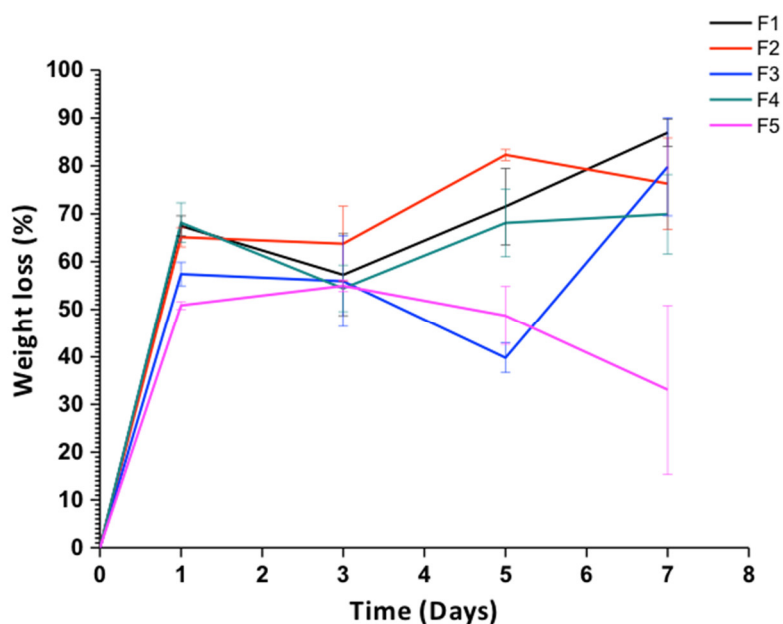


Figure 7. Weight loss vs. time. F1: CS/PVA/GO 20:80:0; F2: CS/PVA/GO 14.75:85:0.25; F3: CS/PVA/GO 19.5:80:0.5; F4: CS/PVA/GO 14.25:85:0.75 and F5: CS/PVA/GO 29:70:1. Data are shown as mean $\pm$ standard deviation of the mean ($n = 3$).

pH Changes

Figure 8 shows the pH changes in the SBF during the immersion period of the samples. The decrease observed in the pH when increasing the time of immersion of the samples in the SBF is associated with the attack to the amorphous zones of the polymers because they retain more acidic species and because they have greater susceptibility to degradation according to the research by Figueira-Maldonado et al. [42]. Besides, the detachment of the degradation by-products typical of CS such as glucosamine and *N*-acetylglucosamine contribute to said pH reduction, so that the formulations with higher CS content (formulations F1 and F5) show more significant reductions in the pH values. These by-products of CS degradation are found in the extracellular matrix of human tissue, making them harmless when released into the body [43]. The pH of the biological media is a fundamental constant for the maintenance of the vital processes since the enzymatic action, and the chemical transformations of the cells are carried out within a pH range between 6 and 8 [44–46]. All the formulations studied showed pH variations within the permissible range in the human body to promote life and maintenance of vital functions.

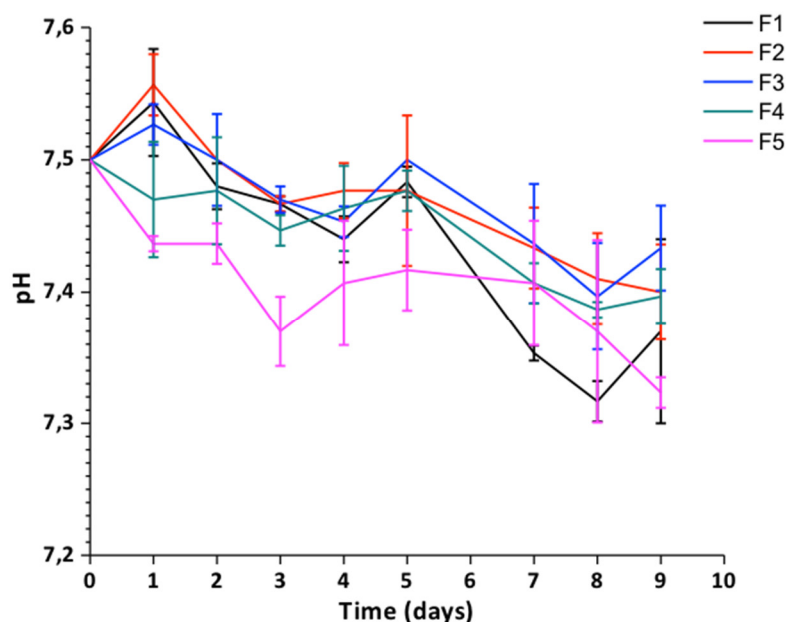


Figure 8. Study of the pH solution vs. immersion time in simulated body fluid (SBF) of the films. F1: CS/PVA/GO 20:80:0; F2: CS/PVA/GO 14.75:85:0.25; F3: CS/PVA/GO 19.5:80:0.5; F4: CS/PVA/GO 14.25:85:0.75 and F5: CS/PVA/GO 29:70:1. Data are shown as mean $\pm$ standard deviation of the mean ($n = 3$).

Scanning Electron Microscopy of the Films after Immersion in Simulated Body Fluid

Figure 9 shows SEM images of the CS/PVA/GO films after their immersion in SBF. The highly rough texture is due to the degradation process of the nanocomposite, which increases with GO content. Calcium phosphate deposition of a layer on the surface films can also be observed, which could be determined by energy dispersive spectroscopy (EDS) with a calcium composition of 36.70% and 16.4% phosphorus for formulation F2 (Supplementary Table S1). The deposition of said apatite layer on the surface (red arrows in Figure 9B) is suitable for a bioactive material since it promotes the interaction between the material and the surrounding tissue [47].

The morphological structure of the deposited layer presents typical morphological characteristics of apatite structures (globular structures), similar to those reported by different authors for materials with bioactive traits [48]. The morphology of the films before and after the immersion in SBF can be seen in Figures 5 and 9, respectively, where an evident difference in the texture of the surface is noted.

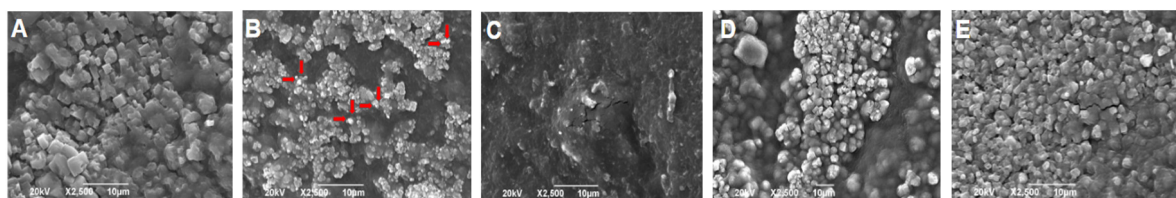


Figure 9. Morphology of CS / PVA / GO films after seven days in the degradation process in SBF. (A) F1: CS/PVA/GO 20:80:0; (B) F2: CS/PVA/GO 14.75:85:0.25; (C) F3: CS/PVA/GO 19.5:80:0.5; (D) F4: CS/PVA/GO 14.25:85:0.75; (E) F5: CS/PVA/GO 29:70:1. All the ratios are wt %.

3.2.5. Antibacterial Activity

As shown in Table 3, no inhibition was observed for films that did not contain GO. Although some authors have demonstrated that chitosan shows activity against Gram-positive and Gram-negative bacteria, due to the loss of intracellular constituents [49,50], our results agree with other

authors [51], which suggest that chitosan must be dissolved to observe antimicrobial effect, because PVA improves hydrogen bonds with chitosan, decreasing its solubility [52].

In the films with added GO, the results at 24 h of incubation showed that the addition contributes to completely inhibit the growth of the strains treated with 0.75% of GO and with a greater inhibition than 1.0%, an effect similar to what happens when essential oils are incorporated [53]. The strain with the least inhibition was *S. aureus*, which showed partial restraint at the concentration of 0.25% of GO, indicating that there is higher resistance by Gram-negative bacteria to inhibition due to CS/PVA effect. The GO effect suggests an interaction at the level of the cell membrane.

The dependence of the antibacterial activity on GO content, coincides with some investigations that show the GO has an inhibitory effect on the growth of Gram-positive bacteria, such as *S. aureus* [54,55], and Gram-negative, such as *E. coli* [54,56–59] and *Pseudomonas aeruginosa* [60], through damage to the cell membrane of bacteria when they come into contact with GO. Damage to the membrane can be caused by the sharp atomic edges of graphene, which can penetrate the cell membrane and physically disturb its integrity and also through lipid peroxidation induced by the oxidative nature of GO [61]. Oxidative stress is considered as an essential component of antimicrobial activity for bacterial cells exposed to GO [62].

Table 3. Inhibition of CS/PVA/GO films against bacterial strains.

Strain	F1	F2	F3	F4	F5
<i>Bacillus cereus</i>	---	++	++	+++	+++
<i>Staphylococcus aureus</i>	---	++	++	+++	+++
<i>Salmonella spp</i>	---	--	++	+++	+++
<i>Escherichia coli</i>	---	++	++	+++	+++

(+++): Complete inhibition of the pathogen; (++) Weak inhibition of the pathogen; (--) Weak pathogen growth; (---) Complete growth of the pathogen. F1: CS/PVA/GO 20:80:0; F2: CS/PVA/GO 14.75:85:0.25; F3: CS/PVA/GO 19.5:80:0.5; F4: CS/PVA/GO 14.25:85:0.75; F5: CS/PVA/GO 29:70:1. All the ratios are wt %.

3.2.6. Biomodel Tests In Vivo

After 30 days of implantation in the biomodels, the recovery of the samples was performed, and in all cases, repair of the surgical defect created was observed. All the biomodels presented hair recovery (Figure 10A) and the absence of injuries and infections in the intervened areas with normal healing of the tissue architecture (Figure 10B,C). The material is initially compatible, an inflammatory reaction to a foreign body is apparent where the cells surround the fragments with a fibrous capsule and the rest of the soft tissues with regular appearance.

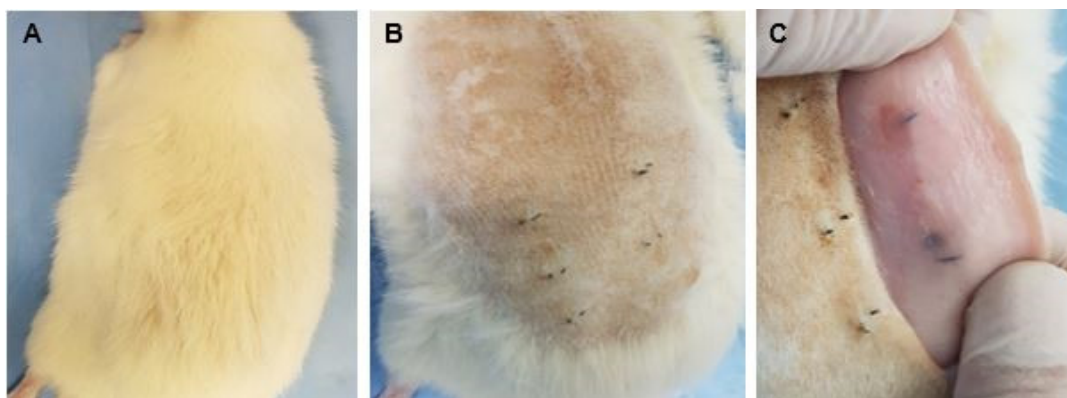


Figure 10. The dorsal area where the implantation was performed after 30 days. (A) Hair recovery; (B) absence of injuries and infections; (C) internal surface of the skin where the implanted samples encapsulated by scar tissue

Figures 11, 12, and 13 correspond to the histological analysis of the subcutaneous cellular tissue (SCT) of the biomodels (Wistar rats) with CS/PVA/GO films implanted. It is apparent that the control sample (porcine collagen) has been wholly reabsorbed and the healing is typical with a recovery of the tissue architecture (Figure 11A–C).

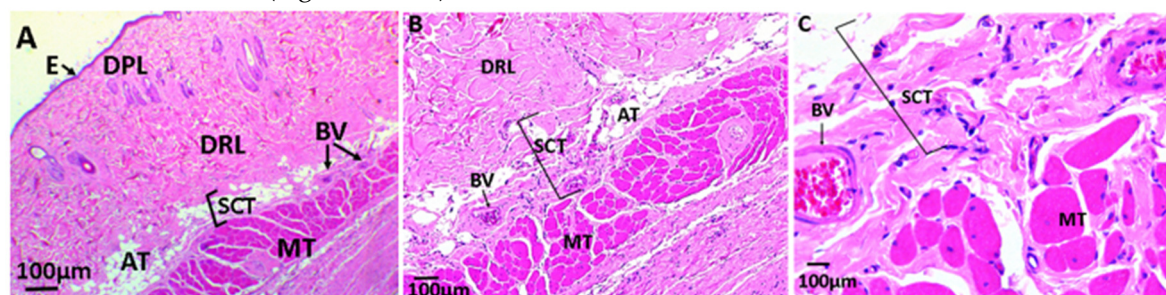


Figure 11. (A–C) Histological analysis of sample control. E: Epidermis; DPL: Dermis papillary layer; DRL: Dermis reticular Layer; BV: Blood vessels; AT: Adipose tissue; MT: Muscular tissue; SCT: Subcutaneous cellular tissue. (A) 4×; (B) 10×; (C) 40×.

In the experimental formulation F2 (CS/PVA/GO 14.75:85:0.25) and F3 (CS/PVA/GO 19.5:80:0.5), normal healing is observed, and small film pieces remain with the presence of inflammatory cells surrounding them (Figure 12).

The samples with GO content at 0.25 and 0.5% showed more significant evidence of degradation, and less inflammatory response, the presence of inflammatory infiltrate being greater in the sample of F3(19.5CS/80PVA/0.5GO) (Figure 12A–C).

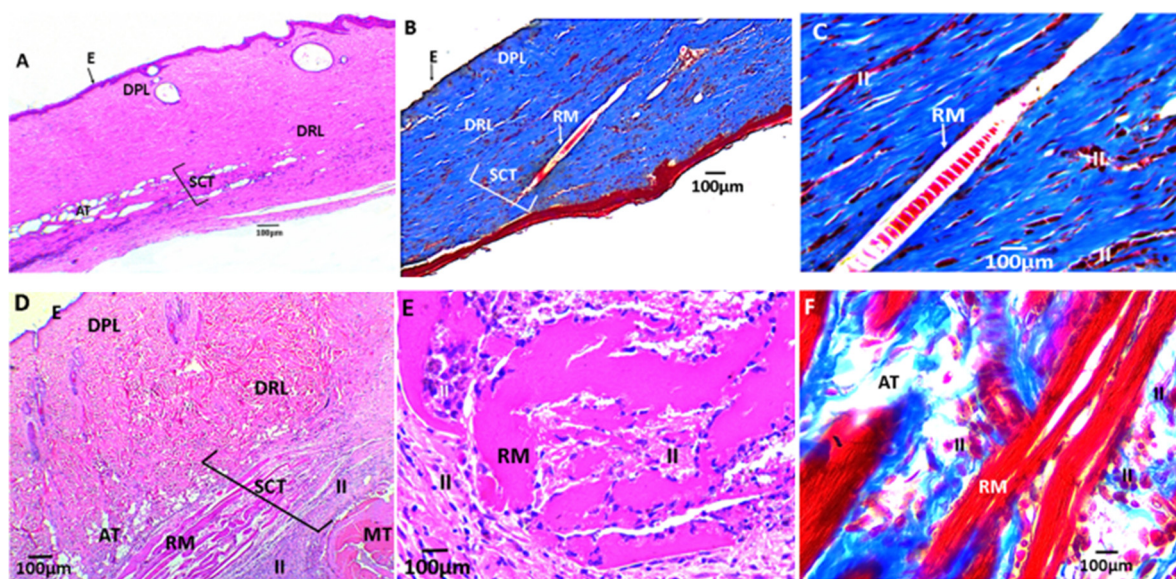


Figure 12. Histological analysis of experimental samples: (A–C) formulation F2 (CS/PVA/GO 14.75:85:0.25) and (D–F) formulation F3 (CS/PVA/GO 19.5:80:0.5). E: Epidermis; DPL: Dermis papillary layer; DRL: Dermis reticular layer; BV: Blood vessels; AT: Adipose tissue; H: Hypodermis; MT: Muscular tissue; SCT: Subcutaneous cellular tissue; RM: Remnant material; II: Inflammatory infiltrate. (A) 4×; (B) 10×; (C) 40×; (D) 4×; (E) 10×; (F) 40×.

The formulations with 0.75 wt % and 1 wt % GO present less evidence of degradation and a greater inflammatory infiltrate. In Figure 13A,B, two zones of degradation are observed: Z1 with poorly degraded films and Z2 with highly fragmented films that are in the process of degradation by inflammatory cells. Figure 13 C corresponds to zone Z1 at 40×.

Figure 13D–F correspond to films with a GO content of 1%. There is very little evidence of degradation, and at 40× the films surrounded by inflammatory cells are observed, starting the reabsorption at their edges.

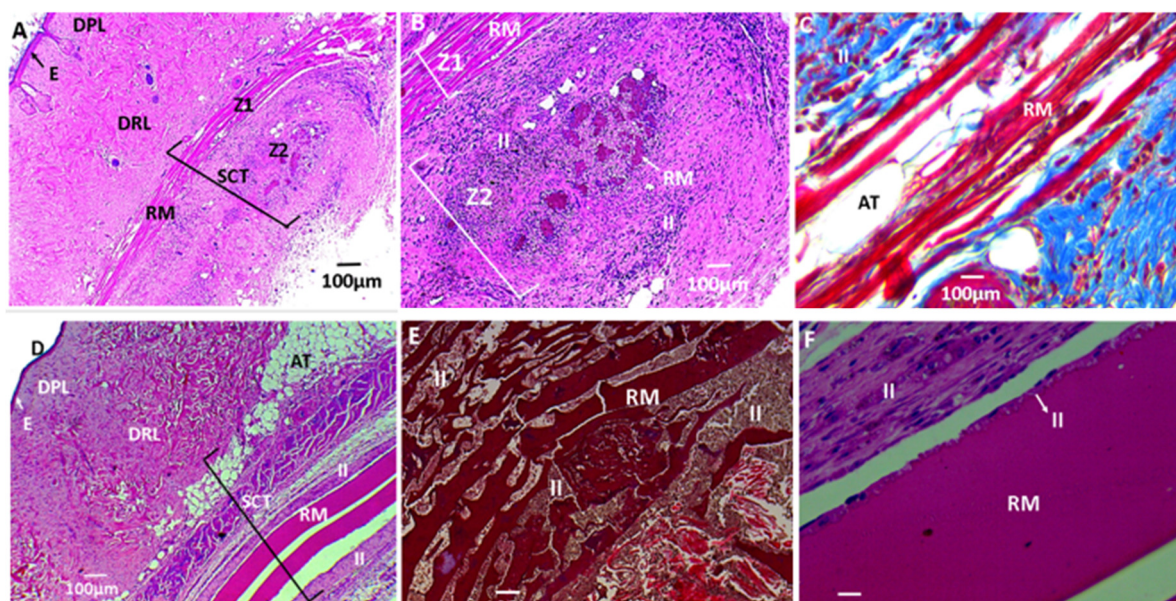


Figure 13. Histological analysis of experimental samples: (A–C) formulation F4 (CS/PVA/GO 14.25:85:0.75) and (D–F) formulation F5 (CS/PVA/GO 29:70:1). E: Epidermis; DPL: Dermis papillary layer; DRL: Dermis reticular layer; BV: Blood vessels; AT: Adipose tissue; H: Hypodermis; MT: Muscular tissue; SCT: Subcutaneous cellular tissue; RM: Remnant material; II: Inflammatory infiltrate; Z1: Zone 1; Z2: Zone 2. (A) 4×; (B) 10×; (C) 40×; (D) 4×; (E) 10×; (F) 40×.

The histological results show that the implanted materials are stimulating a typical foreign body reaction, which is the mechanism typically used by living beings to phagocytose subdermal implanted materials [63–66].

The degradation of the CS/PVA/GO films through fragmentation and phagocytosis is caused by cells belonging to an inflammatory infiltrate and their replacement with healthy tissue. Recovery of the standard architecture (Figures 12 and 13), is an indication of a biocompatible material that eventually will be degraded entirely/reabsorbed by a foreign body reaction, with moderate GO content dependence as previously reported [25,67,68].

4. Conclusions

Films based on CS/PVA/GO nanocomposites were obtained, which showed suitable properties from the mechanical, chemical, and biological point of view for their application in tissue engineering.

The addition of GO to CS/PVA films generated an increase in tensile strength, surface roughness, and degradation time in both SBF and implanted in the biomodels. Also, the antimicrobial activity of the films against the bacteria studied, showed an increase with the content of GO, mainly because the mechanisms of inhibition of the bacterial growth of the GO are generated by contact between the GO and the cell membrane of the bacteria.

After SBF films immersion, a light layer of calcium phosphate was generated on the surface of the material, which is usually related to a bioactive material.

The films with 0.75 wt % of GO were the best formulation with complete growth inhibition of the tested bacteria and advanced degradation in vivo with low foreign inflammatory response of the tissue, demonstrating its potential for application in films for tissue engineering.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Energy dispersive X-ray spectroscopy (EDS) results of the formulation F2.

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and J.D.; writing—review and editing, M.E.V., C.H.V., F.Z. and C.D.G.T.; supervision, F.Z.; funding acquisition, F.Z.

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Supplementary Materials: Antimicrobial Films Based on Nanocomposites of Chitosan/Poly(vinyl alcohol)/Graphene Oxide for Biomedical Applications

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Table S1. Energy dispersive X-ray spectroscopy (EDS) results of the Formulation F2.

Spectrum	In stats.	C (% atom)	O (% atom)	Na (% atom)	Mg (% atom)	P (% atom)	Cl (% atom)	Ca (% atom)	Total
1	Yes	6.32	43.49	2.55	0.74	16.32	0.94	29.65	100.00
2	Yes	15.70	41.21	4.74	-	9.46	10.53	18.36	100.00
3	Yes	10.62	39.46	5.53	-	13.89	3.05	27.44	100.00
4	Yes	8.19	40.11	1.72	0.67	15.66	2.94	30.71	100.00
5	Yes	7.14	35.69	1.29	0.64	16.41	2.12	36.70	100.00
6	Yes	33.38	11.64	13.02	-	-	41.19	0.77	100.00
7	Yes	-	45.29	10.08	-	11.07	11.40	22.16	100.00

ANEXO H: Valencia, C.; Valencia, C.H.; Zuluaga, F.; **Valencia, M.E.**; Mina, J.H.; Grande-Tovar, C.D. *Synthesis and Application of Scaffolds of Chitosan-Graphene Oxide by the Freeze-Drying Method for Tissue Regeneration. Molecules* **2018**, *23*, 2651.

Article

Synthesis and Application of Scaffolds of Chitosan-Graphene Oxide by the Freeze-Drying Method for Tissue Regeneration

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Abstract: Several biomaterials, including natural polymers, are used to increase cellular interactions as an effective way to treat bone injuries. Chitosan (CS) is one of the most studied biocompatible natural polymers. Graphene oxide (GO) is a carbon-based nanomaterial capable of imparting desired properties to the scaffolds. In the present study, CS and GO were used for scaffold preparation. CS was extracted from the mycelium of the fungus *Aspergillus niger*. On the other hand, GO was synthesized using an improved Hummers-Offemmann method and was characterized by Fourier transform infrared spectroscopy (FTIR), Raman spectroscopy, atomic force microscopy (AFM), X-ray diffraction (XRD), and dynamic light scattering (DLS). Subsequently, three formulations (GO 0%, 0.5%, and 1%) were used to prepare the scaffolds by the freeze-drying technique. The scaffolds were characterized by FTIR, thermogravimetric analysis (TGA), and scanning electron microscopy (SEM), to determine their thermal stability and pore size, demonstrating that their stability increased with the increase of GO amount. Finally, the scaffolds were implanted, recollected 30 days later, and studied with an optical microscope, which evidenced the recovery of the tissue architecture and excellent biocompatibility. Hence, these results strongly suggested the inherent nature of chitosan/graphene oxide (CS/GO) scaffolds for their application in bone tissue regeneration.

Keywords: chitosan; graphene oxide; freeze-drying method; scaffolds

1. Introduction

Nowadays, the “gold standard” for repairing of bone injuries included the use of either an autograft or an allograft; however, these have presented some drawbacks associated with limited supply of bone from the host, donor site pain, potential for donor site infection for an autograft, high cost, and the risk of viral and bacterial transmission for an allograft [1–3]. Bone tissue engineering as an effective way to treat bone injuries, has attracted widespread interest from researchers in recent years [4]. Several biomaterials, including natural and synthetic polymers, are used to study cellular interaction, proliferation, and differentiation [5]. Bone tissue recovery is parallel with biochemical

processes that occur at the molecular level and are reflected in cell development. Therefore, it is appropriate to encourage the growth of cells by creating new materials with scaffolds characteristics [6].

A scaffold is a support that allows cellular interactions, which contribute to the formation and repair of functional tissues [7]. Therefore, scaffolds act as supports to facilitate the migration, adhesion, and transport of bioactive cells or molecules in charge of regenerative processes [7]. The scaffolds must, therefore, be three-dimensional and porous structures that serve as a temporary cellular matrix to promote vascular and cellular growth, while the expected tissue regeneration takes place [8].

One of the most promising polymeric materials seems to be chitosan (CS), a deacetylated chitin-derived polysaccharide, reported to be a safe, hemostatic, biocompatible, osteoconductive promoter of the mineralized bone matrix [9], and provider of minimum inflammatory responses after implantation [9]. The similarity between chitosan and glycosaminoglycans structure, a component of the extracellular matrix, facilitates interaction with cells, promoting colonization and encouraging cell differentiation and maturation, [6]. However, the brittle nature and poor dimensional stability of the CS scaffold limits its application.

On the other hand, graphene oxide (GO) is a resistant two-dimensional structure molecule, with the thickness of a carbon atom, but with high strength due to the σ -type bonds and the resonance characteristics in the π -type bonds of the carbons with hybridization sp^2 . GO also presents high stability and properties of a low annular tension aromatic polycycle [10].

The GO exhibits a sp^3 hybridization that is related to the oxygenate groups in the vicinity and the central part of the sheet [11]. The oxygenated groups obtained in the GO by oxidation of graphene consist of hydroxyl (OH), epoxy (COC), and carboxyl (COOH), which gives the GO the possibility of hydrogen bonding to other molecules, such as CS, which in turn presents amine (NH_2) and hydroxyl (OH) groups [11].

Functional moieties such as hydroxyl and carboxyl groups, have been reported to form chemical bonds with CS in scaffold matrices, thereby improving the biological stability and interfacial strength of scaffolds [4,12,13]. Therefore, the introduction of GO into the CS compound should reinforce the mechanical and thermal properties of the scaffold [14].

In the literature, there are only a few reports on the formation of CS/GO composites with a well-organized structure for bone tissue engineering applications [15]. However, to the best of our knowledge, there are no reports including the synthesis and characterization of GO with an improved method, isolation of CS from the mycelia of *Aspergillus niger* and the implantation of the scaffolds in rats' skin, producing normal tissue recovery that evidence biocompatibility of the material.

2. Results and Discussion

2.1. CS Extraction from the Mycelium of *A. niger*

The basic hydrolysis of the vegetative body (mycelium) of the fungus *A. niger* allows the deacetylation of the chitin to prepare chitosan [16]. The extraction yield was 11.5% from 500 g of clean, dry mycelium. Although this yield is similar to those reported in the literature [17], it is low compared to the one obtained from crustaceans [18], since mycelium usually contain, in addition to chitin, different types of polysaccharides, proteins, and fatty acids [19].

Therefore, it is possible to generate CS with higher percentages if the extraction is performed from shells of crustaceans, since more than 60% of its content is chitin; however, there are some protein remnants left that may be allergenic, thus, it is preferred to use mycelium as a source of CS [20]. Moreover, shell extractions require pigment treatment with toxic solutions and enzymatic digestion that could increase the risk of contamination [18]. Mycelium, on the other hand, is a waste product of the industrial production of citric acid and is, therefore, a readily available raw material with lighter extraction conditions for the production of chitosan with lower environmental impact [18].

2.2. Deacetylation Degree and Characterization of CS by ^1H -NMR

Extraction hydrolysis was performed with 40% NaOH, a concentration that generates a degree of chitin deacetylation greater than 50%, without breaking bonds and reducing molecular weight drastically. The deacetylation degree (DD) = 55.7%, was calculated by the potentiometric method (see Supporting Information, Figure S1) using Equation (1) [21].

$$\text{NH}_2 \text{ (DD)} = 16.1(y - x) \times f/w \quad (1)$$

where (y) and (x) represent the second and first volume of equivalence respectively, f is the base concentration, and w is the weight of CS used for titration. This method is not completely accurate, because it allows to manually quantify the free protons in solution, whose concentration varies depending on the ion strength [21]. NMR, on the other side, is a more accurate technique, because it is based on the absorption of all the protons of a molecule subjected to a magnetic field [22].

This method was used for the integration of the signals at $\delta = 4.74$ and $\delta = 1.85$ ppm, corresponding to the amine and methyl hydrogen of the acetamide group of CS, respectively [22], according to Equation (2):

$$\text{DD} = [100 - (\text{IH(D)}/(\text{IH(A)} - \text{IH(D)}))](100) \quad (2)$$

where IH(D) corresponds to the proton integral in the amine of the deacetylated monomer and IH(A) corresponds to the proton in the methyl of the acetylated acetamide monomer. The calculation by this method gave a DD of 66.7%.

Finally, the ^1H -NMR technique was used for the characterization of CS. In the spectrum (Figure S2), a signal appears at $\delta = 3.69$ ppm for the anomeric proton of the deacetylated monomer and at $\delta = 3.48$ ppm another signal appears for the anomeric proton of the acetylated monomer; in addition, two more signs are observed at $\delta = 4.74$ and $\delta = 1.85$ ppm, corresponding to the amine and methyl hydrogen of the acetamide group of CS, respectively [22].

2.3. Characterization of CS by FTIR Spectroscopy

The IR spectrum (Figure S3) shows the characteristic bands of the CS [22], for example, the band at 3360 cm^{-1} with a shoulder of 3273 cm^{-1} corresponding to the O-H stretching vibration of the glucosamine overlapped by the N-H vibration of the amine; in addition, at 1552 cm^{-1} , the band due to the N-H stretching vibration of the primary amine of the deacetylated structure is observed. At 1639 cm^{-1} the band corresponding to C=O tension of the carbonyl of the acetylated monomer appears and finally, at 1076 cm^{-1} , the band corresponding to the tension vibration of the C-O-C bond of the anomeric carbons [22].

2.4. Molecular Weight Determination of CS

To obtain the molecular weight, a capillary viscometer and a gel permeation chromatography (GPC) were performed on chitosan as it forms viscous solutions, allowing the measurement of the average viscous molecular weight (M_v) [23]. For this purpose, the drop times of 25 mL solutions were measured with a Ubbelohde viscometer, prepared from a standard solution (see supporting information). All the concentrations of the samples are shown in Table S1. The densities of the solutions and the constant β of the viscometer (3.31×10^{-5}) were also calculated. From these data, the specific viscosity was calculated and plotted as a function of concentration (Figure S4).

The intrinsic viscosity is the intercept value of the specific viscosity curve and is symbolized by $[\eta]$. The Mark Houwink-Sakurada equation relates this viscosity to the constants k (1.81×10^{-3}), and α (0.93), previously established for these conditions [24].

$$M_v = ([\eta]/K)^{1/\alpha} \quad (3)$$

The M_v calculated for the CS was 18,715 g/mol, as expected, lower than that contained in crustaceans [25].

The United States Pharmacopeial Convention (USP) guide for the chromatographic measurement of CS was used for the GPC analysis [26].

The peak corresponding to the CS appears at 22.3 min and with the calibration curve (Figure S5), the number average molecular weight (M_n) calculated was 6481.87 g/mol. This value is lower than the M_v , since M_n represents the total weight of all the molecules presented in the polymer sample divided by the total number of moles, while the M_v is related to a smaller quantity of moles: only those of low molecular weight [23].

2.5. Synthesis of the GO

An improved Hummers-Offemmann method was used to obtain GO [27]. Typically, in the modified methods, solutions of $H_2SO_4:HNO_3$ are used, and reactions are not too exothermic. For that reason, the reaction can take 1–2 weeks [27]. Marcano et al. found that using a 9:1 $H_2SO_4:H_3PO_4$ solution not only improves reaction performance, but also decreases reaction times to 6–12 h [27]. The use of the acid mixture allows the $KMnO_4$ used for oxidation to react completely with the graphite in an exothermic reaction, and thus, increasing the performance and reaction speed. Any remaining of the oxidation is removed with H_2O_2 [27].

2.6. Characterization of the GO by FTIR Spectroscopy

The IR spectrum (Figure 1) shows the characteristic bands of the GO [28]. The band at 3363 cm^{-1} corresponds to the O-H bond tension vibration, which is complemented by the C-OH band at 1151 cm^{-1} due to the hydroxyl groups of the GO. At 1651 cm^{-1} the tension band C=C characteristic of the double bonds in the polycyclic aromatic graphene ring appears. At 1078 cm^{-1} the C-O-C band characteristic of epoxy appears. The C=O band is displaced at a lower frequency by the interactions of the intramolecular hydrogen bond, which causes overlapping with the C=C band. Moreover, the band is weakened because the carbonyl groups are only around the sheet [28].

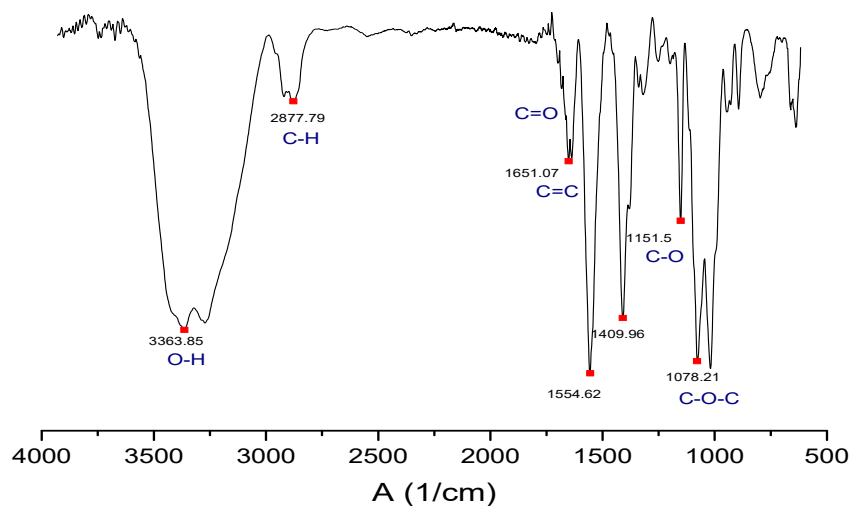


Figure 1. Fourier transform infrared spectroscopy (FTIR) spectrum of the graphene oxide (GO) synthesized in the study.

2.7. Characterization of the GO by Raman Spectroscopy

Raman spectrum (Figure S6) shows the two characteristic bands of the GO. The D = 1358 band shows the sp^2 - sp^3 interactions between the GO carbons and the oxygen-rich groups, while G = 1598 band shows the sp^2 - sp^2 interactions of the C=C links. The degree of oxidation of the GO was also measured by relating the intensity of both bands [29]. The degree of oxidation was found to be

80%, demonstrating that the procedure used to prepare GO was enough to obtain a high percentage of oxidation [29].

2.8. Characterization of GO by X-ray Diffraction (XRD)

The GO diffractogram (Figure 2) shows the characteristic scattering peak at an angle of 9.75° . Usually, this peak appears at 10° [28] and represents the scattering caused by the -COOH groups that give the GO sheets an increase in size compared to graphite [28]. In the XRD spectrum, there are also other peaks between $20\text{--}40^\circ$ which are characteristic of graphite oxide [28]; this indicates that there may be between two and ten overlapping GO layers when the GO is not dispersed in an aqueous solution. This factor loses relevance when preparing the scaffolds as the graphite oxide is dispersed, separating the sheets and reshaping the GO. This was verified by atomic force microscopy (AFM) studies, where measurements are made by dispersing the GO in solution.

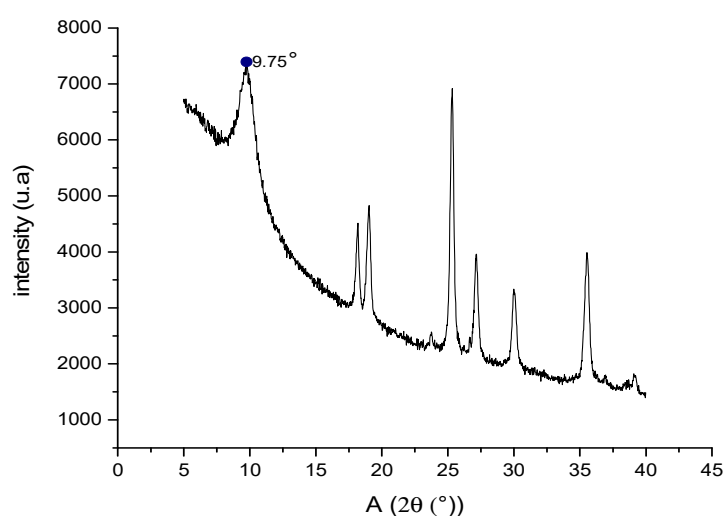


Figure 2. X-ray diffractogram of the GO synthesized in our study.

2.9. Atomic Force Microscopy (AFM) Studies of GO

Figure 3, shows microscopic images of three different GO plates of the sample, with $5.0\text{ }\mu\text{m}$ and $2.0\text{ }\mu\text{m}$ area, dispersed in aqueous solution. The thickness profiles (Figure 3b) of one section of each sheet are shown. The thickness approaches to zero nm along the section since the thickness of a GO sheet must not exceed the thickness of a carbon atom [30]; however, there may be some agglomerations where thicknesses are of up to 20 nm. Independently, each film is individually dispersed in the solution.

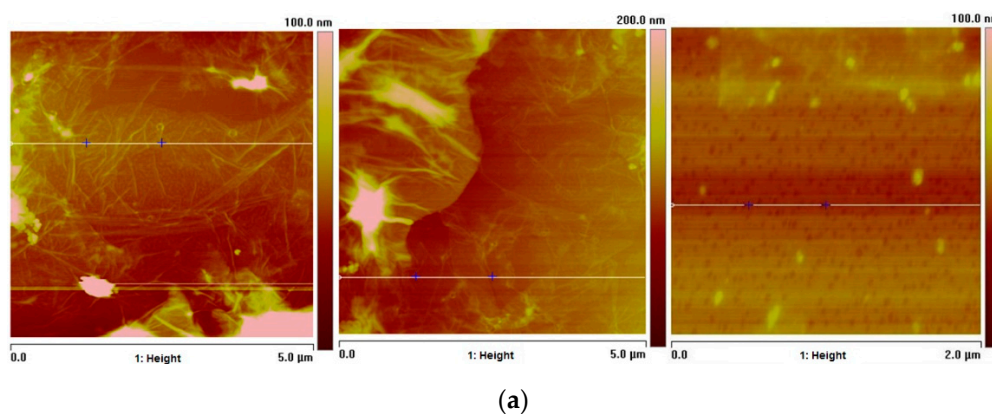


Figure 3. Cont.

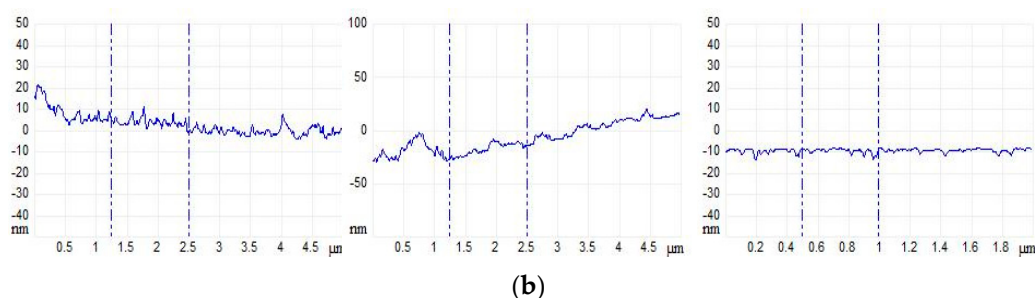


Figure 3. (a) Atomic force microscopy of sectioned GO plates (b) sectional thickness of each plate according to its height.

2.10. Particle Size Study of GO by Dynamic Light Scattering

The particle size distribution of GO sheets was determined using Dynamic Light Scattering (DLS) (Figure S7). The average diameter of the sheets is 531.2 nm. Due to that, the size is low; the sheets do not overlap when dispersed in a solution allowing the construction of scaffolds without GO agglomerations.

2.11. Preparation of CS/GO Scaffolds by Freeze-drying Method

The scaffolds were prepared following a protocol similar to that of Mohandesa et al. [31]. Because the molecular weight of the CS obtained is low, the amount of CS used for scaffolding must be high [31]. For the same reason, the solution must be subjected to considerable agitation for the complete dispersion of the compounds.

The GO, on the other hand, is subjected to sonication to separate the overlapping layers (as shown in the XRD) to eliminate the agglomerations of the compound. This results in an efficient interaction between the CS and the GO where the roughness of the nanoscale surface of the GO, and the two-dimensional structure, establish an interaction that adheres the GO films to the CS chains, providing to the CS, strong mechanical properties that characterize the individual GO films [10].

Three formulations were used for the preparation of the scaffolds. One without GO and the other two with 0.5% and 1.0% GO to compare the morphology, thermal stability, biological potential of each scaffold, and to evaluate the effect of increasing the GO on the properties.

After that, with the freeze-drying process, samples were frozen at low pressure, and the solvent was sublimated, producing a solid and stable structure (Figure 4) due to the strong interfacial adhesion between the well dispersed GO sheets and the polymeric matrix of CS [14]. The two-dimensional nanometric structure of GO finds it easy to align itself between the polymer chains. The hydrophilic characteristics of these films and CS give them a favorable compatibility with regard to the formation of hydrogen bonds evidenced in the FTIR [14], which results in homogeneous structures that are resistant to aqueous solvents, as shown by the hydrolytic degradation test, and whose porosity and roughness provide them with good scaffolding properties that stimulate cell growth [7].

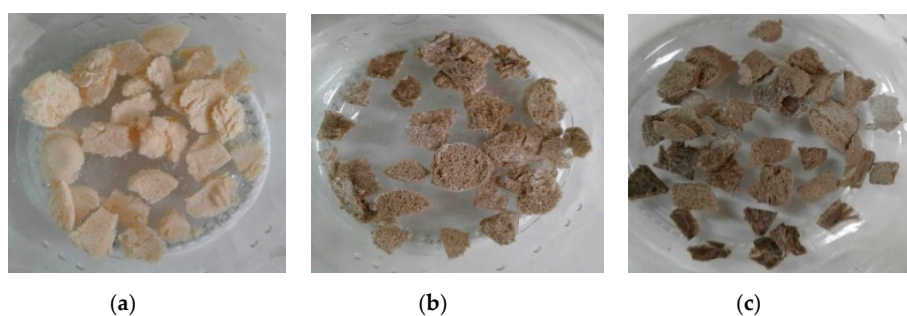


Figure 4. Pictures of the freeze-dried scaffolds of chitosan (CS) (a) without graphene oxide (GO), (b) with 0.5% of GO and (c) with 1.0% of GO.

2.12. Characterization of Scaffolds by FTIR Spectroscopy

The IR spectrum of scaffolds (Figure 5) shows characteristic bands of both compounds. The signal of the band at a higher frequency of about 3360 cm^{-1} , which for both compounds represents the O-H bond tension vibration, is weakened by the increase in the amount of GO.

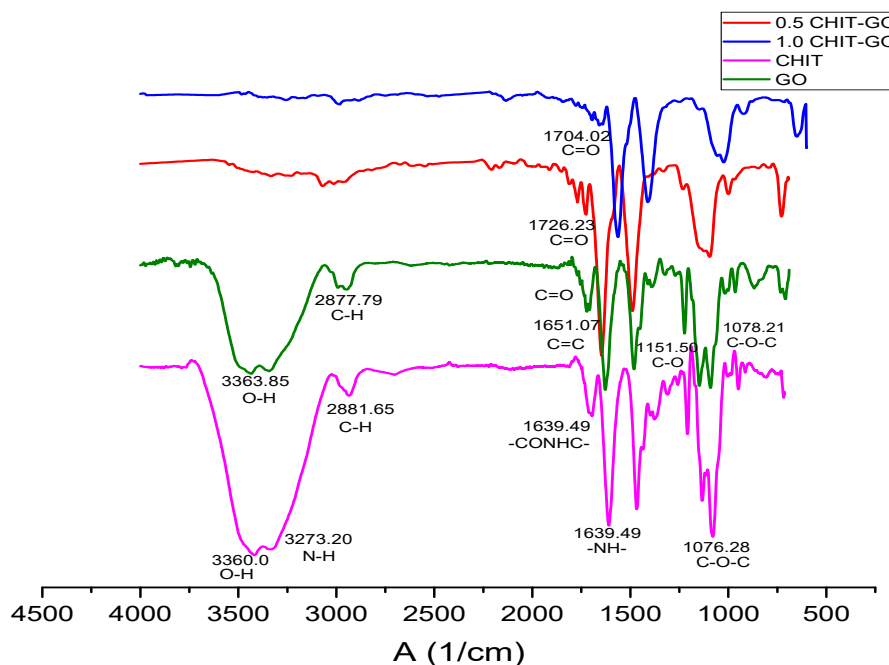


Figure 5. Fourier transform infrared spectroscopy (FTIR) spectrum of the chitosan/graphene oxide (CS/GO) scaffolds synthesized.

This results from the interactions between the molecular vibrations of the hydroxyl of both compounds where the intensity of the overtones increases and the intensity of the fundamental band decreases [32]. This decreasing in the frequency of the band shows that there is a hydrogen bonding interaction between the hydroxyl of CS and GO [14]. The same occurs at 1070 cm^{-1} where the tension band of the C-O-C bond is located. Additionally, the epoxy groups interact at the same location with the OH groups of the CS [14]. This trend is not observed in the hydroxyl band, because the C-O-C band is also part of the glycosidic bond and these oxygenate groups are not susceptible to interaction by this type of bond [14].

In turn, between 1704 cm^{-1} and 1726 cm^{-1} the band corresponding to the C=O appears at a lower frequency due to the intramolecular hydrogen bonds of the GO, which was previously weakened [28]. However, for the scaffolds, carboxyl oxygen is intermolecularly bonded with the oxygenated molecules of the CS and therefore its frequency increases and no longer overlaps with the C=C band, leaving the carboxyls of the GO evident [14].

The other bands that are not part of the hydrogen interactions such as the C-C bond tension band and the -CONHC- of the substituted N- amide remains intact [14].

2.13. Scanning Electron Microscopy (SEM) of Scaffolds

Figure 6 shows microscopic images of the scaffolds prepared with the three formulations, respectively.

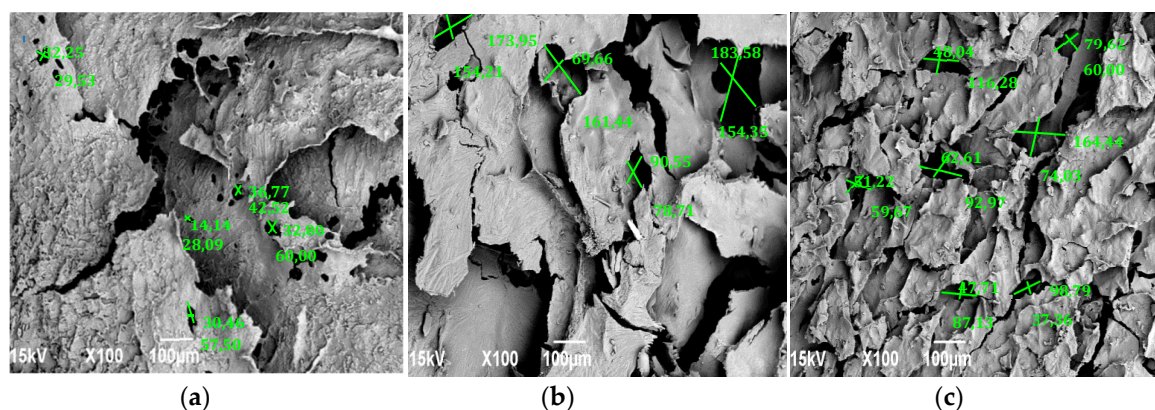


Figure 6. Scanning electron microscopy of chitosan (CS) scaffolds (a) without graphene oxide (GO), (b) with 0.5% GO and (c) with 1.0% GO.

Each composition shows porosity and interconnection, however, the morphology varies as the amount of GO is increased. Figure 6a which corresponds to the CS in the absence of GO, less porosity and roughness is perceived as compared to the other samples. Additionally, most of these pores do not exceed 50 μm , a size necessary for cell proliferation [33]. For this reason, cells are less likely to clump together in this structure; Figure 4b,c, containing 0.5% and 1.0% GO, have more pores and flakes. The formulation containing 1.0% of GO shows more homogeneity with the porosity with an average size of 78.38 μm , calculated using the pore diameter Equation (4) [34],

$$d = ((l)(h))^{(1/2)} \quad (4)$$

where l and h are the maximum and minimum pore size, exceeding the threshold for cell growth. This homogeneous structure, which could promote optimal cell proliferation, is the result of the interaction of CS and GO confirmed by the FTIR and SEM, whose hydrophilic interactions [14] and three-dimensional shape provides stability as confirmed by other authors [7,34]. This was verified with the thermogravimetric analysis and the degradability test.

2.14. Thermogravimetric Analysis of Scaffolds

Thermogravimetric analyses (TGA) of the scaffolds are shown in Figure S8. The thermogram of the CS scaffold without GO (a) shows two mass losses during the temperature rise, as normally occurs in the TGA of the non-lyophilized polymer [35]. The first loss (26.5%) occurs between 71.1 $^{\circ}\text{C}$ and 128.6 $^{\circ}\text{C}$ and corresponds to the remaining mass of water in the CS that is not dry, even after freeze-drying. This loss is also noticeable in scaffolds with 0.5% GO Figure S8b and 1.0% GO Figure S8c; however, in (c), less than half of the water mass degraded in (a) is lost, due to the fact that as the temperature increases, these nanocomposites tend to behave like hydrogels that retained water molecules in their matrices [36]. The hydrogen bonds between the two compounds were weakened, allowing CS chains to stretch freely, which increased the possibility of interacting with other GO sheets, generating greater intercrossing and a water-related three-dimensional network. Thus, it was not easily eliminated [36].

The second loss (33.8%) of the mass of the scaffold without GO (a) with a maximum degradation of 253 $^{\circ}\text{C}$ corresponds to the degradation of the CS chains [35]. In comparison, for the scaffolds containing GO, the decomposition temperature was 270.5 $^{\circ}\text{C}$ and 273.9 $^{\circ}\text{C}$ for (b), and (c), respectively. This shows that there was a strong interaction, due to the hydrogen bonds between the chains of CS and GO [10], which gives thermal stability of a polymer with a higher molecular weight [36].

For scaffolds with GO content, a third degradation temperature with losses of 19.3% and 17.6% at 485.6 $^{\circ}\text{C}$ and 481.8 $^{\circ}\text{C}$ for (b) and (c), respectively, were observed. This is attributed to the removal of the stable oxygenated functional groups of GO [29] and the removal of aromatic remains [26]. Thus, this degradation did not occur in scaffolds without GO content [29]. It is important to remark that

most of the weight loss occurred at temperatures above human body temperature, which indicates that all these structures will be very stable under body conditions.

2.15. Degradability Test of Scaffolds on Physiological Serum

Figure S9 shows the scaffolds added in aqueous medium sterilized at 0.90% *w/v* NaCl. The scaffold degradability (solubility) in the serological medium was evaluated qualitatively in this environment. Usually, increased stability with the introduction of GO, an essential fact for cell proliferation, is expected in *in vitro* biological tests.

The scaffold of CS without GO (a) was completely dissolved in the physiological medium within five minutes, while the scaffolds whose compositions contain GO did not dissolve in the medium, as a result of the improved mechanical properties that GO provides [7]. The introduction of GO increased stability with the increase of GO percentage (b and c).

2.16. Biological Tests

After remaining implanted for 30 days, the samples were recovered and studied macroscopically and by using optical microscopes.

When the dissection was performed, it was observed that the skin had generally healed with hair formation and, inside it, the material remained encapsulated, without the presence of granulation tissue or purulent exudate (Figure 7).

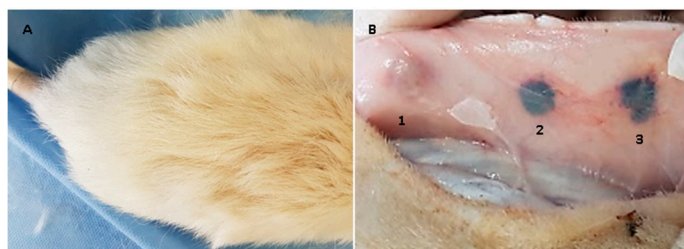


Figure 7. Image (A) shows the dorsal area where the surgical preparation was made; image (B) shows the internal surface of the skin, the three samples encapsulated by a scar tissue are appreciated. 1: chitosan/graphene oxide (CS/GO) 0%; 2: CS/GO 0.5% and 3: CS/GO 1%.

The samples were processed for histological analysis by Hematoxylin and Eosin (HE) and Masson trichromacy (MT) techniques, where a porcine collagen film of 5 mm diameter by one mm in thickness was used as control material [37]. The studies with HE showed the presence of a normal histological architecture in the implantation zone (subcutaneous cellular tissue); additionally, there was no control material present (Figure 8).

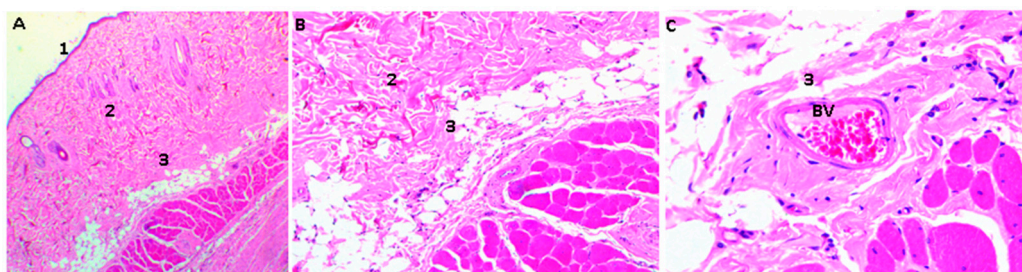


Figure 8. Rat skin sample implanted with collagen film. 1: Epidermis, 2: Dermis, 3: Subcutaneous cellular tissue. Three corresponds to the implantation area, BV: Blood vessel. Hematoxylin-Eosin Technique. Image (A) is at 4× magnification, Image (B) at 10×, and Image (C) at 40×.

Unlike the samples containing the control material, CS and CS/GO scaffolds were found in different resorption states in all the experimental samples. It is evident that the higher the GO content, the less evidence of degradation is observed, as expected for tissue regeneration applications [37].

Figure 9 shows the histological analysis of a CS/GO 0% scaffold. CS scaffolds were observed in an evident degradation/resorption process; there was also the presence of mixed inflammatory infiltrate, responsible for the resorption.

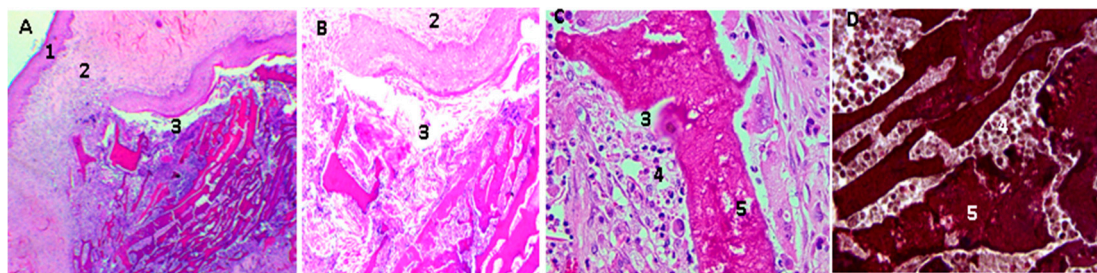


Figure 9. Chitosan/graphene oxide (CS/GO) 0% films implanted in rat skin; in the pictures (A,B), movies are seen at 4 $\times$ and 10 $\times$, respectively, using the Hematoxylin-Eosin Technique; in the images (C,D), at 40 $\times$, the films in the process of and surrounded by a mixed inflammatory infiltrate are observed, the D image is realized using Masson's Trichromacy technique. 1: epidermis, 2: Dermis. 3: subcutaneous cellular tissue (implantation zone), 4. Mixed inflammatory infiltrates, 5. Scaffolds of CS/GO 0%.

In Figure 10, scaffolds of CS/GO 0.5% are shown after 30 days of implantation: there was less evidence of resorption/degradation than in the case of films of only CS. Additionally, there was abundant inflammatory infiltrate.

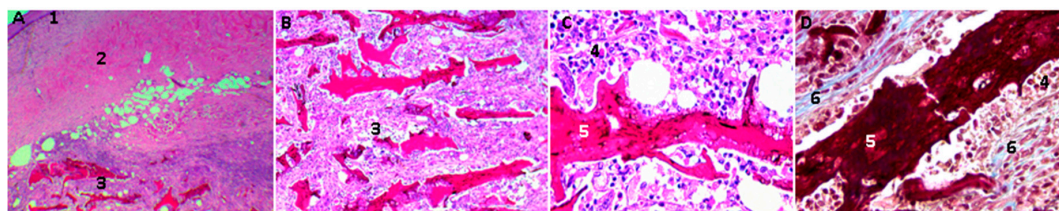


Figure 10. Scaffolds of chitosan/graphene oxide (CS/GO) 0.5% implanted in rat skin; in the images, (A,B) scaffolds are appraised to 4 $\times$ and 10 $\times$, respectively, using the Hematoxylin-Eosin Technique. Images (C,D), at 40 $\times$, are scaffolds in the process of degradation and are surrounded by a mixed inflammatory infiltrate. Image (D) is made using Masson's trichromacy technique; in this image we can see a fibrous tissue capsule surrounding the CS/GO scaffolds. 1: epidermis, 2: Dermis. 3: subcutaneous cellular tissue (implantation zone), 4. Mixed inflammatory infiltrates, 5. Scaffolding CS/GO 0.5%, 6: Fibrous capsule. Technique. Image (A) is at 4 $\times$ magnification, Image B at 10 $\times$. Image (C) at 40 $\times$.

The CS/GO, 1% scaffolds present less evidence of resorption/degradation as shown in Figure 11. There is also an abundant inflammatory infiltrate and a fibrous capsule surrounding the particles.

The macroscopic findings (normal scarring and formation of new skin hair) and the encapsulation of the embedded materials without signs of granulation tissue, or the presence of purulent exudate, but also with the recovery of standard tissue architecture, are evidence of the initial biocompatibility of the material [38].

The presence of a fibrous capsule surrounding the material in the process of resorption, as well as the inflammatory infiltrate observed in all cases, indicate a process of cell-mediated resorption, which is in the parameters of normality of a standard resolution process for these types of materials, mediated by a reaction response to a foreign body [38].

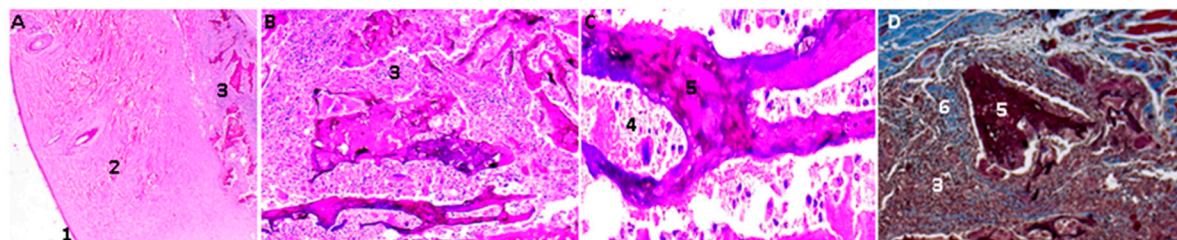


Figure 11. Scaffolds of CS/GO 1% implanted in rat skin. In the images (A,B), the scaffolds are appreciated at 4× and 10×, respectively, using the Hematoxylin-Eosin Technique. In the image (C) at 40×, the film is observed in the process of resorption and surrounded by a mixed inflammatory infiltrate. The image (D) at 10× is made using Masson's trichromacy technique. In this image, a fibrous tissue capsule is seen surrounding the scaffold of CS/GO. 1: Epidermis, 2: Dermis. 3: subcutaneous cellular tissue (implantation zone), 4. Mixed inflammatory infiltrates, 5. CS/GO 1%, 6: Fibrous capsule.

Although there is no greater availability of research where histological studies of graphene oxide implanted in biomodels were carried out, it is possible to find works with *in vivo* characterizations, similar to the one used in this research, for different materials considered biocompatible, such as calcium phosphate, hydroxyapatite, and chitosan.

Cruz et al. and Gonzales et al. determined the biocompatibility of a tricalcium phosphate and chitosan bio-composite implanted in wistar rats' subdermal tissue for 20 days. They found an inflammatory infiltrate and a connective tissue surrounding the material [39]; similarly, Figueiredo et al. compared, in an eight-day study, the intramuscular inflammatory response in Wistar rats, from a xenograft (porcine origin) and an alloplastic (hydroxyapatite), finding a moderate inflammatory response and forming a fibrous capsule [40]. Broon et al. inserted a bio-ceramic material in wistar rats subdermal tissue, observing a mild to moderate inflammatory response with the presence of collagen fibers at 20 days [41].

Additionally, Balanta, Zuluaga, and Valencia found that after 21 days of implantation of chitosan films in wistar rats, subdermal tissue remained without reabsorbing surrounded by a fibrous tissue capsule [42]. Moura et al. implanted chitosan hydrogels, finding a chronic inflammatory response at 30 days with the presence of mixed inflammatory infiltrate and a fibrous capsule surrounding the material [43].

The results presented some materials as biocompatible (calcium phosphate, hydroxyapatite, and chitosan), which indicates that the most common inflammatory response for biomaterials implanted in subdermal tissue of rats is the chronic inflammatory reaction, characterized by an inflammatory infiltrate between mild and moderate, with presence of a fibrous capsule surrounding the material.

The toxicity of graphene oxide has been studied mainly in cell cultures, finding that some cell lines are more sensitive than others to the effect of the nanoparticle (intracellular localization and toxicity of graphene oxide and reduced graphene oxide nanoplatelets to mussel hemocytes *in vitro*) [44]. According to Lalwani et al., the cytotoxicity of the GO is influenced by the size of the particular, dose, distribution, obtaining technique, etc. [45]. In the case of this investigation, large particles were used, in a range between 50 and 1000 µm. This could have influenced the response to the foreign body that was present and in the encapsulation of the particles by a fibrous capsule while the process of cell-mediated resorption was carried out [44], as well as the functionalization with the CS on what improved the biocompatibility of the compound.

Thangave et al. used a dorsal skin design of murine models, and found eight days of skin scarring with collagen formation stimulus when using scaffolds of reduced graphene oxide ((RGO)-Isabgol in normal and diabetic rats) [37]. In this investigation, with 30-day results, the normal healing process was observed with the formation of a collagen capsule surrounding the particles and the presence of blood vessels.

3. Experimental

3.1. Materials

The mycelium of the *Aspergillus niger* fungus was obtained from Sucroal (Cali, Colombia). All other reagents were used as obtained from Sigma-Aldrich (Palo Alto, CA, USA) unless otherwise stated.

3.2. Synthesis

3.2.1. Extraction and Purification of CS

Of the mycelium of the *A. niger*, 500 g was placed in three beakers, and 2 L of deionized water was added. Each solution was mixed using a mechanical mixer, Heidolph RZR 2020 (Heidolph Instruments, Schwabach, Germany), to wash the mycelium. After that, it was filtered off. The mycelium was harvested, and the washing process was repeated six times to remove impurities that were not part of the mycelium.

The mycelium resultant was dispersed in two trays and allowed to dry at 90 °C for 48 h in an oven. Then, it was added to a 5 L nozzle balloon, and after that, it was added to a 40% solution of NaOH in a 5:1 ratio relative to dry mycelium. This solution was mechanically stirred at 1300 rpm for 5 h at 120 °C and then removed and filtered after completion of the reaction.

The brownish filtrate corresponding to hydrolyzed mycelium was collected and washed according to the washing protocol described above until it reached a pH close to 8.

The mycelium obtained was introduced in a beaker and 3 L of water were added. The solution was stirred at 1300 rpm, and glacial acetic acid was added dropwise until a pH of 3.8 was reached to dissolve the CS. This solution was filtered again, and the process was repeated until a pH of 9.8 was reached to precipitate the CS.

The CS was filtered and dried for 24 h at 60 °C. To purify it, it was solubilized again with acetic acid and precipitated with NaOH, then filtered again (11.5% yield) [13].

3.2.2. GO Synthesis

In a 500 mL Erlenmeyer, placed in a refrigerator with ice, three grams of graphite, nine grams of KMnO_4 and 100 mL of an $\text{H}_2\text{SO}_4\cdot\text{H}_3\text{PO}_4$ solution, were added slowly with constant magnetic agitation and mixed together. After that, this solution was sonicated for 3 h in a sonicator, Branson 2800, (Branson Ultrasonics Corporation, North Olmsted, OH, USA) with cold water reflux to prevent the temperature from exceeding 25 °C. Then, another nine grams of KMnO_4 was added and sonication was continued for three more hours without allowing the temperature to exceed 40 °C. A 0.013 mM solution of H_2O_2 was added until effervescence was observed, after which it was washed with H_2O -miliQ in a centrifuge until a neutral pH was reached. A final washing with $\text{CH}_3\text{OH} \geq 99\%$ was carried out and it was then dried in a vacuum furnace, (Precision Scientific CO, Chicago, IL, USA) at 60 °C for 2 h to obtain 5 g of GO [27].

3.2.3. CS/GO Scaffold Preparation

The CS was pulverized and sieved to achieve uniform particle size with a D-500 Success Technic disperser (Success Technic Industries, Selangor, Malaysia). GO, on the other hand, was dispersed in water and sonicated for 30 min to cause exfoliation and separation of the slices. Both compounds were dispersed and agitated for 1 h at 10,000 rpm in dispersing equipment to make three solutions: 19.5% CS/GO and 2% CH_3COOH by adding 0, 0.5 g, and 1 g GO respectively [31].

For each formulation, two falcon tubes were completed with 30 mL of the solution, which were then frozen in a freezer at $-9.8\text{ }^\circ\text{C}$ for 24 h.

The six tubes were then placed in a freeze-dryer Labconco Freezone 4.5 (Labconco Corporation, Kansas City, MO, USA) at a pressure of 12 Pa and $-52\text{ }^\circ\text{C}$ for 48 h to sublimate the CH_3COOH solvent and generate the scaffolds completely. Finally, these were cut with a scalpel to 1 mm thick and

approximately 5 mm in diameter for characterization, for performing the degradability test in aqueous serum at 0.90% *w/v* NaCl, and for the insertions in bone and rat skin.

3.3. Characterization

¹H-NMR measurements were performed on a 400 MHz NMR Bruker Ultra Shield (Bruker Corporation, Billerica, MA, USA) using DMSO-d₆ as a solvent. FTIR measurements were performed on an infrared equipment, Thermo brand model Nicolet 6700 (ThermoFisher Scientific, Waltham, MA, USA), using KBr tablets. GPC measurements were performed using a gel permeation chromatograph Agilent 1200 (Agilent Technologies, Santa Clara, CA, USA) with 2 intercrossed polymer columns Shodex ohpak (Showa Denko, Tokyo, Japan) as the stationary phase and NaNO₃ 0.15 M /HCOOH 0.5 M as the mobile phase, using pullulan standards for the calibration curve (Figure S4), and a refractive index detector. Raman determination was performed on a Confocal Raman Microscope (Renishaw InVia Reflex, Wotton-under-Edge, UK). XRD measurements were run on an diffractometer X'pert PRO (Malvern PANalytical, Jarman Way, Royston, UK) Radiation: k(alpha1) 1.540598 and k(alpha2) 1.544426, from a copper anode with a 45 kV electron accelerator voltage and current to generate 40 mA electrons, with 0.25° and 0.125° incident beam optical grid, 75 mm diffracted beam grid, 0 mm soler grid.04 rad and a detector PIXel (Malvern PANalytical, Jarman Way, Royston, UK) in Scanning mode with an active length (°) of 2.5108, step size 0.0197°, range of 2θ from 4°–90°, and time per step 304.390 s. The AFM analysis was performed in a multimode AFM, Veeco Instruments (Santa Barbara, CA, USA) equipped with a nanoscope Iva control system version 6.14r1 (Veeco Instruments, Santa Barbara, CA, USA; particle size measurements. DLS was measured on a particle size determiner, Zetasizer Nano Z (Malvern Panalytical, Jarman Way, Royston, UK) Scanning electron microscopy (SEM) was performed on a scanning electron microscope (JEOL JSM-6490LA, Musashino, Tokyo, Japan) and each sample was coated with a copper bath. The Thermogravimetric analysis was performed on a TGA-2050 thermogravimetric analyzer (TA instrument, New castle, DE, USA) adjusted in a working temperature range between 25–400 °C.

3.4. In Vivo Compatibility Tests

Following the ISO 10993 standard recommendations, subdermal implantation tests were performed with three samples of each formulation on the dorsal surface of three adult Wistar rats. For the implantation, a surgical preparation was made on the skin, 10 mm long by 15 mm deep and 0.02 g of each formulation were deposited. Finally, the tissues were closed with silk suture 4-0. We then waited for 30 days, to observe the tissue reaction to the implanted materials. This research was approved by the animal ethics review committee of Universidad del Valle in Cali, Colombia, according to the endorsement of the ethics committee CEAS 001-016.

4. Conclusions

The degree of deacetylation of 55.7% and 66.7%, calculated by potentiometric and ¹H-NMR method showed that the chitin contained in the mycelium of the *A. niger* reacted, producing chitosan with a yield of 11.5% based on the initial dry weight.

The graphene oxide was synthesized by an improved Hummers-Offemann method. The improvements get an increase in the weight of the GO, higher than the weight of the starting material. The FTIR and the Raman spectra demonstrated that the oxidation was completed. The AFM and XRD images showed that graphene oxide was obtained after the freeze-drying method.

Scaffolds were successfully elaborated using the freeze-drying method. Their FTIR spectrum showed a decrease in the intensity and the frequency of the characteristic bands of the oxygen groups of both compounds. This indicates that interaction by hydrogen bonds between both molecules occurs as the amount of GO increases.

The TGA and serum degradation test corroborated this interaction by showing that scaffolds with larger GO compositions tend to degrade less; this also showed that absorption in dermal tissues is

greater once the scaffolds are implanted. SEM images showed that the morphology, interconnection, and uniform porosity of the scaffolds with higher GO content allow them to have the potential to generate cell growth of osteoblasts so that they can be used for future biocompatibility tests and insertions in living organisms.

The three formulations behaved as biocompatible in the 30 days that the materials were implanted. Histological analysis showed a decrease in resorption and degradation related to an increase of the GO in each scaffold. Macroscopically, the material presented compatibility, which is evidenced in the formation of new hair, in wound healing, and the recovery of tissue architecture. Moreover, there was no presence of granulation with tissue damage or the presence of pus, which evidenced the initial biocompatibility of the material.

The implanted materials were surrounded by a fibrous capsule, in a typical reaction to a foreign body and resorption of the material mediated by inflammatory cells.

All these results suggest the potential that CS-GO scaffolds obtained have in tissue engineering for cell regeneration starting from a CS obtained from the *A. niger* mycelium, derived from the residue of a company producing citric acid.

Supplementary Materials: The following are available online. Table S1: Concentrations and drop times of solutions taken in an Ubbelohde viscometer, Figure S1: Potentiometric titration of the CS, Figure S2: ¹H-NMR of the CS, Figure S3: FTIR of the CS, Figure S4: Viscosity curve. Specific viscosity vs. concentration, Figure S5: Calibration curve of GPC with pululan standards, Figure S6: Raman of the GO, Figure S7: DLS of the GO, Figure S8: The thermogravimetric curve of CS scaffolds (a) without GO, (b) with 0.5% GO and (c) with 1.0% GO, Figure S9: Degradability test of CS scaffolds in physiological serum (a) without GO, (b) with 0.5% GO and (c) with 1.0% GO added.

Author Contributions: Conceptualization, C.D.G.-T., C.H.V., J.H.M., and F.Z.; Methodology, C.H.V., C.D.G.-T., and C.V.; Investigation, C.H.V., M.E.V., and C.V.; Writing-Original Draft Preparation, C.D.G.-T., C.H.V. and C.V.; Writing-Review & Editing, C.D.G.-T., C.H.V., C.V., J.H.M., M.E.V. and F.Z.

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Conflicts of Interest: The authors declare no conflict of interest.

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Sample Availability: Samples of the compounds are not available from the authors.



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Article

Synthesis and application of scaffolds of Chitosan-Graphene oxide by the Freeze-drying method for tissue regeneration

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1. Experimental

1.1. Deacetylation degree and characterization of CS by potentiometric method

For the DD calculation, a potentiometric titration of CS with a WTW pHmeter pH 3310 was performed, a NaOH solution of 0.101 M previously standardized with C₈H₅KO₄ as titrating agent was used and a solution of 0.253 g of CS in 20 ml HCl 0.3M.

1.2. Capillary viscosimetry for the calculation of the Mv.

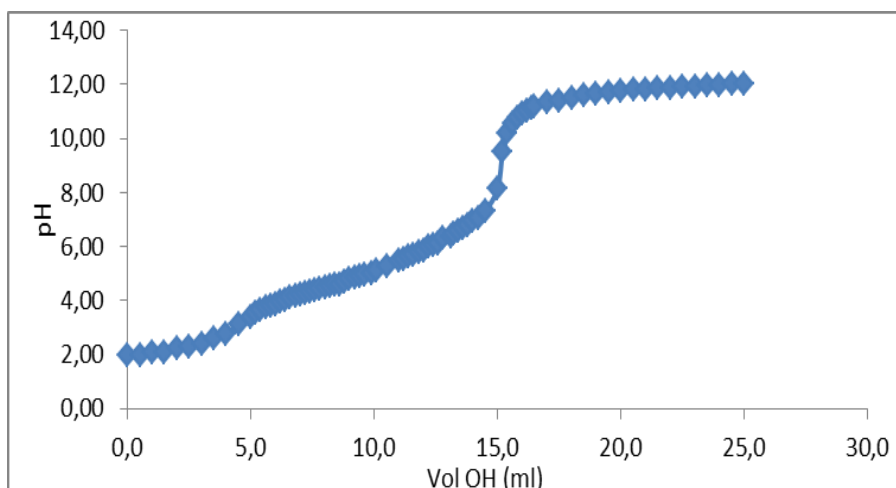
A standard solution of 100 mL of CH₃COOH 0.1 M, NaCl 0.2 M and 0.02 g/mL of CS was prepared for the calculation of the Mv of CS. From this 5 solutions of 25 mL were prepared with the concentrations shown in figure 3 and the density was measured with a pycnometer of 1 mL and the fall time with a viscometer of ubbelohde viscometer with constant β of 3.31×10^{-5} that is calculated with the time of fall of the water.

1. Supporting tables

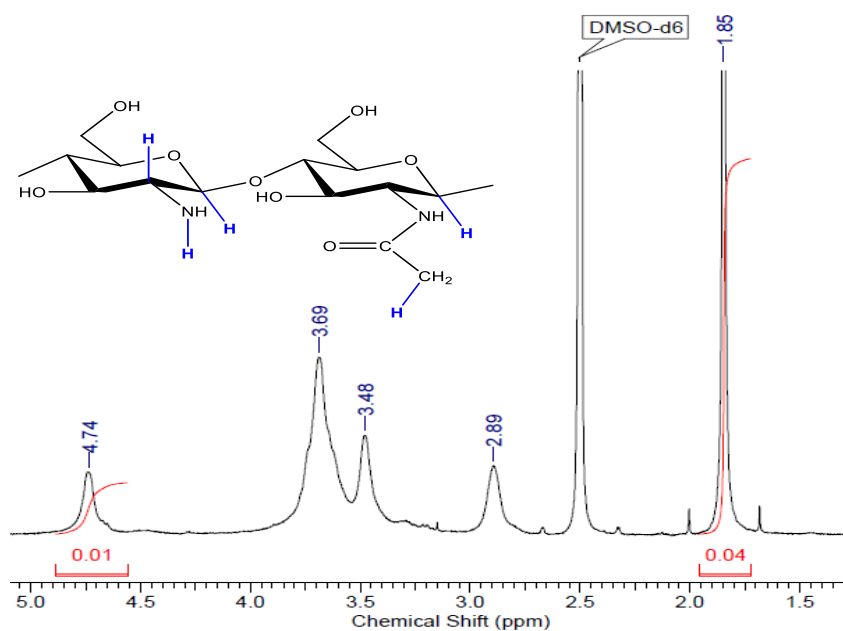
Table S1. Concentrations and drop times of solutions taken in an ubbelohde viscometer.

Concentration (mL/g)	Time (s)	ρ (g/mL)
Solvent	277	1.0320
0.0008	279	1.0324
0.0010	282	1.0327
0.0013	283	1.0332
0.0015	284	1.0348
0.0018	285	1.0360
0.0020	286	1.0368

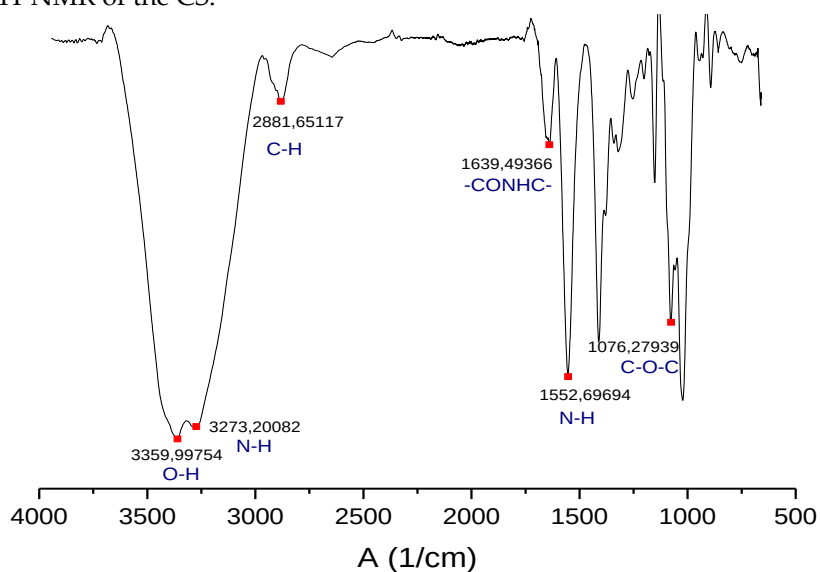
2. Supporting images



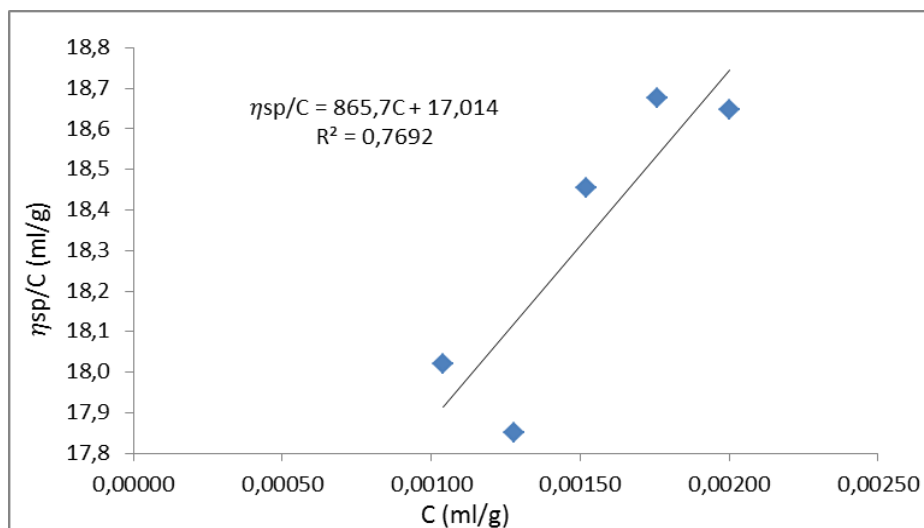
31 **Figure S1.** Potentiometric titration of the CS.



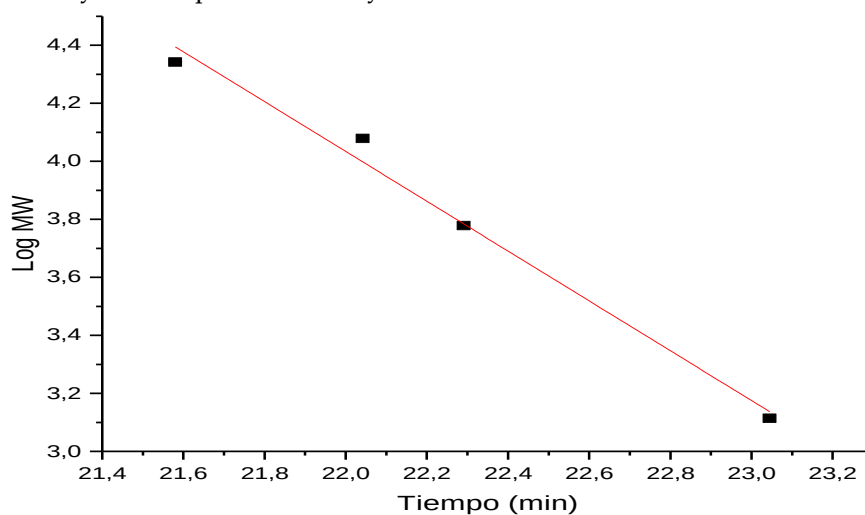
32 **Figure S2.** ^1H -NMR of the CS.



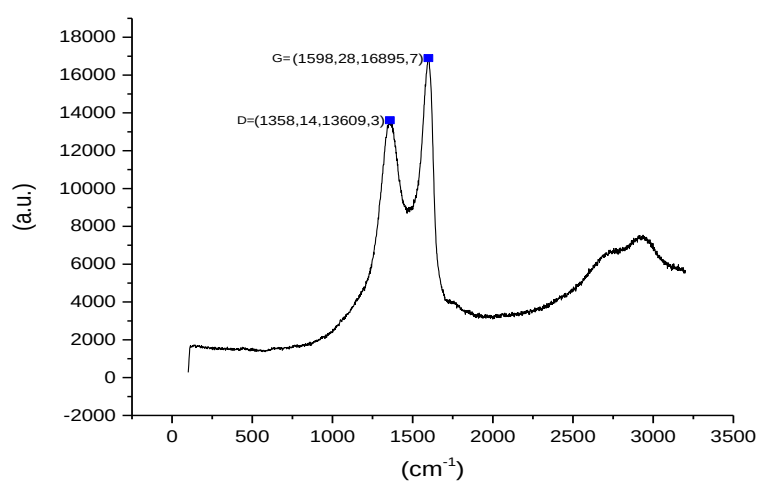
33 **Figure S3.** FTIR of the CS.



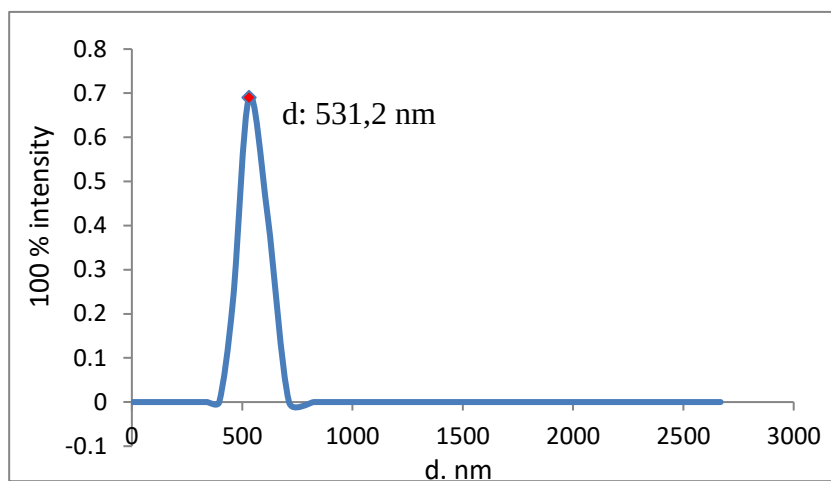
34 **Figure S4.** Viscosity curve. Specific viscosity vs concentration.



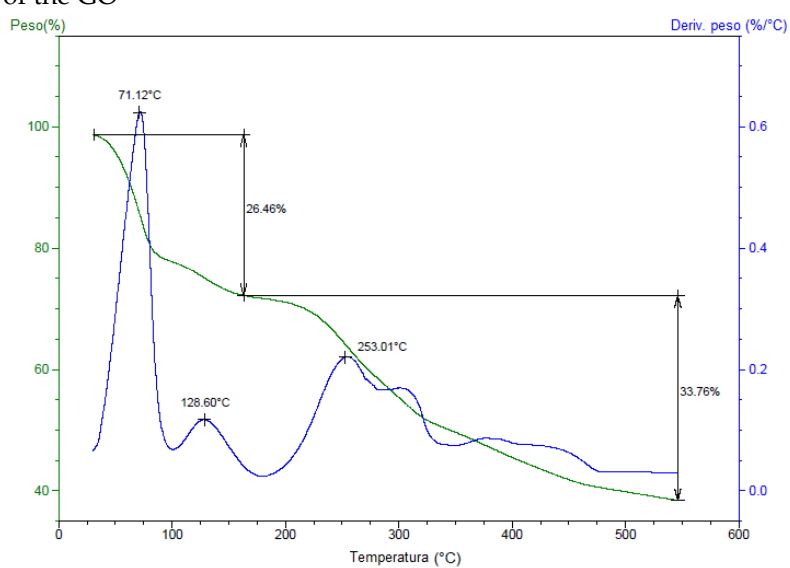
35 **Figure S5.** Calibration curve of GPC with pululan standards.



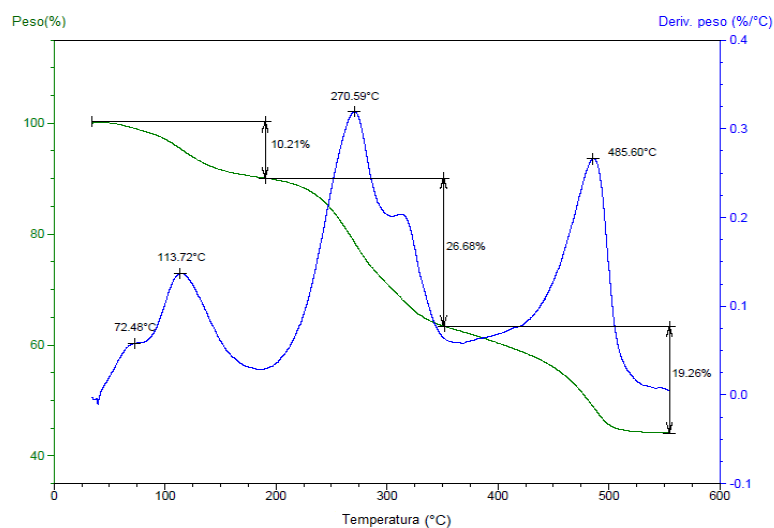
36 **Figure S6.** Raman spectrum of the GO



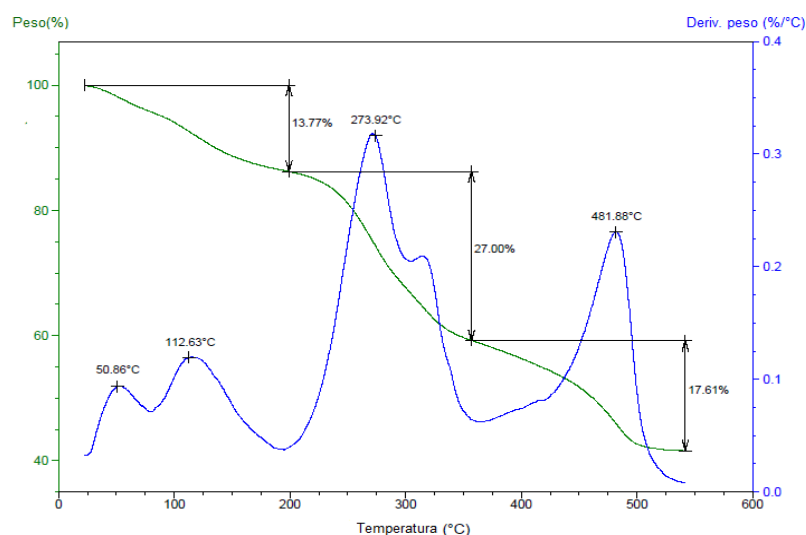
37 Figure S7. DLS of the GO



(a)

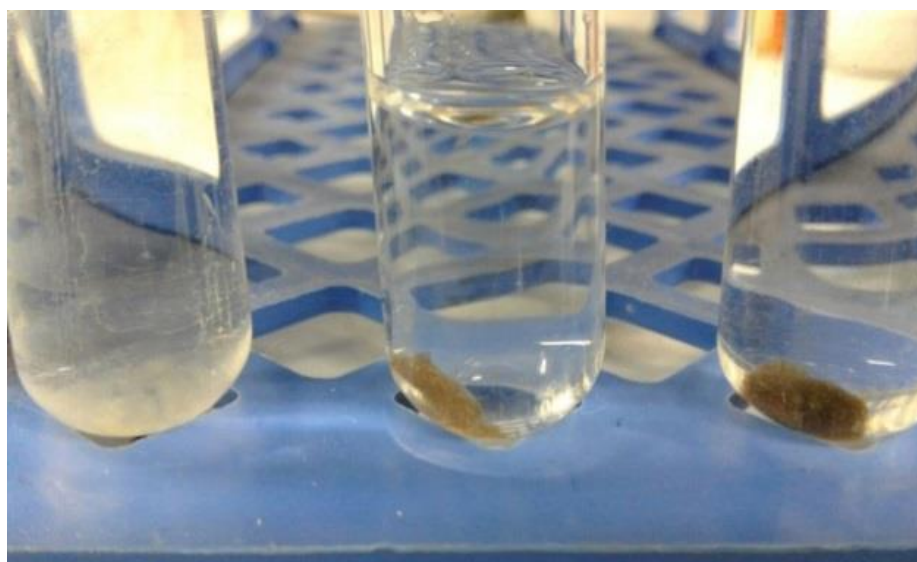


(b)



(c)

38 **Figure S8.** Thermogravimetric curve of CS scaffolds (a) without GO, (b) with 0.5% GO and (c) with
 39 1.0% GO.



(a)

(b)

(c)

40 **Figure S9.** Degradability test of CS scaffolds in physiological serum (a) without GO, (b) with 0.5%
 41 GO and (c) with 1.0% GO added.