

Electrophoretic separation of proteins on capillary columns grafted with
non-fouling, stable films prepared by controlled radical polymerization

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Publications derived from this thesis work

1. J. H. Espinal, J. E. Gómez, J. E. Sandoval, Closer look at the operating definition of protein recovery in CE. *Electrophoresis* **34**, 1141-1147 (2013).
2. F. H. Navarro, J. E. Gómez, J. H. Espinal, J. E. Sandoval, Neutral hydrophilic coatings for capillary electrophoresis prepared by controlled radical polymerization. *Analytica Chimica Acta* **948**, 104-112 (2016).

Abstract

Proteins are complex macromolecules that are inherently active towards a variety of surfaces. The active sites of the surface can enable structural alteration and destabilization of many proteins leading to their adsorption. Capillary electrophoresis (CE) is one analytical application where the consequences of adsorption can be especially catastrophic when the protein solute becomes stuck on the capillary before reaching the detector. Chemical modification of the fused-silica capillary has been aimed at eliminating unwanted interactions between the inner wall of the capillary tube and the sample undergoing separation. The goal of this project is to provide a hydrolytically stable organic film that coats the inner-wall of the fused-silica tubes used in CE. We used a surface-confined grafting procedure based on atom transfer radical polymerization (ATRP) which was also surface-initiated from α -bromoisobutyryl groups. Immobilization of the initiator was achieved by hydrosilylation of allyl alcohol on hydride silica followed by esterification of the resulting propanol-bonded surface with α -bromoisobutyryl bromide. The modified surfaces were characterized by IR and solid-state NMR spectroscopies, atomic force microscopy, contact-angle measurements, etc. The focus of this thesis work was to quantitatively assess the extent of surface deactivation (non-fouling) upon grafting by examining the CE migration profile of several chemical probes, including proteins. Additionally, a systematic assessment of the stability of all new bonded materials was also an essential part of this work. Our results showed that the coated capillary tubes exhibit (i) a complete (100%) recovery of lysozyme; *i.e.*, there is no adsorption of this challenging solute on the coated surface, and (ii) a high long-term hydrolytic stability under the inherently harsh conditions of capillary isoelectric focusing. As a by-product of the present work, we have come up with an improved analytical methodology to determine solute recoveries in CE.

Presentation of the thesis

The main objective of this project is to advance in the solution of the analytical problem associated with the still unsatisfactory performance of current organic films that coat the inner-wall of the fused-silica tubes used in capillary electrophoresis (CE). Although considerable advances have been made in the development of chemically modified silica surfaces for analytical separations, there are still two major limitations to be overcome in CE; namely, surface adsorption of certain solutes, such as proteins, and hydrolytic instability of the film. The effectiveness of a given surface modification for wall-deactivation is routinely assessed by inspection of the CE peak profiles of selected probes, including basic proteins. A hydrolytically stable polymeric layer is also of great importance because the prolonged contact of the coating with harsh buffer solutions during electrophoresis eventually leads to the gradual loss of the bonded material and results in increased active site exposure, deterioration of separation and contamination of one or more fractions with the column's degradation products. The long-term removal of the undesirable protein-silica interactions should result in clear-cut separation profiles that provide better characterization of complex biological samples. The advances of biotechnology and the advent of proteomics, the most active field of the post-genome era, have brought a tremendous demand on high resolution analytical tools for the separation of proteins in very complex matrices. Current necessities in present CE practice are certainly there.

To address such challenge, we have developed a novel synthetic approach to make thickness-controlled polymeric CE coatings that simultaneously exhibit non-fouling behavior (due to its highly hydrophilic structure) along with superior resistance to hydrolysis (resulting from polymer composition and its attachment to the silica surface). This thesis work makes emphasis on two critical aspects of capillary film performance: surface deactivation of the fused-silica capillary and coating resistance to hydrolysis under severe electrophoretic conditions. Developing an appropriate tool to quantitatively assess solute recovery in CE run was necessary to address the issue of surface deactivation. This part of the project is described in Chapter 1 of this document. Chapter 2 addresses the evaluation of the capillary coating performance in terms of protein recovery (applying of the new measuring tool) and long-term hydrolytic stability under the severe electrolyte conditions prevailing in capillary isoelectric focusing.

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Closer look at the operating definition of protein recovery in capillary electrophoresis

Abstract

Analyte recovery is an important figure to assess protein adsorption on fused-silica capillaries. In 1991 Regnier and coworkers estimated recovery by assuming the loss of analyte from adsorption and thus the decrease in peak area measured by two detectors to be proportional to the length of the capillary section between them. In this report, we closely examine this concept and its adaptation to commercial CE instruments to determine protein recovery. We hypothesize that, once a steady-state migration is reached, protein adsorption is a first order process with respect to protein concentration and surface density of adsorbing sites. This hypothesis is shown to be valid over a reasonably wide range of capillary effective length and, as a result, protein recovery decreases exponentially with the migrated distance. However, unlike the traditional recovery figure obtained through a conventional spike process, protein recovery measured by this approach does not have the same merit since it is strongly dependent from capillary dimensions and applied electric field. Nevertheless, protein recovery and the slope of the logarithmic protein peak area *vs.* length plot are useful figures to compare protein adsorption on different capillary surfaces. Several literature reports dealing with the application of Regnier concept to calculate protein recovery are discussed.

Key words: Protein recovery

Introduction

The advent of proteomics, one of the most hectic areas of the omics field, demands high resolution analytical tools for the separation of proteins in very complex matrices (1,2). CE is becoming increasingly important for high throughput data generation in biological research because of its high speed, separation efficiency, selectivity and versatility, low sample size requirement and easy automation (3,4). Unfortunately, a full exploitation of the superb separation efficiency of CE in the proteomics field has been hindered by protein adsorption on the fused silica capillary surface (5). A variety of methods has been devised to minimize such interaction between proteins and the silica surface. One of the most common approaches involves the incorporation of suitable additives to the BGE to dynamically coat the inner surface of the capillary (6). Such additives range from oligoamines and simple surfactants (mostly cationic, but also non-ionic and zwitterionic detergents) to neutral and cationic polymers. Most additives work as competing agents for cation exchange sites, mainly ionized silanol groups, on the silica surface, so that they are no longer accessible to protein interactions. Because they are cost-effective, reproducible and very easy to implement, these dynamic coatings have

become increasingly popular. So, by simply masking the undesirable protein-silica interactions, dynamic coatings have significantly improved separation profiles that enable better characterization of complex proteome samples. A more elaborate but permanent approach to deal with protein adsorption is to chemically modify the inner capillary wall with, for instance, a cross-linked hydrophilic polymer. However, permanent coatings usually exhibit low hydrolytic stability that limits their useful lifetime, and high cost (money, effort, or both). While significant advances have been made, truly effective and long-lasting surface deactivation toward protein adsorption by means of dynamic or permanent covalently-bound coatings remains one of the greatest challenges in the current practice of CE.

Performance parameters such as analyte recovery, separation efficiency, peak asymmetry, electroosmotic mobility (EOM) change and baseline shifting have been used to monitoring protein adsorption in CE (6). From an analytical point of view, method accuracy can be measured from an analyte recovery experiment (7). A known amount of the analyte is added ("spiked") into the sample, prior of the analysis. The amount –usually concentration– of analyte in the unspiked and spiked samples is then measured and the recovery or recovery factor, R , is determined by the expression

$$R = \frac{Q_{meas}}{Q_{theor}} \quad (1)$$

where Q_{meas} is usually the difference in concentration between the spiked and unspiked samples, and Q_{theor} refers to the known or "theoretical" concentration from the spike. Because of the extremely small surface area of the inner wall of a capillary tube, protein recovery experiments are difficult to implement and require high sensitivity detection. For instance, Righetti and coworkers used a fluorescence-labeled model protein to measure analyte loss due to adsorption on the fused silica surface (8). After saturating the whole length of the capillary with the fluorescent protein, they desorbed it by electrophoretically flushing the capillary with an SDS-containing electrolyte. The eluted protein was then quantified by an off-line fluorescence technique. Notice that, while this method led to important conclusions regarding the adsorption process, it relies on a relatively long static protein-silica contact time to ensure reproducible data. Under regular CE conditions, however, a short plug of the protein sample traveling through the capillary tube experiences a much shorter contact time with the silica surface. Regnier and coworkers were the first to report an on-capillary electrophoretic method to measure protein recovery (9). Using a home-made CE instrument equipped with two detectors in series on a single capillary tube, they estimated recovery by assuming that the loss of analyte from adsorption and hence the decrease in peak area measured by the two detectors was proportional to the length of the capillary section in between. Later on, Lucy and his research group adapted Regnier concept to commercial CE instruments with a single detector (10). They determined the percent recoveries of proteins on the same capillary tube by comparing the peak areas before and after shortening the column. Subsequently, Taverne and coworkers simplified the experimental procedure by using the long and short portions of an intact capillary tube, *i.e.*, no cutting (11). Unfortunately, neither Regnier and coworkers (9,12) nor Lucy and coworkers (10,13-18) provided explicit equations to calculate protein recovery. Specifically, they never mentioned which reference they used to express the decrease in peak area as a percentage, something which is essential to the recovery equation. Such lack of details has resulted in diverse interpretations of the original concept. Some authors appear to

interpret the method concept in a manner presumably similar to that of the original work (19,20), while others do so in a quite different fashion, as we will discuss below (11,21-23).

In this report, we closely examine the concept as well as the adaptation to a single detector of Regnier's method to assess protein recovery. The hypothesis that the amount of protein adsorbed is proportional to solute concentration as well as migrated distance is experimentally tested in this work using a pentamine to dynamically coat the inner wall of a capillary. The different CE-based approaches to calculate protein recovery are discussed.

Materials and methods

CE experiments were performed in an Agilent model 3D CE System (Palo Alto, CA, U.S.). Fused-silica capillaries of 50 μm i.d. were purchased from Biotaq Inc. (Silver Springs, MD, U.S.). Piperazine-N,N'-bis(3-propanesulfonic acid) (PIPPS) was purchased from GFS Chemicals (Columbus, OH, U.S.); tetraethylenepentamine (TEPA), lysozyme (chicken egg white), benzyl amine and benzyl alcohol were purchased from Sigma-Aldrich (St. Louis, MO, U.S.). All solutions were prepared in 18-M Ω deionized water from a Millipore Corp. (Bedford, MA, U.S.) model Milli-Q system fed with distilled water. 25 mM PIPPS buffer containing 1.5 mM TEPA and adjusted to pH 4.00 ± 0.03 with sodium hydroxide was used as BGE. A solution of 1.0 mg/mL lysozyme and 10.0 mM benzylamine was prepared in the BGE and used for protein assays. An 8-m fused silica capillary tube was etched with a 1.0 M NaOH solution at 30°C for 2 h by forcing the solution through with 7-bar N₂ pressure (24). EOM determinations followed a procedure previously described (24). A 5.0-mM solution of benzyl alcohol in the PIPPS BGE was used as neutral marker. Benzylamine was used as internal standard to correct lysozyme peak areas for injection volume variations. Protein recoveries were determined using the CE method of Regnier's modified by Lucy and coworkers (10). In this procedure, replicate injections of the lysozyme/benzylamine mixture were performed on each of five adjacent pieces of NaOH-etched capillary with total lengths of 35.0 to 75.0 cm. Removal of a 1-2 mm section of the polyimide cladding to obtain a detection window at a fixed 8.5-cm distance from one end of the capillary column was carried out by a procedure previously described (24). In this manner, capillary tubes with effective lengths of 26.5 to 66.5 cm were obtained. Separations were carried out under constant electric field strength of 320 V/cm by adjusting the applied voltage accordingly. Likewise, injection and rinse pressures were also adjusted to allow for constant pressure gradients of 0.67 and 50 mbar/cm respectively. In all cases, the injection time was 2.0 s, and the rinse time between runs was 5 min. Prior to use, all capillaries were conditioned with the BGE under high pressure for 30 min after which a plateau EOM value was obtained (24). Again, the pressure applied during BGE-conditioning was adjusted to obtain a constant pressure gradient of 115 mbar/cm. The equations used to calculate protein recovery are described below. A separate 35-cm piece of the same capillary was also used to measure lysozyme peak areas after injecting the sample mix from the inlet (26.5-cm effective length) and then the outlet (8.5-cm effective length) of an intact capillary tube, according to the approach devised by Taverna *et al.* (11). A reversed applied potential was used in the later case. In another experiment, a 50-cm length of the same tube (41.5 cm to detection) was used to measure protein peak areas (again, referred to benzyl amine as internal standard) under various applied potentials; the experiment was repeated after cutting the tube to a 35.0-cm total length (8.5 cm to detection), according to Lucy's approach (10).

Results and discussion

The fundamental concept of Regnier method relies on the assumption that the decrease in peak area measured by the two detectors is solely proportional to the capillary length separating them. In contrast, while protein adsorption on silica substrates is an extremely complex and controversial subject, the simplest model depicts a bimolecular process involving attachment of the protein to the surface-active sites. Accordingly, we assume that the surface composition of the inner wall of a fused silica capillary is uniform, and that protein adsorption is a first order process with respect to its concentration as well as the surface concentration of adsorption sites. Under steady-state migration conditions, as a sample plug travels a distance dl along the column its peak area decreases in an amount dA which should be proportional to the concentration of protein (hence its peak area, A) and to the number of active adsorption sites (5,25):

$$-dA \propto A \, dn_{ads} \quad (2)$$

where dn_{ads} represents the surface sites available for adsorption. As the protein plug reaches the detector, the total number of adsorption sites to which it has been exposed is

$$n_{ads} = 2\pi r l \Gamma_{ads} \quad (3)$$

where r is the capillary inner radius, l is the travel distance to the detection point (the effective capillary length) and Γ_{ads} is the surface density of adsorption sites. Hence, the number of adsorption sites per unit length of capillary is given by

$$\frac{dn_{ads}}{dl} = 2\pi r \Gamma_{ads} \quad (4)$$

Therefore,

$$dA = -2\phi\pi r \Gamma_{ads} A dl = -k A dl \quad (5)$$

This equation integrates to

$$\ln \frac{A}{A_0} = -kl \quad (6)$$

Here, the intercept A_0 ($l = 0$) corresponds to the peak area of the protein plug sample before any adsorption takes place. In other words, A_0 is the peak area the protein sample would have had at the capillary inlet. The slope, k , is proportional to the surface density of adsorption sites and the inner diameter of the tube (Eq. 5).

When Eq. 1 is applied to protein recovery in CE, Q_{meas} stands for the number of protein molecules that reach the detector (*i.e.*, recovered after traveling a distance l through the capillary), a quantity which is directly proportional to its peak area A . Q_{theor} represents the original number of protein molecules that are injected at the capillary inlet which, in turn, is proportional to the theoretical or "initial" peak area A_0 :

$$R = \frac{Q_{meas}}{Q_{theor}} = \frac{A}{A_0} \quad (1a)$$

Combined with Eq. 6, this expression leads to

$$R = e^{-kl} \quad (7)$$

Notice that, according to Eqs. 5 and 7, recovery is not only a function of the surface density of active adsorption sites but also of capillary dimensions. This is a matter of significant importance. Strictly speaking, protein recovery as calculated from Eq. 7 (based on a CE profile experiment) is not an intrinsic feature of the inner surface of the capillary only and, consequently, does not have the same universality as the analyte recovery figure calculated from Eq. 1 (typically, a spike experiment), as generally thought. Unless $k = 0$, the protein percent recovery value obtained will never, in principle, be equal to 100% (l , in practice, can never be equal to zero) and hence capillary dimensions as well as CE conditions must be fully reported. Naturally, a k -value not significantly different from zero would be a strong evidence of an adsorption-free CE surface. Nevertheless, protein recovery based on the equations presented here is still a valid figure to compare, for instance, different BGE additives or varying capillary chemistries run on the same system and under the same conditions.

In contrast with the operating definition of analyte recovery (Eq. 1), there is no spiking in this case and, therefore, R can only be obtained indirectly. A formal way to do this is by measuring the peak areas on several pieces of capillary tube (or a single long piece shortened several times), followed by linear fitting of $\ln A$ vs. l data to Eq. 6. Figure 1 (curve with blue circles) shows that, within the range of effective length considered, a plot of the logarithm of protein peak area vs. migrated distance is indeed a straight line:

$$\ln A = \ln A_0 - kl \quad (6a)$$

Linear regression furnishes the slope and recovery figures shown in Table 1.

It should be pointed out that application of the method assumes that essentially irreversible adsorption occurs, which –within the present context– means that any adsorbed protein can be completely removed by simple rinsing between runs with protein-free BGE. In this manner, a renewed (and reproducible) capillary surface should be obtained and reliable peak area measurements can be made. If, on the other hand, truly irreversible protein adsorption takes place, the tube can no longer be used and no useful data result (12). In fact, since application of the method depends on peak area integration measurements, protein profiles with moderate to severe tailings would not provide reliable results. This is why we used a fused-silica capillary dynamically modified with TEPA as opposed to an untreated one. It is also worth mentioning that, within the experimental uncertainty, benzyl amine exhibited insignificant adsorption on the silica surface and, hence, its use as internal standard did provide a better precision of the lysozyme peak areas when compared to the non-ratioed values. See Supporting Information for details.

Obtaining the regression parameters from Figure 1 may seem a time-consuming and expensive process. A simpler approach to estimate protein recovery could be a two-point straight line approximation by which peak areas are measured either before and after cutting the capillary (according to Lucy *et al.*) (10,13-18), or after separately injecting the sample at both ends of a single and intact capillary tube (according to Taverna *et al.*) (11,21,22). In either case, the slope k can be approximated to

Table 1. Comparison of analytical parameter values based on the modified Regnier method as calculated by linear regression and by a two-point linear approximation.

	Five-point linear regression	Two-point linear approximation
Slope, k	0.0108 ^a (3.0%) ^b	− 0.0038 ^c (27%)
%Recovery ($l = 26.5$ cm)	75.2 ^d (0.9%)	90 ^e (3%)

^a Estimated from linear fitting to Eq. 6. ^b Percent relative standard deviation in parenthesis. ^c Estimated from Eq. 8 with $l = 41.5$ cm and $s = 26.5$ cm. ^d From Eq. 7 with $l = 26.5$ cm (the shortest effective length attainable with our CE instrument). ^e Calculated from Eq. 9 with $l = 41.5$ cm and $s = 26.5$ cm.

$$k = \frac{1}{l-s} \ln \frac{A_s}{A_l} \quad (8)$$

where A_l and A_s are the measured peak areas for the effective lengths l and s respectively, and s represents either the effective length of the capillary after the cutting, or the shorter segment of the uncut tube. In this case $s = L - l$, where L is the total length of the capillary, and it is implicit that $s < l$ and $A_s \geq A_l$. Recovery can be estimated from

$$R = \left(\frac{A_l}{A_s} \right)^{\frac{l}{l-s}} \quad (9)$$

It appears that this two-point-straight line approximation has been the approach used by all researchers in the past (10,11,13-23).

When we attempted to apply Taverna's approach using the long and short portions of an intact capillary tube to estimate protein recovery, interesting results arouse. Our CE instrument can accommodate capillaries with a minimum total length of 35 cm, with a fixed distance (8.5 cm) from the point of detection to the outlet. This represents an effective length of 23.5 cm under "regular" CE conditions (this is, injection at the anodic inlet). Contrarily to what is expected from a backward extrapolation of the fitted line in Figure 1, the lysozyme peak area obtained after separation from the short ($s = 8.5$ cm) end of the capillary was smaller than that from the longer end ($l = 23.5$ cm), as shown by the red square data points in Figure 1. This means that apparent recoveries above 100% were obtained (typically $107 \pm 2\%$), as calculated from Eq. 9. Clearly, Eq. 6 does not apply for lysozyme at such short effective length on our system. There are several facts relevant to the experimental remark in question. First, adsorption of strongly cationic proteins such as lysozyme occurs preferentially at the capillary inlet (12). Second, such initial protein adsorption process occurs immediately after injection, typically within micro- to milli-seconds and, most likely, as multilayered aggregates (5). Finally, desorption of the reversibly bound protein molecules from the silica surface follows, until a steady state migration is reached (5,25). Of course, such desorption starts right after voltage application: as the protein plug starts moving along the tube, it is continuously preceded and followed by protein-free

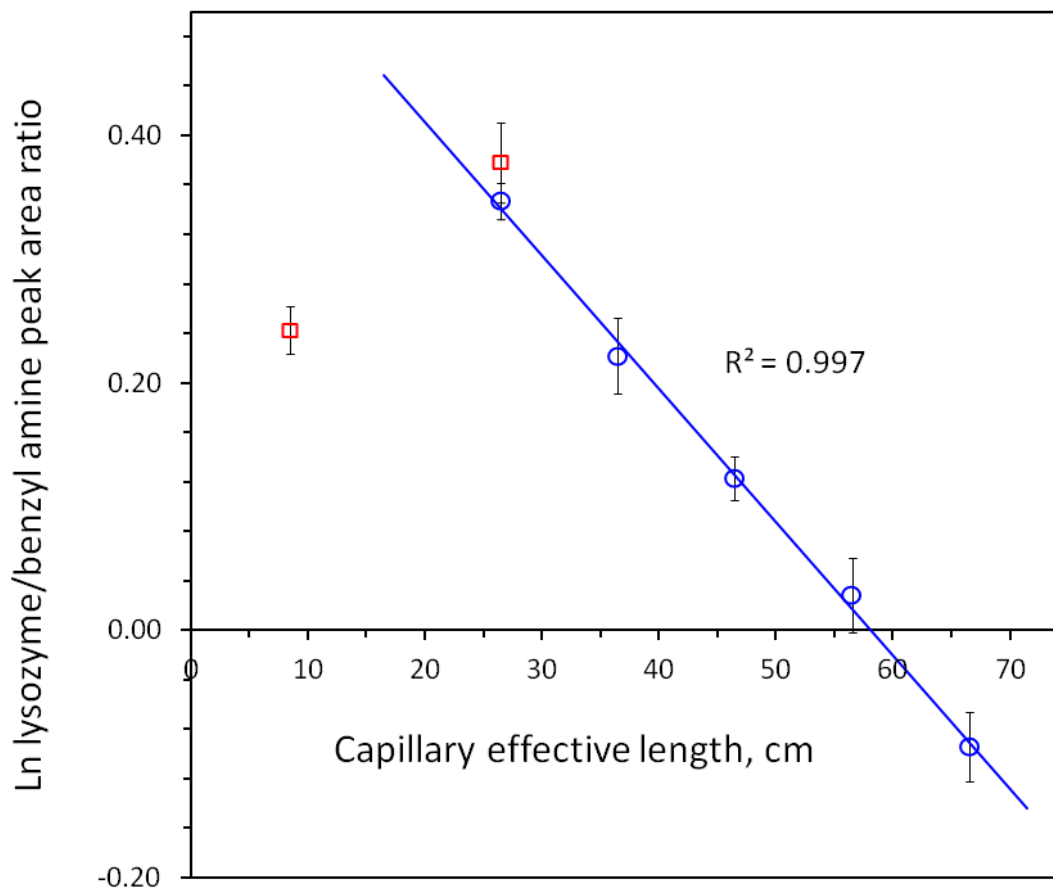


Figure 1. Logarithm of peak area of lysozyme as a function of migrated distance. CE conditions: BGE, 25 mM PIPPS buffer containing 1.5 mM TEPA, adjusted to pH 4.00 ± 0.03 ; sample, 1.0 mg/mL lysozyme and 10 mM benzylamine (internal standard) in BGE; injection, 2 s at 0.67 mbar/cm; applied electric field, 320 V/cm for 10-20 min; 5-min BGE-rinse between runs at 50 mbar/cm; detection at 200 nm. Blue circles: Separate capillary tubes with varying effective lengths and injections at the inlet. Error bars represent ± 1 SD ($n = 10$). Red squares: A single 35.0-cm piece of the same capillary tube after injection of the same sample mix at the inlet ($l = 26.5$ cm), and then at the outlet ($s = 8.5$ cm) with reversed applied potential. Error bars represent ± 1 SD ($n = 5$).

BGE, which results in surface rinsing during which a net desorption occurs. Notice that protein peak area is undoubtedly increasing in the length interval between 8.5 and 26.5 cm; which means that desorption prevails at $l = 8.5$ cm. Shortly after (definitely before the upper end of this length interval), a new condition is reached under which adsorption dominates and Eq. 6 applies. So, one possible qualitative interpretation of our unusual observation is that after just 8.5 cm of protein traveling, desorption has not yet counteracted the initial adsorption effect that led to a substantially decreased peak size; *i.e.*, a steady-state migration condition has not been reached at this point. In contrast, at 26.5 cm and beyond, such steady state has already been attained and the delayed protein molecules originate a second wave that manifests itself as significant peak tailing, a higher baseline after the

passage of the peak, or even a secondary peak (26). The effect is illustrated in Figure 2 for a typical pair of electropherograms from the short and long portions of a single capillary tube. The highly symmetrical lysozyme peak coming out from the short effective length deteriorates significantly at the long effective length owing to the tailing that results, at least in part, from the delayed initial desorption. A direct inference from this observation is that the minimum effective length to which Eq. 6 applies must be determined for a CE system prior to calculate protein recovery from this method. When we applied Lucy's experimental approach consisting of repeating peak area measurements after shortening the capillary tube total length from 50 to 35 cm, no anomalous behavior was observed. This alternate two-point straight line experiment gives k and R values which are moderately comparable with those from the regression (see Table 1). Estimated precision, however, is poorer for the two-point straight line approximation, particularly for k . Precision data were estimated from conventional random error propagation analysis. Details are given as Supporting Information.

Electric field effects

The dependence of recovery with the extent of protein exposure to the silica surface suggests that a brief exposure may lead to an improved recovery. Besides using a short effective length, the time of residence of the protein in the capillary tube can be further shortened by increasing the applied electric field strength. In this work, we use PIPPS, a zwitterionic buffer whose low conductivity enabled us to apply high voltage gradients without significant overheating. As shown in Supporting Information, Ohm's law applies fairly well up to 500 V/cm. The percent recovery of lysozyme increases rapidly at low to moderate strengths, while the effect is progressively less pronounced at higher field strengths, as illustrated in Figure 3. Needless to say, this result emphasizes our former suggestion that all CE conditions must be fully documented in a protein recovery report.

Comments on selected literature reports

Regnier and coworkers as well as Lucy and his research group determined protein loss on a capillary tube by subtracting peak areas either between two detectors (Regnier) or between an original and shortened capillary (Lucy). To the best of our knowledge, they never provided an explicit equation for their calculations. More recently though, Liu *et al.* (19) and afterward Jurcic and Yeung (20) applied Regnier concept to assess protein recovery in coated capillaries while clearly stating that recovery, R' , was calculated from the peak area ratios obtained from the long (A_l) and the short (A_s) capillaries:

$$R' = \frac{A_l}{A_s} \quad (10)$$

Unlike Eq. 1a, this expression should result in a biased protein recovery value. In order to present a more meaningful comparison, let us express R' in terms of R by combining Eq. 10 with Eq. 9 and rearranging:

$$R' = R^{\frac{l-s}{l}} \quad (11)$$

Clearly, Eqs. 9 and 10 should deliver different results, except in the extremes cases where $R=0$ or $R=1$. Otherwise R' is always greater than R , the "true" value, as shown in Figure 4 (continuous red curve).

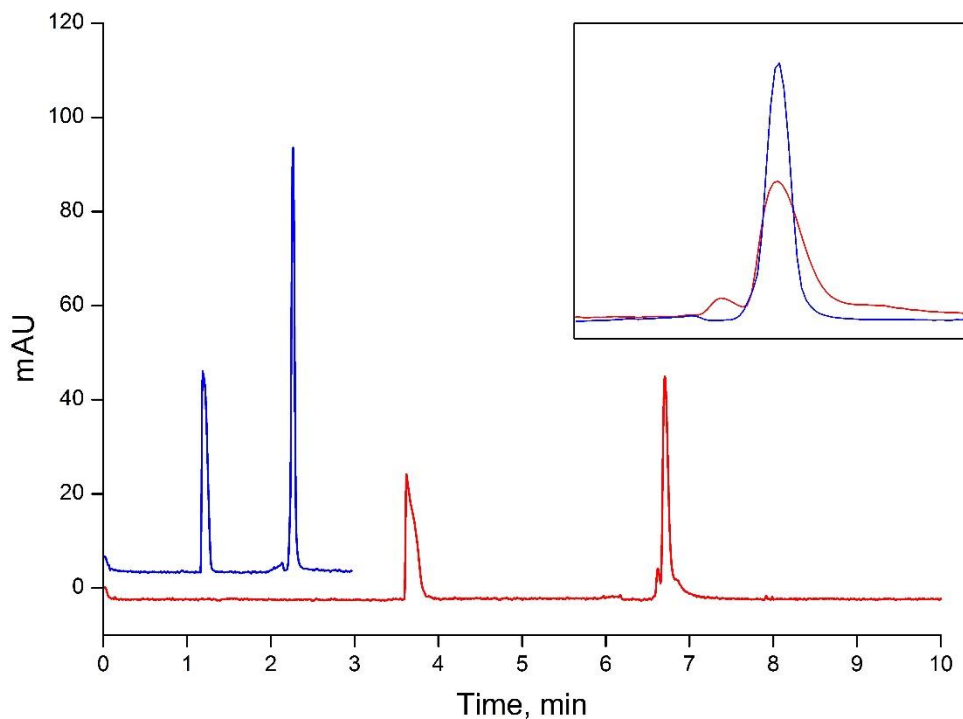


Figure 2. Typical pair of electropherograms obtained from a benzyl amine/lysozyme sample injected at the short ($s = 8.5$ cm, blue curve) and then the long ($l = 26.5$ cm, red curve) length of a single capillary tube. A negative voltage was applied in the later case. In both electropherograms the early peak corresponds to benzyl amine. The insert compares the lysozyme peak profiles on expanded time scale.

Furthermore, Taverna *et al.* made a different interpretation of Regnier concept (11,21,22). They acknowledged the necessity of including the theoretical A_0 parameter as a reference in their recovery percentage calculation:

$$\frac{A_0 - A_s}{A_0} = \frac{A_s - A_l}{A_s} \quad (12)$$

They arrived at a different expression for recovery, R'' :

$$R'' = \left(\frac{A_l}{A_s} \right)^2 \quad (13)$$

Again, when Eq. 13 is combined with Eq. 9 and rearranged, one obtains

$$R'' = R^{\frac{2(l-s)}{l}} \quad (14)$$

This equation leads to the dot-dashed green curve of Figure 4. It appears that Eq. 13 exhibits a similar bias, although to a larger extent, in comparison to Eq. 10.

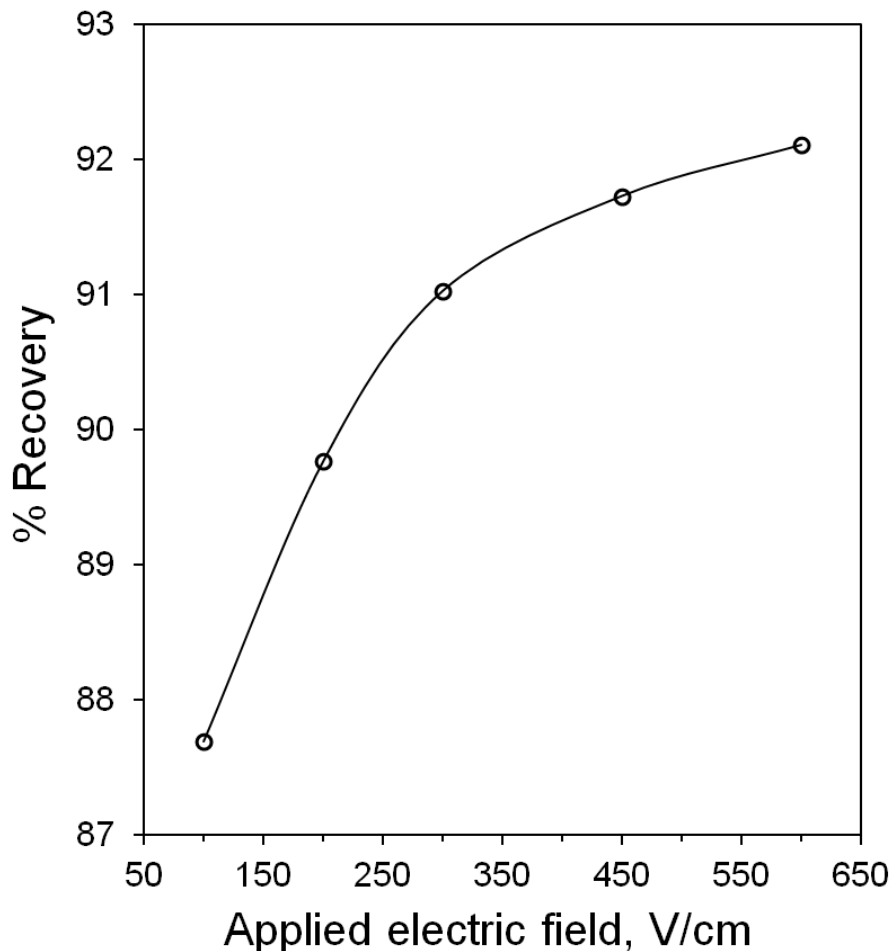


Figure 3. Percent recovery of lysozyme as a function of applied electric field strength. Five replicate injections were performed on a 50.0-cm ($l = 41.5$ cm) capillary. The tube was then shortened to 35.0 cm ($s = 26.5$ cm) and the injections were repeated; rinse and injection pressures were shortened accordingly. Other experimental conditions as in Figure 1.

Yet, another interpretation of Regnier method can be found in the recent literature. Descroix and coworkers apparently assumed the decrease in peak area to be proportional to the migrated distance alone (that is, they applied Regnier original concept) but somehow utilized Eq. 5 instead, and ended up with an expression equivalent to Eq. 9 (23). Although these authors had previously reported this equation, a systematic description of the underlying mechanism was still missing from their paper.

Interestingly, when one tests the original Regnier assumption that the decrease in peak area was proportional to the effective capillary length only (9), a reasonably straight line (within the experimental uncertainty) also turned out, as shown in Figure S-5 of the accompanying Supporting Information. This experimental "evidence" of a zero-order for the absorption process with respect to protein concentration would mean that protein peak area decreases at a constant rate as the plug travels along the capillary:

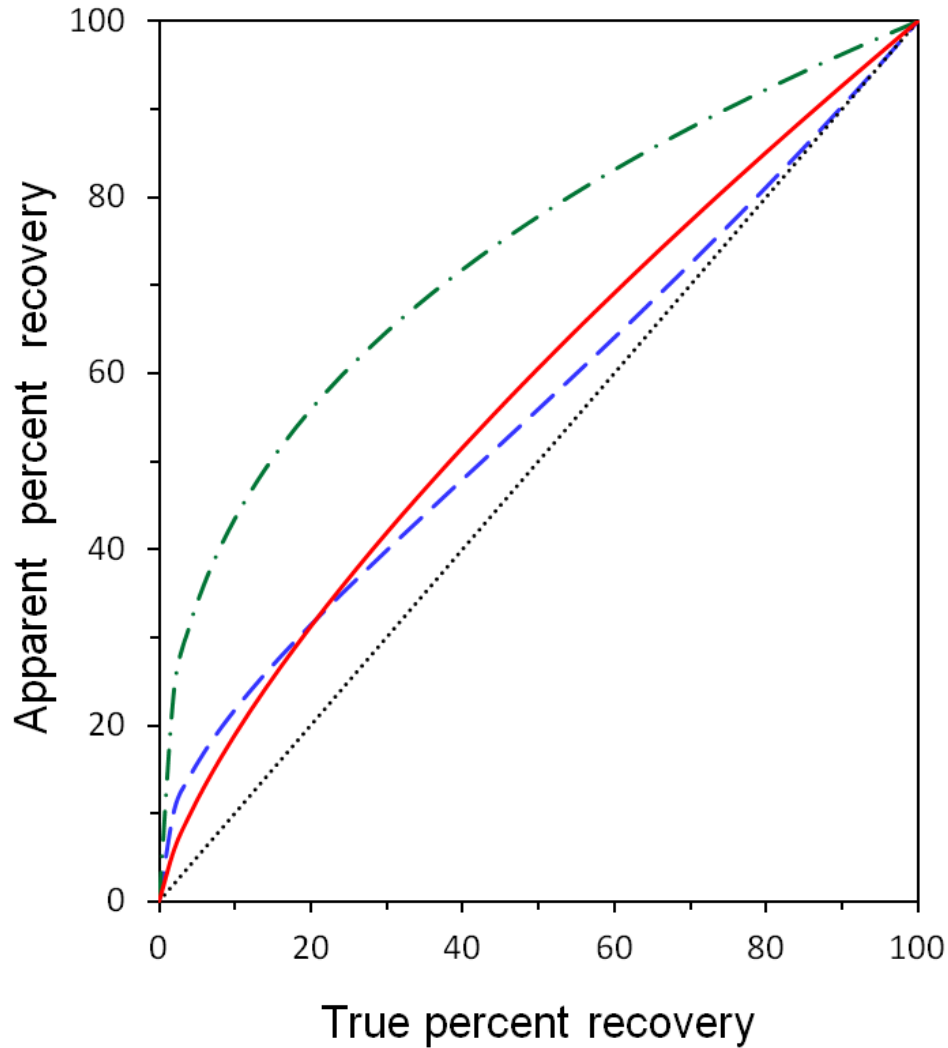


Figure 4. "Apparent" percent recoveries from three different interpretations of Regnier's procedure to evaluate protein adsorption, as a function of the true recovery values. The dotted line indicates unbiased results, according to Eq. 9 (this work); the continuous red, dot-dashed green and dashed blue curves represent percent recoveries results in terms of the true recovery value, according to Eqs. 11, 14 and 19, respectively. In all cases $l = 41.5$ cm and $s = 26.5$ cm.

$$dA = -kdl \quad (15)$$

Whereas Eq. 5 makes more physical sense, let us for a moment go along with Eq. 15 and allow it to lead us to the following equation for recovery:

$$R''' = \frac{A}{A_0} = \frac{A_0 - kl}{A_0} \quad (16)$$

After applying the two-point straight line approximation, one obtains

$$R''' = \frac{A_l}{A_0} = \frac{(l-s)A_l}{lA_s - sA_l} \quad (17)$$

Finally, the following equation yields R''' in terms of R , as illustrated by the dashed blue curve in Figure 4:

$$R''' = \left(\frac{lR}{l-s+sR} \right)^{\frac{l}{l-s}} \quad (19)$$

Notice also that, unlike R –that moves asymptotically toward zero (Eq. 7), the R''' figure can become negative for a sufficiently long capillary. On the other hand, at recoveries above 80%, R''' -values closely approach those of the true value, a fact that is consistent with the apparent linearity of the peak area *vs.* effective length plot. Nevertheless, such presumed proportionality should not justify the use Eq. 16 or 17, which rely on an unproven hypothesis for protein adsorption.

Concluding remarks

We have experimentally confirmed that the hypothesis considering the amount of protein adsorbed to be proportional to protein concentration as well as the migrated distance is a valid one over a reasonably wide range. The lack of an explicit equation for protein recovery during its initial stage has resulted in different interpretations of Regnier concept. Protein recovery, as determined by this method, does depend on the capillary dimensions as well as the applied electric field; consequently, it is not an intrinsic property of the capillary silica surface, as usually thought. Thus, such protein recovery does not have exactly the same worth as the established analyte recovery figure obtained from sample spiking. Nevertheless, the slope k should be a useful figure to comparatively assess protein adsorption on different capillaries or BGE additives. Moreover, it is important to experimentally determine the minimum effective length to which Eq. 6 applies for a given CE instrument prior to quantify protein adsorption. This is a consequence, at least in part, of the delayed protein desorption that occurs at the beginning of the capillary tube.

Acknowledgments

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Supporting information

Fig S-1 shows the benzyl amine peak area *vs.* injection number for a series of capillaries of increasing effective length, indicating that the internal standard did not undergo any significant adsorption during the whole experiment described in Figure 1 of main text.

Fig S-2 shows the logarithm of benzyl amine peak area as a function of effective length. The lack of correlation between the variables and a slope not significantly different from zero confirm the absence of significant adsorption of the internal standard on the capillary inner surface.

The effect of using benzyl amine as internal standard on relative peak precision is presented in Figure S-3.

Figure S-4 shows a typical Ohm's plot which supplements the information in Figure 3 of main text.

Figure S-5 shows that when the same peak area data points that produced Figure 1 of main text are used to plot peak area *vs.* effective length a reasonably straight line also results.

Table S-1 relates the equations describing the various analytical parameters of Table 1 (k and R) in main text with those resulting from conventional random propagation analysis.

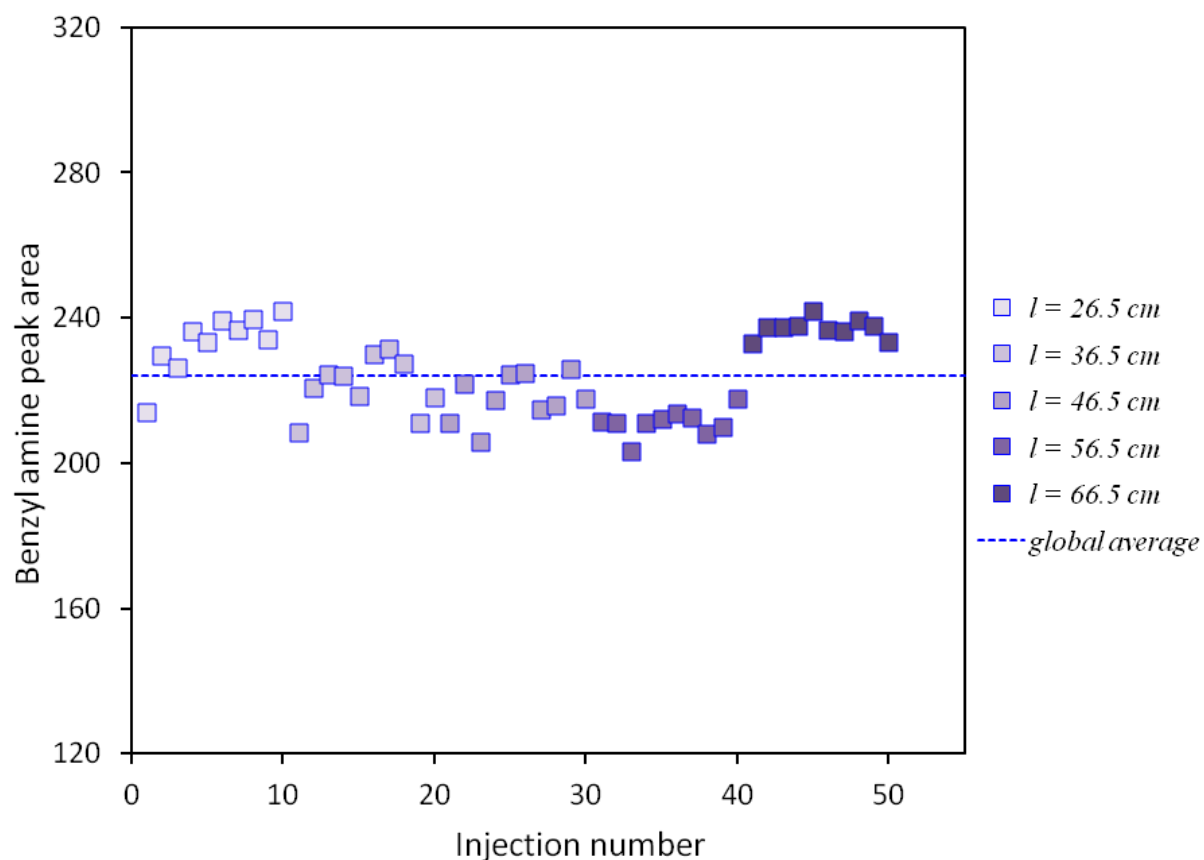


Figure S-1. Plot of benzyl amine peak area *vs.* injection number for a series of capillaries of increasing effective length. The peak area of the internal standard remained essentially constant, within the experimental uncertainty. CE conditions as described in Figure 1 of main text.

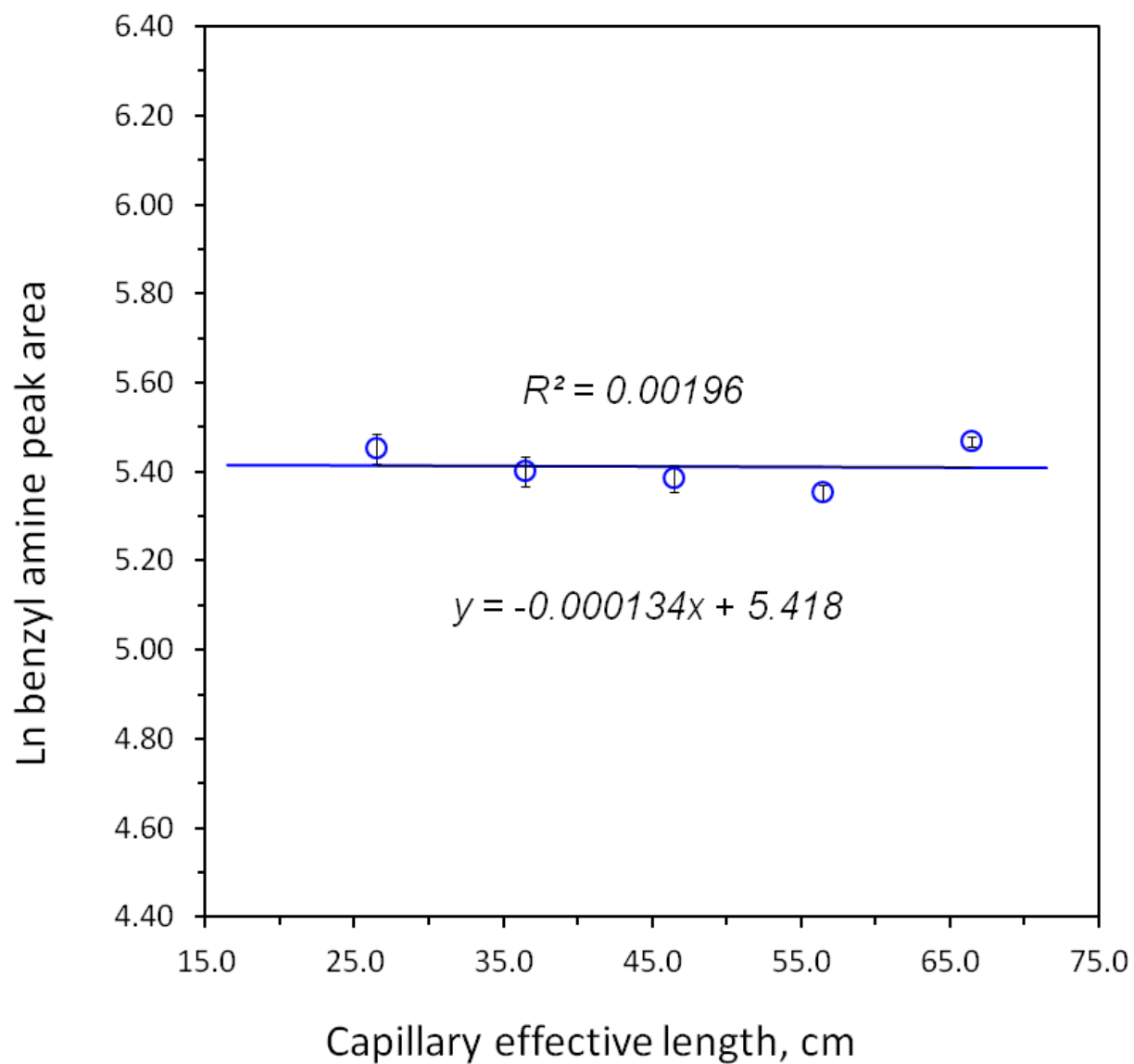


Figure S-2. Logarithm of benzyl amine peak area as a function of effective length. Notice the lack of correlation between the variables and a slope not significantly different from zero. Experimental conditions as in Figure 1 of main text.

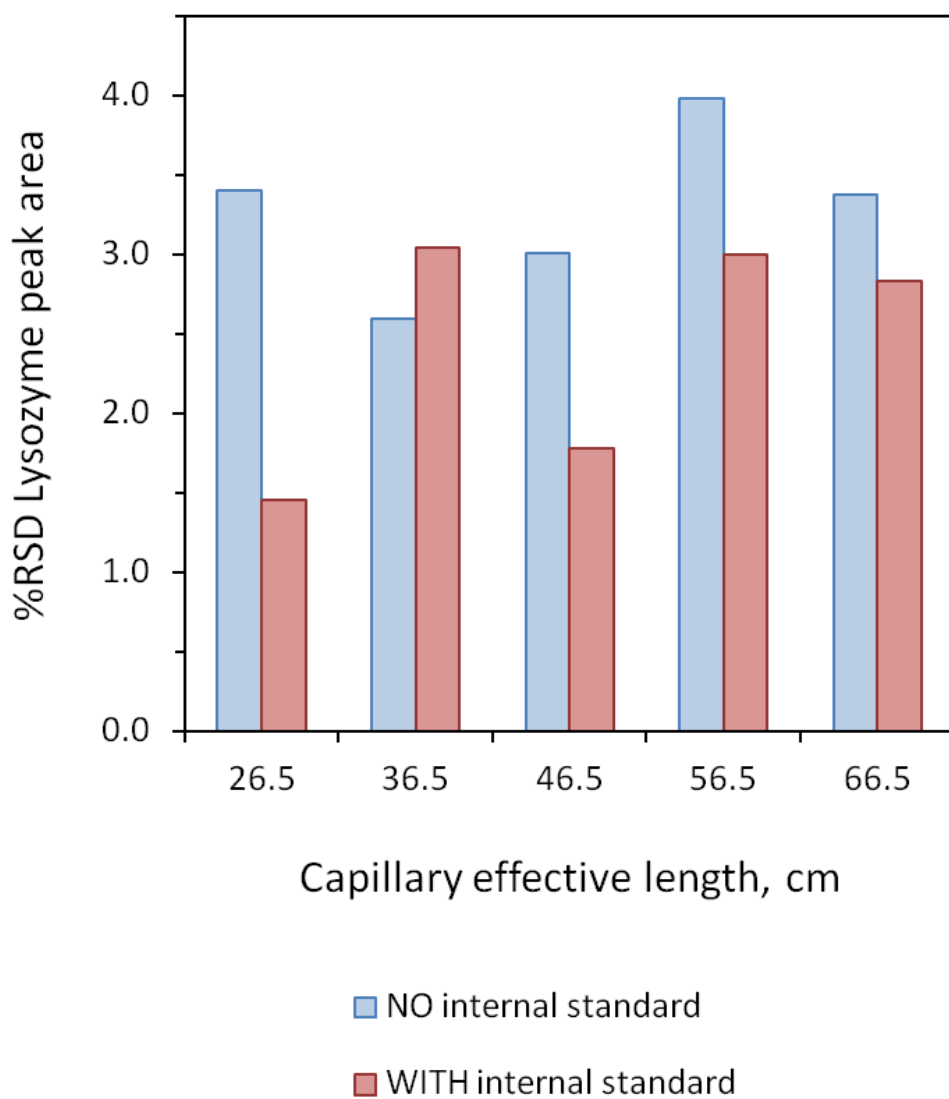


Figure S-3. Comparison of the relative precision ($n = 10$) obtained without and with benzyl amine as internal standard. Experimental conditions were as described in Figure 1 of main text.

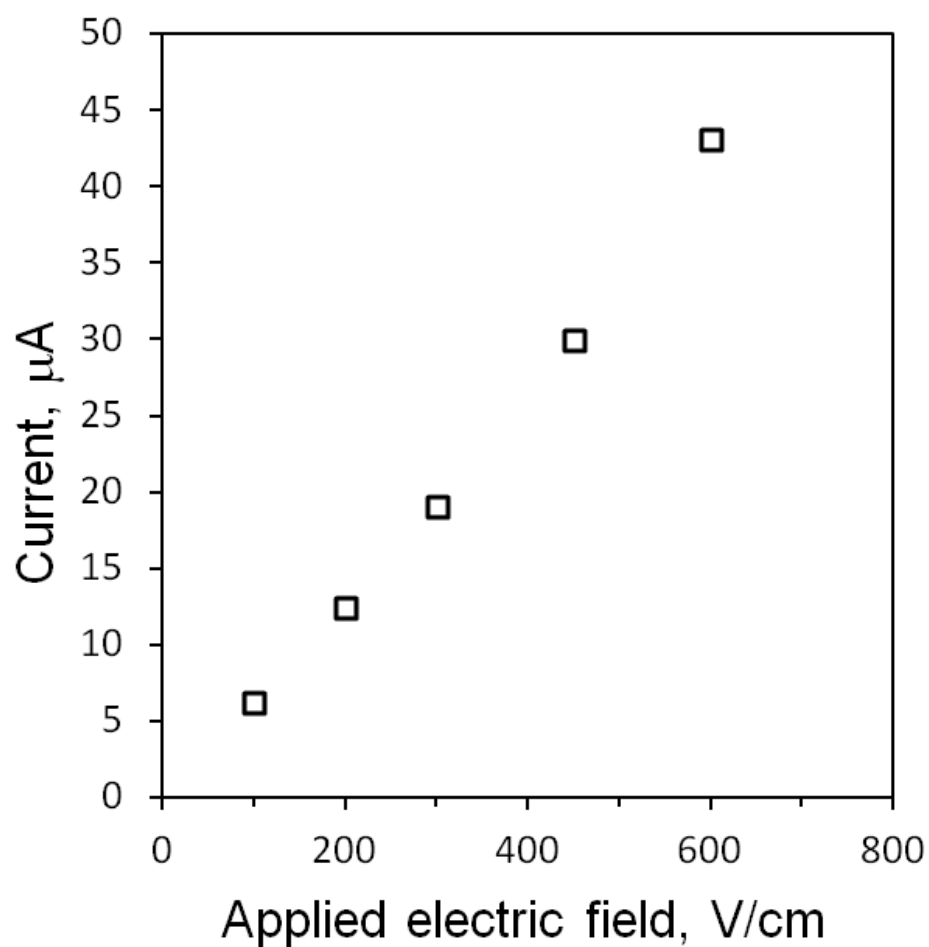


Figure S-4. Electric current through the BGE solution as a function of applied electric field strength. Measurements were performed on a 50.0-cm ($l = 41.5$ cm) capillary. No significant differences were observed when the capillary was shortened to 35.0 cm ($s = 26.5$ cm) and the measurements were repeated. Experimental conditions as described in Figure 3 of main text.

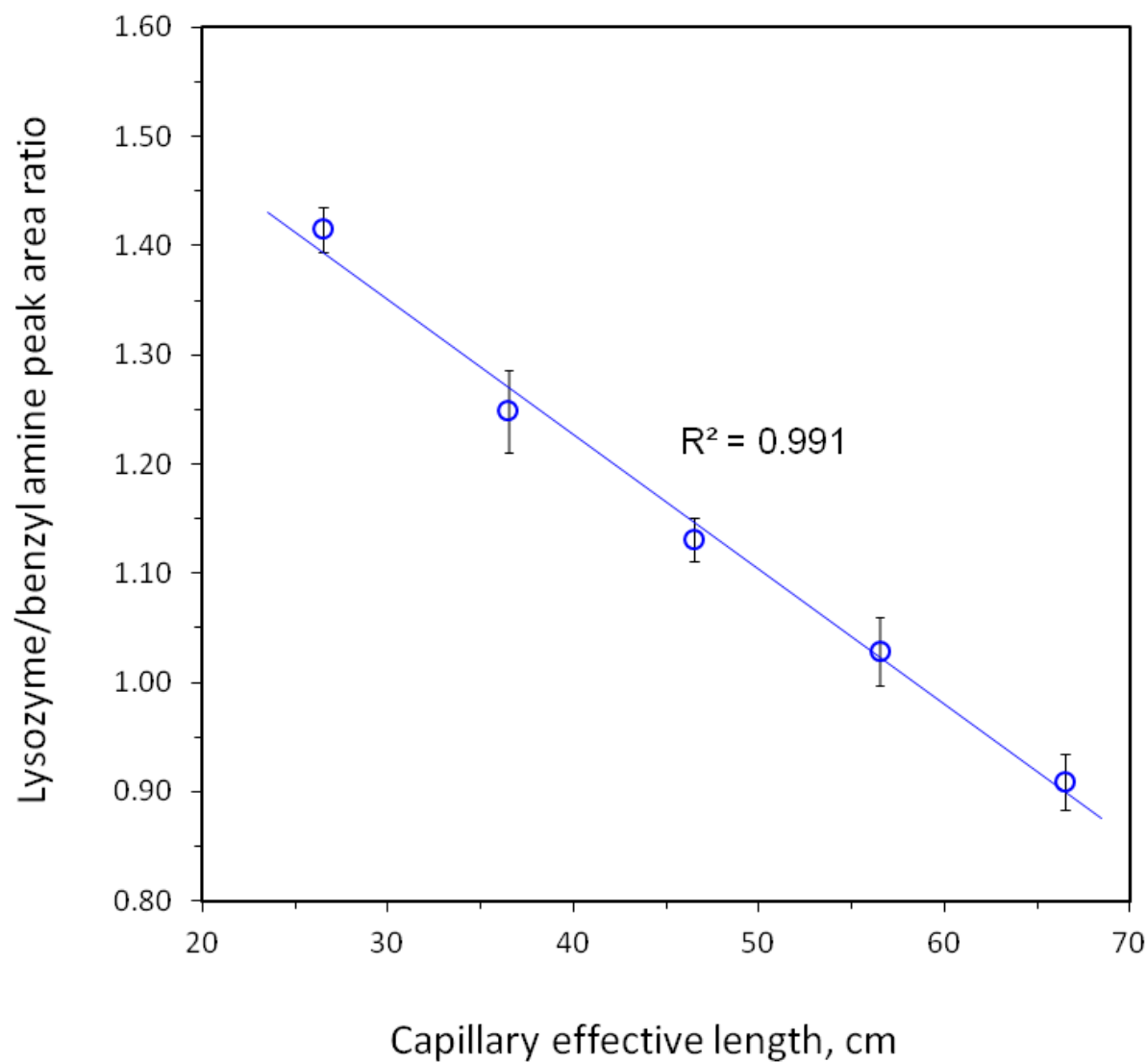


Figure S-5. Peak area of lysozyme as a function of its migrated distance. Experimental data points are the same for Figure 1 of main text.

Table S-1. Equations used to estimate precision of important analytical figures obtained from a five-point linear regression and a two-point linear approximation.*Five-point linear regression*

	Parameter	Relative standard deviation
Slope, k ^a	k	$\frac{s_k}{k}$
Recovery fraction, R	$R(s) = e^{-ks}$	$\frac{s_{R(s)}}{R(s)} = s \cdot s_k$

Two-point linear approximation

	Parameter	Relative standard deviation ^b
Slope, k	$k = \frac{1}{l-s} \ln \frac{A_s}{A_l}$	$\frac{s_k}{k} = \frac{\sqrt{\left(\frac{s_{A_l}}{A_l}\right)^2 + \left(\frac{s_{A_s}}{A_s}\right)^2}}{\ln \frac{A_s}{A_l}}$
Recovery fraction, R	$R(s) = \left(\frac{A_l}{A_s}\right)^{\frac{s}{l-s}}$	$\frac{s_{R(s)}}{R(s)} = \frac{s}{l-s} \sqrt{\left(\frac{s_{A_l}}{A_l}\right)^2 + \left(\frac{s_{A_s}}{A_s}\right)^2}$

^a From regression. ^b Assuming uncertainties in length measures (l and s) to be insignificant compared to those in peak area measures (A_s and A_l).

Neutral hydrophilic coatings for capillary electrophoresis prepared by controlled radical polymerization

Abstract

In the present study, porous silica particles as well as impervious fused-silica wafers and capillary tubes were modified with hydrophilic polymers (hydroxylated polyacrylamides and polyacrylates), using a surface-confined grafting procedure based on atom transfer radical polymerization (ATRP) which was also surface-initiated from α -bromoisobutyryl groups. Initiator immobilization was achieved by hydrosilylation of allyl alcohol on hydride silica followed by esterification of the resulting propanol-bonded surface with α -bromoisobutyryl bromide. Elemental analysis, IR and NMR spectroscopies on silica micro-particles, atomic force microscopy, ellipsometry and profilometry on fused-silica wafers, as well as CE on fused-silica tubes were used to characterize the chemically modified silica substrate at different stages. We studied the effect of monomer concentration as well as cross-linker on the ability of the polymer film to reduce electroosmosis and to prevent protein adsorption (*i. e.*, its non-fouling capabilities) and found that the former was rather insensitive to both parameters. Surface deactivation towards adsorption was somewhat more susceptible to monomer concentration and appeared also to be favored by a low concentration of the cross-linker. The results show that hydrophilic polyacrylamide and polyacrylate coatings of controlled thickness can be prepared by ATRP under very mild polymerization conditions (aqueous solvent, room temperature and short reaction times) and that the coated capillary tubes exhibit high efficiencies for protein separations (0.3 -0.6 million theoretical plates per meter) as well as long-term hydrolytic stability under the inherently harsh conditions of capillary isoelectric focusing. Additionally, there was no adsorption of lysozyme on the coated surface as indicated by a complete recovery of the basic enzyme. Furthermore, since polymerization is confined to the inner capillary surface, simple precautions (*e.g.*, solution filtration) during the surface modification process are sufficient to prevent capillary clogging.

Key words: ATRP grafting from, hydride silica, hydrosilylation, hydroxylated monomers, hydrolytic stability

Introduction

Fused-silica capillary tubes used in GC and micro-bore LC have also found extensive use in the modern version of electrophoresis, CE (1, 2). In contrast with chromatography, chemical modification of the fused silica capillary has been aimed primarily at eliminating unwanted interactions between the inner wall of the CE tube and the sample undergoing separation. Many chemical modifications schemes used

to modify silica-based chromatographic substrates have been readily extended to electrophoretic capillaries (3-5). Strictly speaking, the type and number of active surface functionalities on silica surfaces (silanols, siloxanes, etc.) depend on factors such as synthesis method, thermal history and the presence of humidity. Additionally, the irregular porous structure of silica micro-particles governs the accessibility of reagents to the active silica sites during surface modification. This kinetic factor aside, the chemistries of porous and flat silicas are essentially equivalent. It is also accepted that wafer surfaces are geometrically and chemically equivalent to the inner surface of fused-silica capillaries; they are both flat at the molecular level. Surface coverage resulting from organic groups grafted on a flat silica surface should be significantly denser than that of the same groups on a curved surface (particulate porous silica) since the later is sterically more constrained resulting in dissimilar group conformations (6).

One of the greatest challenges in the current practice of CE is the requirement of surface deactivation of capillaries toward protein adsorption. Such unsolved bottleneck arises from the nonspecific interactions (ion exchange, hydrogen bonding, dispersion, etc.) between the inner capillary wall and the solute. Such unwanted interactions are responsible for excessive peak tailing, incomplete solute recovery and unreliable quantification. As a result, the high speed, separation efficiency, selectivity and versatility, minimal sample size requirement and easy automation of CE cannot be fully exploited in the case of many proteins. Researchers have suggested that a polymeric film that furnishes a hydrophilic, stable and adsorption-resistant (non-fouling) barrier between the capillary wall and the solute is required in protein analysis (7, 8). Such materials are also of great interest in many other fields such as medical sciences, biosensors, contact lenses technology, enzyme-based immuno assays, to name just a few (9).

The attachment of polymers to solid surfaces via chemical bonding is an important strategy to modify the substrate properties in such a manner that the nonspecific interactions between the protein molecules and silica surface are minimized (7, 10). Anchoring of a polymer onto a silica surface has been achieved by two main strategies, the "grafting-to" and the "grafting-from" methods. The grafting-to approach involves the attachment of a prefabricated polymer from solution, via the formation of a covalent bond between polymer active groups and matching groups on the substrate surface. Water-soluble polymers such as poly(ethylene glycol) and poly(vinyl alcohol) are typical examples of preformed polymers used in the grafting-to technique. Hydroxyl groups of the polymer react with surface-immobilized active species, such as glycidyl (11). Despite its experimental simplicity, the grafting-to strategy is limited by strong steric hindrance effects that worsen with increasing polymer size and eventually hinder contact of incoming polymer with surface reactive sites (10). In the grafting-from approach, propagating polymer chains grow from surface-immobilized initiator groups. Among the various procedures for the grafting-from method, atom transfer radical polymerization (ATRP) is especially attractive for its remarkable control over the molar mass of the grafted polymer, great versatility (works well with a variety of functionalized monomers), compatibility with water and the possibility of mild polymerization temperatures (12-14). In the ATRP-based grafting-from approach, chain transfer and thermal self-initiation processes are essentially negligible and polymeric chains grow exclusively from the surface; *i.e.*, the process is both surface-initiated and surface-confined. The initiator moieties are most usually anchored to the silica surface via silane coupling chemistry. Halogenated ATRP initiator groups, such as benzyl chloride or, to a much greater extent, 2-bromo-isobutanoyl have

been used in the past to grow neutral hydrophilic films on fused silica capillaries. Wirth and her research group reported the first covalent bonding of a polymer film for CE by ATRP in 1998 (15). Surprisingly, very few papers have been published about ATRP applied to CE since then (16-18).

Although acrylamide has been the most commonly used monomer to make polymeric coatings for CE (4, 7), the limited stability at moderately high pH of polyacrylamide has been a well-known fact from traditional slab gel electrophoresis (19). The slow deamidation of the polymer under this condition leads to a considerable deterioration of the coating, evidenced by the formation of fully-dissociated carboxylic groups that cause strong electroosmosis, polymeric layer swelling and analyte band distortion. The use of N-substituted acrylamide derivatives such as N-acryloylamino-ethoxyethanol, has resulted in coated capillaries with superior resistance to hydrolysis and hence improved long-term CE separations at alkaline pH (20). N-acryloyl-aminoethanol (AAE) (21-23) and its relative N-acryloyl-aminopropanol (AAP) (24, 25), also N-substituted acrylamide derivatives, should provide durable coatings as well. It appears that the N-substitution with hydroxyl-terminated chains produces polymeric coatings that are not only hydrolytically more stable, but have also higher hydrophilicity compared to polyacrylamide (19, 24, 25). When it comes to electrophoretic performance, hydrophilicity turns out to be of paramount importance since a high hydrophilicity of the polymer effectively precludes proteins to compete with water for its potentially adsorptive sites (26). It has been suggested that any monomer less hydrophilic than acrylamide should be considered unsuitable to produce a good quality gel “since acrylamide is itself already at the border-line between hydrophilicity and hydrophobicity” (25).

There is a definite need for hydrophilic coatings of improved hydrolytic stability that enable the use of CE at its full potential, and such improved materials are frequently targeted at protein separations. In the present work, we explore the combination of several promising synthetic schemes to modify the fused-silica surface of capillaries for a lasting resistance to protein adsorption. More specifically, our work attempts to bring together the best of three worlds: (i) a stable anchorage of the polymerization initiating group to the inner wall of the capillary tube by means of Si–C linkages formed by hydrosilylation; (ii) a stable polymeric film whose strength arises from the N-substitution on acrylamide; and (iii) a surface-confined *in-situ* polymerization method (ATRP) that prevents clogging of the capillary tube while permitting an easy control of the coating thickness.

Materials and Methods

Instrumentation

IR spectroscopy was performed on a Shimadzu, Model FTIR-8400 spectrometer (Columbia, MD, U.S.) equipped with a diffuse reflectance infrared Fourier transform (DRIFT) accessory. Solid state NMR characterization was carried out using a Bruker Advance II-400 MHz NMR spectrometer (Rheinstetten, Germany) equipped with a Bruker MAS II probe. Electrophoretic separations were performed on Agilent model 7100 or 1600 CE Systems (Palo Alto, CA, U.S.). Atomic force microscopy (AFM) images were obtained with an Asylum Research model MFP-3D-SA (Santa Barbara, CA, U.S.) instrument.

Carbon percent measurements were carried out on a FlashEA 1112 elemental analyzer Thermo Inc. (Waltham, MA, U.S.).

Materials

Fused-silica capillaries (50- μm id, 360- μm od and 20- μm polyimide coating) were purchased from Biotag Inc. (Silver Springs, MD, U.S.) and Polymicro Technologies (Phoenix, AZ, U.S.). UV-grade fused-silica wafers (25.4 x 9.0 x 1.0 mm) were purchased from Laser Optex Inc. (Beijing, China). Silica micro-particles (Nucleosil, 7- μm diameter and 91.0- $\text{m}^2 \text{g}^{-1}$ surface area, and YMC Gel, 10- μm diameter and 288- $\text{m}^2 \text{g}^{-1}$ surface area) were obtained from Macherey-Nagel (Düren, Germany) and YMC Co. (Kioto, Japan) respectively. Piperazine-N, N'-bis(3-propanesulfonic acid) (PIPPS) was purchased from GFS Chemicals (Columbus, OH, U.S.). N-acryloyl-aminoethanol, N-acryloyl-aminopropanol, 2-hydroxyethyl methacrylate, α -bromoisobutyryl bromide, tris[2-(dimethylamino)ethyl]amine (Me_6TREN), bipyridine (bpy), allyl alcohol, platinum(0)-1,3-divinyl-1,1,3,3-tetramethyldisiloxane (Karstedt's catalyst, ~2 % Pt in xylene), hexachloroplatinic acid, cuprous chloride, 2,5-di-tert-butylhydroquinone, potassium cyanide, EDTA, 3-(N-morpholino)propanesulfonic acid (MOPS), methylcellulose, Ampholytes pH 3-10 and pH 6-8 (40% aqueous solutions) and DMSO were purchased from Sigma-Aldrich (St. Louis, MO, U.S.). Analytical grade solvents were obtained from various vendors; they were freshly distilled from sodium shavings before use.

Surface modification

Prior to modification, fused-silica capillaries (typically, a 6.0-m length) and wafers were conditioned by etching with NaOH followed by leaching with HCl, according to procedures described elsewhere (27). Porous silica particles did not undergo any conditioning. Hydride silica substrates were prepared as described previously (28). 3-hydroxypropyl (propanol) bonded silica phase was prepared by hydrosilylation of allyl alcohol on hydride silica, as described in a recent report (29). Esterification of the bound propanol groups was carried out by treatment for 6 h at room temperature ($20 \pm 2^\circ\text{C}$) with a solution containing 0.25 M of α -bromoisobutyryl bromide and 0.25 M of pyridine in dry DMF under a nitrogen blanket (30-32). This solution was prepared in a nitrogen-purged glove bag. The product was sequentially rinsed with 10%v/v water in DMF and THF, and then dried under nitrogen. Polymerization of 2-hydroxyethyl acrylate (HEA), AAE and AAP was catalyzed with $\text{Cu}^{\text{I}}(\text{Me}_6\text{TREN})^+\text{Cl}^-$ in 4:1 v/v ethanol/water solvent (23). 2-Hydroxyethyl methacrylate (HEMA) was polymerized with $\text{Cu}^{\text{I}}(\text{bpy})_2^+\text{Cl}^-$ in 1:1 v/v methanol/water solvent (33). For a typical polymerization in a capillary, a solution containing 1.0 M monomer and 20 mM Cu(I) catalyst in alcohol/water solvent was passed through the column for 3 h at room temperature (18, 30). This solution was prepared with helium-degassed solvent in a nitrogen-purged glove bag. At the reaction end, the column was sequentially rinsed with solvent and water for 30 min each. The column was emptied with nitrogen gas for a few minutes. Fused-silica wafers were modified by immersion in the reagent solution with gentle shaking, while 65 mg of porous silica were used per mL of reagent solution with magnetic stirring.

Spectroscopy and microscopy

DRIFT, solid state NMR and AFM measurements were obtained accordingly to previously described procedures (28). An Uvisel 2 Spectroscopic Phase Modulated ellipsometer was used for film thickness measurements at Horiba Scientific laboratories (France). Perfilometry measurements were carried out with a KLA Tencor D-120 stylus profiler (U.S.) at the Materials Engineering School of Universidad del Valle.

Surface coverage

The concentration, Γ_R , of surface-bonded R-groups on porous silica was obtained from elemental analysis of the bonded material along with the BET specific surface area of the hydride intermediate substrate (34).

CE experiments

Prior to use, capillaries were conditioned with the electrolyte (typically, 25 mM PIPPS, adjusted to pH 4 with NaOH) under high pressure (7 bar) for 20 min, after which a stable electroosmotic mobility (EOM) value was obtained (27). EOM determinations followed methodology previously described in the literature (35, 36). CIEF separation conditions were adapted from a method devised by Hempe *et al.* (37, 38).

Results and discussion

Silica substrate modification

The synthetic scheme used to chemically modify the inner capillary surface with a polymeric film is outlined in Figure 1. Initiator immobilization is accomplished by hydrosilylation of allyl alcohol on hydride silica followed by esterification of the resulting propanol-bonded surface with α -bromoisobutyryl bromide. The first step is based on the formation of a stable Si–C bond by the catalytic addition of silicon hydride to an olefin (hydrosilylation). We have recently demonstrated that allyl alcohol can also be anchored to the hydride silica surface and that it does so with a high coverage (3–5 $\mu\text{mol m}^{-2}$) (29). In the esterification of the anchored alcohol groups with α -bromoisobutyryl bromide, we found that using DMF as solvent and pyridine as HBr-binding base maximize the solubilization of the bromide salts produced during the esterification process. This is important to minimize clogging of the tube as the reaction is performed on fused-silica capillaries. When this two-step procedure is applied on porous silica micro-particles, ligand density information from the primary (hydrosilylation) as well as the secondary (esterification) modifications can be readily estimated from percent carbon contents along with specific surface area and structural information (molar mass and number of carbon

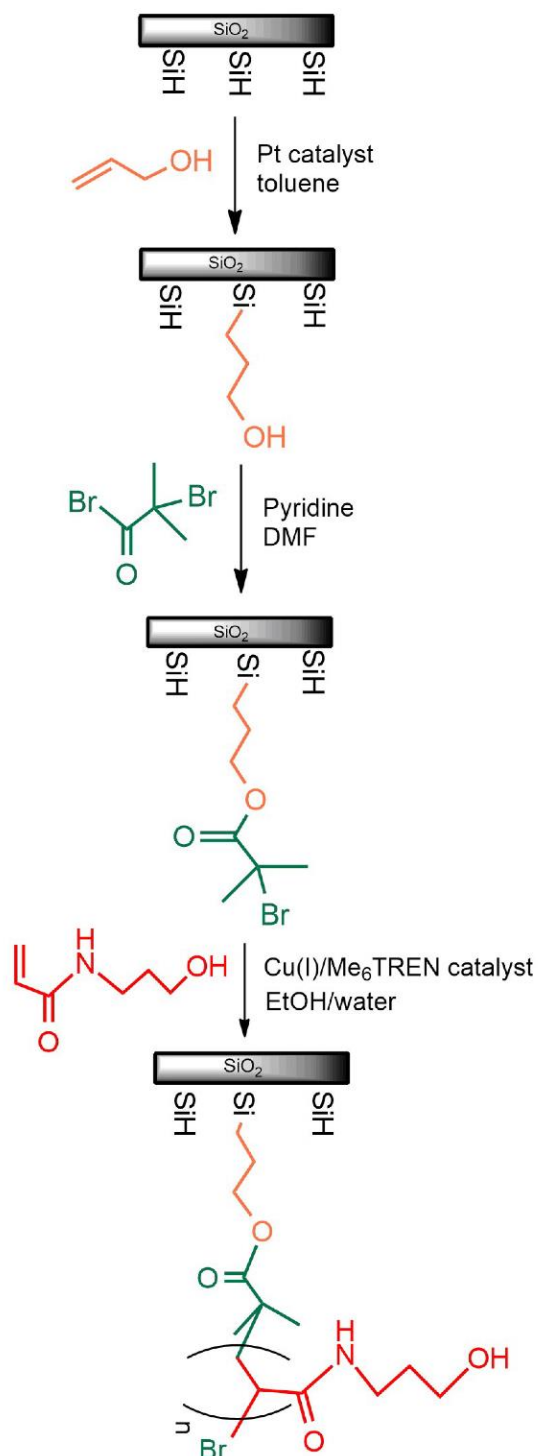


Figure 1. Schematic diagram illustrating the hydrosilylation of allyl alcohol on the Si-H surface, followed by esterification of the propanol-functionalized silica surface to immobilize the α -bromoisobutyryl initiator, and then by surface-initiated and -confined polymerization of N-acryloyl-aminopropanol via ATRP.

atoms) of the anchored groups (34). Our results indicate that a typical propanol-modified substrate with a surface coverage of $3.5 \mu\text{mol m}^{-2}$ ($2.1 \text{ chain nm}^{-2}$) produced an α -bromoisobutyryl coverage of $1.1 \mu\text{mol m}^{-2}$ ($0.75 \text{ chain nm}^{-2}$), which means that about 30% of the surface propanol groups were esterified. One can think of the resultant functionalized silica surface as consisting of active α -bromoisobutyryl initiator moieties surrounded by a dense layer of inactive propanol groups. Naturally, denser surface coverages of the α -bromoisobutyryl groups should be expected on a flat silica surface in comparison to the same groups on a curved surface such as that of porous silica. Since the average cross sectional area of a polymer chain ($\sim 1.8\text{--}2.0 \text{ nm}^2$) is much larger than that of a typical initiator group ($\sim 0.20 \text{ nm}^2$) (39, 40), our relatively low α -bromoisobutyryl coverage ($1.5 \text{ nm}^2 \text{ chain}^{-1}$) should provide the footprint surface area required for a dense polymer grafting quite well, as described below. Previous studies show that such modest α -bromoisobutyryl group density favors an efficient polymer growth (41).

When we applied our ATRP-based grafting-from approach to several hydroxylated monomers –namely, HEA, HEMA, AAE, and AAP– efficient polymerization took place. Monomer structures are depicted in Figure S-1 of the Supplementary Material. It is important to point out that we used mild conditions for the polymerization reaction: aqueous solvent, room temperature and short reaction times, as suggested by Armes (31, 33) and Kakuchi (23). These conditions represent a significant departure from those proposed by Wirth in her original report (100°C , dry DMF solvent, and 40-h reaction time) (15). Undoubtedly, the milder conditions are more attractive while still favoring good grafting density of the polymer. Furthermore, we used a much more common and (about 500 times) more active α -bromoisobutyryl group as initiator. Figure 2 shows IR spectroscopic evidence that confirms polymerization from the immobilized α -bromoisobutyryl initiating groups. A substantial increase of stretching bands in the $2950\text{--}2850 \text{ cm}^{-1}$ region is observed, which is indicative of symmetric and asymmetric aliphatic C-H stretching, along with the appearance of characteristic C=O stretching bands at 1655 and 1730 cm^{-1} for amides (curves B and C) and esters (curves D and E) respectively. Most likely, the bands at 3085 and 1550 cm^{-1} (curves B and C) can be assigned to the amido N–H stretching and bending modes respectively. Furthermore, ^{13}C CP-MAS NMR spectra clearly verifies chemical bonding of the polymeric film to the silica surface, as shown in Figures S-2 to S-4 of the Supplementary Material. To further characterize the polymeric film on the silica surface, AFM was used on coated silica plates by probing the surface after polymerization. As shown in Figure 3, the surface is uniformly coated with the polymeric film and exhibits a more rugose surface topography (rugosity = 3.16 nm), in comparison to an unmodified plate ($\sim 0.3 \text{ nm}$) (28). Thickness measurements with ellipsometry revealed a uniform film with a layer thickness of $53 \pm 2 \text{ nm}$. Additionally, profilometry measurements provide some thicknesses of $59 \pm 8 \text{ nm}$, which is quite consistent with that from ellipsometry. One intrinsic feature of ATRP-based grafting-from process is that the polymerization is confined to the substrate surface. Since free initiator is absent in the monomer/catalyst mix, no polymer is formed in the bulk of the solution, avoiding blockage of the capillary column. Additionally, a simple rinsing procedure provides adequate film cleanup. These details are of critical importance in the preparation of chemically modified CE tubes.

Electrophoretic performance

Chemical modification of CE tubes is accompanied by a drastic drop of electroosmosis, the electrokinetic bulk flow of electrolyte originated at the wall/solution boundary. We evaluated the effect

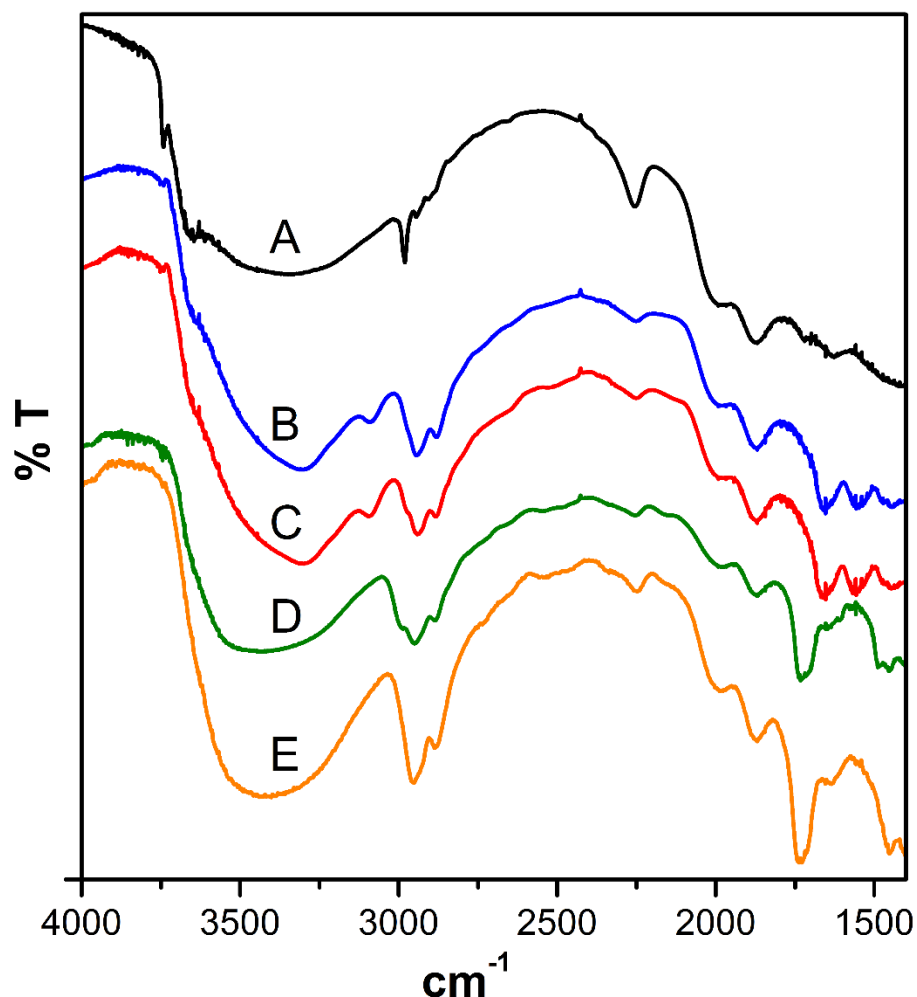


Figure 2. DRIFT spectra of porous silica particles (Nucleosil) functionalized with (A) α -bromoisobutyryl initiator, and the polymerization products of (B) AAP, (C) AAE, (D) HEA and (E) HEMA monomers. Monomer concentrations, 1.0 M; catalyst concentrations, 20.0 mM; temperature, 25°C; reaction time, 3 h. Catalyst and solvent (B) through (D), $[\text{Cu}^{\text{I}}(\text{Me}_6\text{TREN})]^+\text{Cl}^-$ and ethanol/water 4:1 v/v, (E), $[\text{Cu}^{\text{I}}(\text{bpy})_2]^+\text{Cl}^-$ and methanol/water 1:1 v/v.

of monomer concentration on the ability of the polymer film to reduce electroosmosis and it was found to be very small, as expected, and essentially insensitive to variations of monomer concentration. This is shown in the Supplementary Material for polyAAE, polyHEA and polyHEMA coatings (Figure S-5). We next examined the CE performance of polymer-coated capillary tubes with respect to their ability to prevent protein adsorption (*i. e.*, its non-fouling capabilities). Peak asymmetry and separation efficiency of two strong silanol-sensitive compounds, $\text{Ru}(\text{bpy})_3^{3+}$ and lysozyme, were used as indicators of the extent of adsorption on the coated capillaries upon varying monomer concentration. The Ru-complex is very sensitive to electrostatic interactions with silica surfaces, while lysozyme is also sensitive to other nonspecific interactions such as hydrogen bonding, hydrophobic effects, etc. The effect of increasing monomer concentration is evident in the case of polyAAE (1-2 M-levels appear to favor low asymmetry

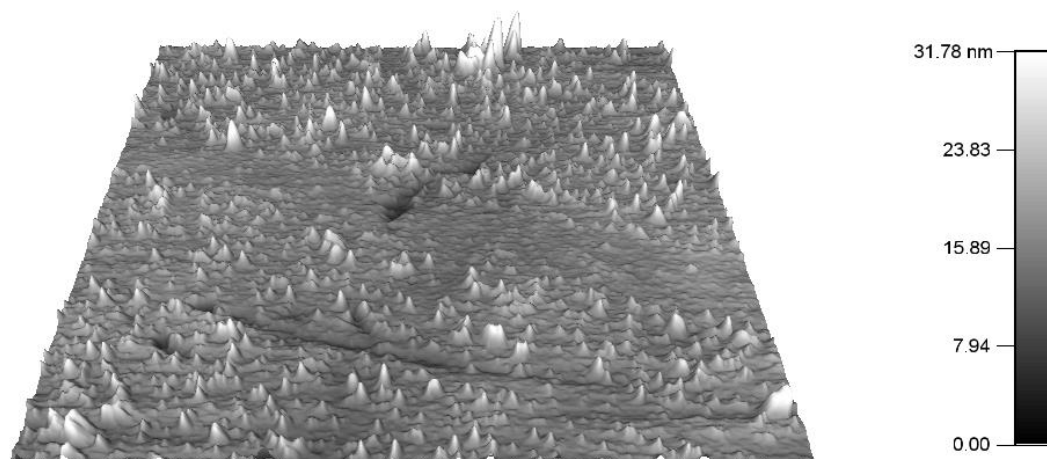


Figure 3. Typical AFM image of a silica plate modified with a polyAAE film. The plate was probed in the intermittent noncontact mode with a force constant of 42 N/m and resonance frequency of 300 kHz. A rectangular silicon cantilever with tetrahedral tip and nominal spring constant of 0.06 N/m was used. The scan size was 3 μm $\times$ 3 μm . Preparation conditions as described in Figure 2.

values and high plate count) and essentially absent in the case of polyHEMA (This is shown in Figure S-6 of the Supplementary Material). In the case of polyHEA, a concentration ≥ 2 M appears to favor high plate counts. Similar observations, albeit to a lesser extent, apply to the Ru probe (not shown). We also examined the effect of cross-linker concentration on polymer film behavior (chemical structures of the cross-linkers used are also depicted in Figure S-1 of the Supplementary Material) and found that, similarly to monomer concentration, there is no evident effect on EOM (see Figure S-7 of the Supplementary Material). On the other hand, low concentration of cross-linker (0.1% molar) appear to furnish lower asymmetry and higher plate count in the case of polyAAE, while there was very little or slightly detrimental effect for polyHEA and polyHEMA (see Figure S-8 of the Supplementary Material). Results are summarized in Figure 4, where it appears that, generally speaking, the presence of cross-linker improves the symmetry, separation efficiency and time-corrected area of lysozyme when the amide monomers, AAE and AAP, are used. In contrast, when the two ester monomers, HEA and HEMA, are used, the presence of cross-linker appears to be somewhat detrimental to the electrophoretic performance of the basic enzyme. It should be pointed out that there does not seem to be a most favorable monomer among the ones studied. PolyAAE exhibits the largest corrected peak area, which suggests maximum solute recovery. Cross-linked polyAAP shows the greatest separation efficiency, while uncross-linked HEMA has a remarkably low peak asymmetry.

Several proteins with high pI were separated to test the capability of the polymeric coating to resist adsorption. Figure 5 shows a typical electropherogram for a mixture of four of these proteins (cytochrome c, lysozyme, trypsin and ribonuclease A) at pH 4.0, with separation efficiencies comparable to those of former reports (15). Whereas separation efficiencies are very high (typically 0.25 to 0.65 million plates per meter; see Table S-1 of the Supplementary Material), CE performance at its full potential –a few million plates per meter– seems to be elusive, and some residual fouling appears

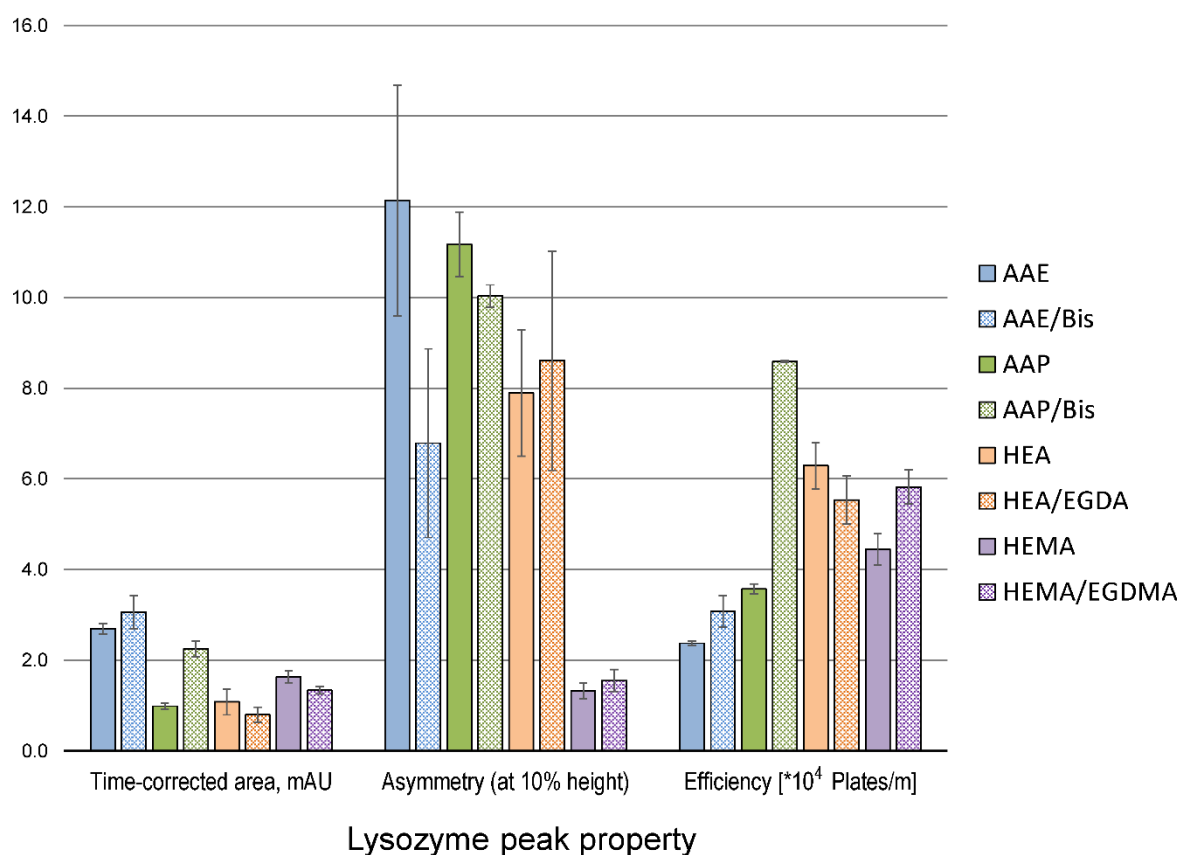


Figure 4. Comparison of lysozyme CE peak features on several polymeric coatings with and without their corresponding cross-linkers. Error bars represent ± 1 SD (n = 3). ATRP conditions equivalent to those in Figure 2. Cross-linker concentration, 0.1% molar with respect to the monomer. CE conditions: capillary, 35.0 cm (effective length, 26.5 cm); electric field, 400 V cm⁻¹ (9.8 μ A); electrolyte, 25.0 mM PIPPS buffer with pH 4.0; protein concentration, 0.5 mg mL⁻¹ each in buffer; injection, 3 s at 50 mbar; detection, 210 nm.

to be an inevitable limitation of our hydrophilic coatings at this stage of development. The graphical abstract shows another example of the great selectivity obtained for two cytochromes C on a polyAAE-Bis modified capillary under the same CE conditions used in Figure 5. To further complement our observations, such residual adsorption can be quantitatively assessed by analyte recovery measurements. This is accomplished by linear fitting of logarithmic peak area *vs.* effective length data (42). For a given tube id the slope, k , is proportional to the surface density of adsorption sites and, hence, is a useful figure to comparatively assess protein adsorption on different capillary coatings. Preliminary experiments, summarized in Table 1, show that the k -values for both polyHEMA and polyAAP coatings are statistically undistinguishable from zero which, in turn, indicates a complete (100%) recovery of the basic enzyme; *i.e.*, there is no adsorption on the coated surface. In contrast, the oligoamine (tetraethylenepentamine) used to dynamically coat a native capillary, while quite effective

Table 1

CE recovery of lysozyme as a function of the nature of the coating grafted on the inner wall of the capillary (42).

coating	k , cm ⁻¹ ^a	S_k	%Recovery ^b ($l=26.5$ cm)	$S\%_{recovery}$
polyHEMA ^c	0.001 ₅	0.009 ₂	96	23
polyAAP ^d	0.002 ₉	0.004 ₄	93	11
none (native) ^e	0.005 ₅	0.001 ₈	86	4

CE conditions: electrolyte, 25.0 mM PIPPS buffer adjusted to pH 4.00 ± 0.05 with 12.5 mM γ -aminobutyric acid; also containing 1.5 mM tetraethylenepentamine in the case of the native capillary tube. Sample: 1.0 mg mL⁻¹ lysozyme and 3.0 mM histidine as internal standard in electrolyte (10.0 mM benzylamine as internal standard with the native capillary tube). Applied field, 400 V cm⁻¹ (current, 5.8 μ A). Five replicate injections (5 s at 0.67 mbar cm⁻¹) were performed on a 50- μ m capillary tube with varying effective length.

- a) Estimated from linear fitting of the logarithm of lysozyme peak area (referred to the internal standard) *vs.* effective length, l .
- b) Estimated from the equation $R = \exp(-kl)$ with R as the analytical recovery of lysozyme and 26.5 cm being the minimum possible l in our instrument.
- c) Uncross-linked. Other preparation conditions as described in Figure 2.
- d) Cross-linked with 0.10% molar Bis with respect to the monomer.
- e) Native tube etched with 1.0 M NaOH, leached with 1:1 v/v HCl and conditioned with electrolyte.

to reduce lysozyme adsorption under the same CE conditions, exhibits some measurable analyte lost during its passage through the capillary tube as indicated by a k -value which is statistically different from zero. With regards to precision, while the repeatability of migration times was 0.2-0.7 %RSD, the typical peak area precision was 5-13 %RSD ($n = 10$ successive injections). The later is likely due to the difficulties associated with the various isoforms that accompany the enzyme samples, as illustrated in Figure 5.

Long-term hydrolytic stability

We have chosen the very demanding electrolyte conditions of capillary isoelectric focusing (CIEF) to assess long-term hydrolytic stability of our polymeric coatings. While we used a hydrolytically durable N-substituted amide as the starting monomer along with a stable Si-C link to the silica surface, when a sodium hydroxide solution is used as cathode electrolyte –as it is done in CIEF, the underlying siloxane bridges that hold the silica backbone will eventually collapse. Although to a lesser extent, the phosphoric acid solution used as anode electrolyte will also be very aggressive towards the polymeric

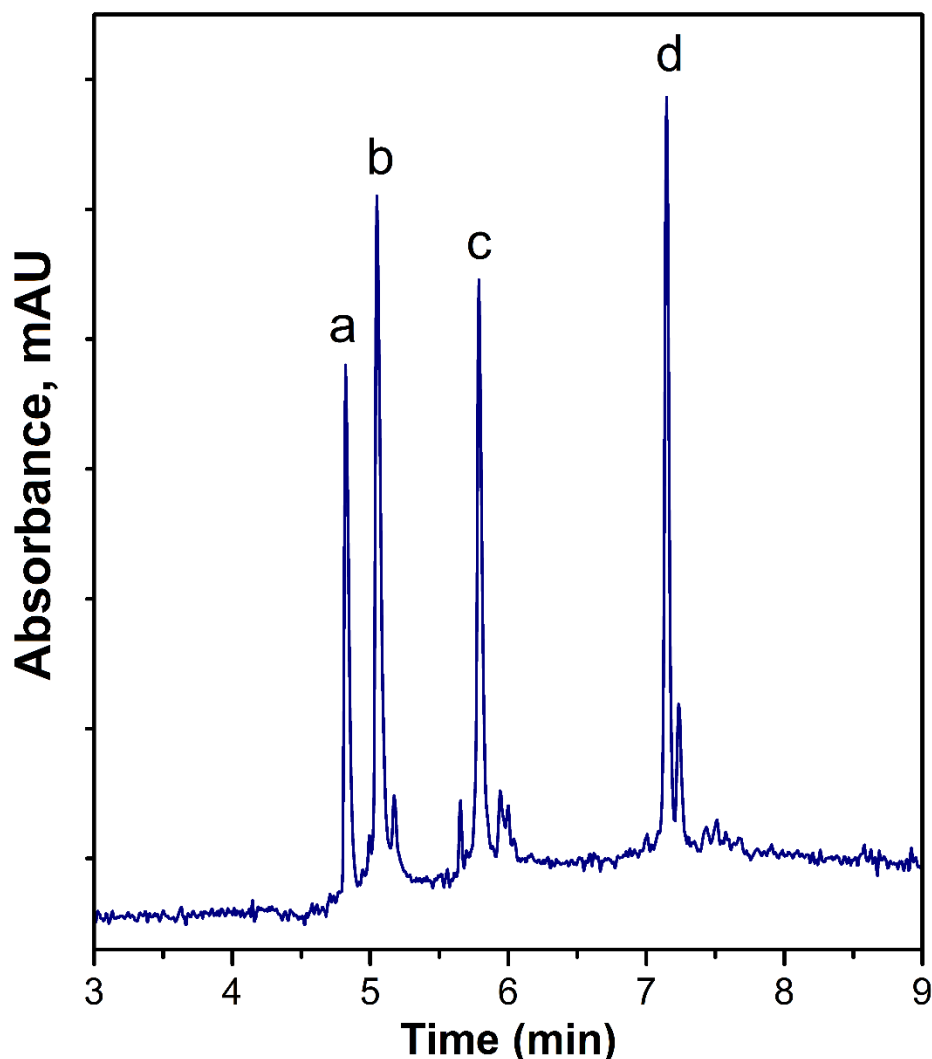


Figure 5. CE separation of a mixture of basic proteins in a polyAAE-coated capillary. Conditions: capillary, 35.0cm (effective length, 26.5 cm); electric field, 400 V cm^{-1} ($8.1 \mu\text{A}$); electrolyte, 25.0 mM PIPPS buffer with pH 4.0; protein concentration, 0.1 mg mL^{-1} each in 10-fold diluted buffer; injection, 15 s at 20 mbar; detection at 200 nm. Solutes: (a) cytochrome C, equine, (b) lysozyme, chicken, (c) trypsin, bovine, (d) α -chymotrypsinogen A, porcine.

coating. Figure 6 shows typical CIEF separation profiles of blood samples on a polyAAP-Bis coated capillary tube. The two upper panels of Figure 7 illustrate measured migration time and area for two hemoglobin (Hb) peaks of a hemolyzed blood sample mix as a function of injection number during a reiterating CIEF experiment. Within the experimental error of this several-day-long experiment, peak migration time and, to a lesser extent, corrected peak area do not seem to deteriorate significantly. It should be pointed out that there is a certain trend associated with each 10-injection cycle (we renewed the sample after 10 consecutive injections) which contributes to the overall variability and may mask the long-term profile of the parameter under study. This effect of sample renewal is more pronounced for the peak area (mid panel of Figure 7) and for polyAAE coatings (not shown). Long-term precision

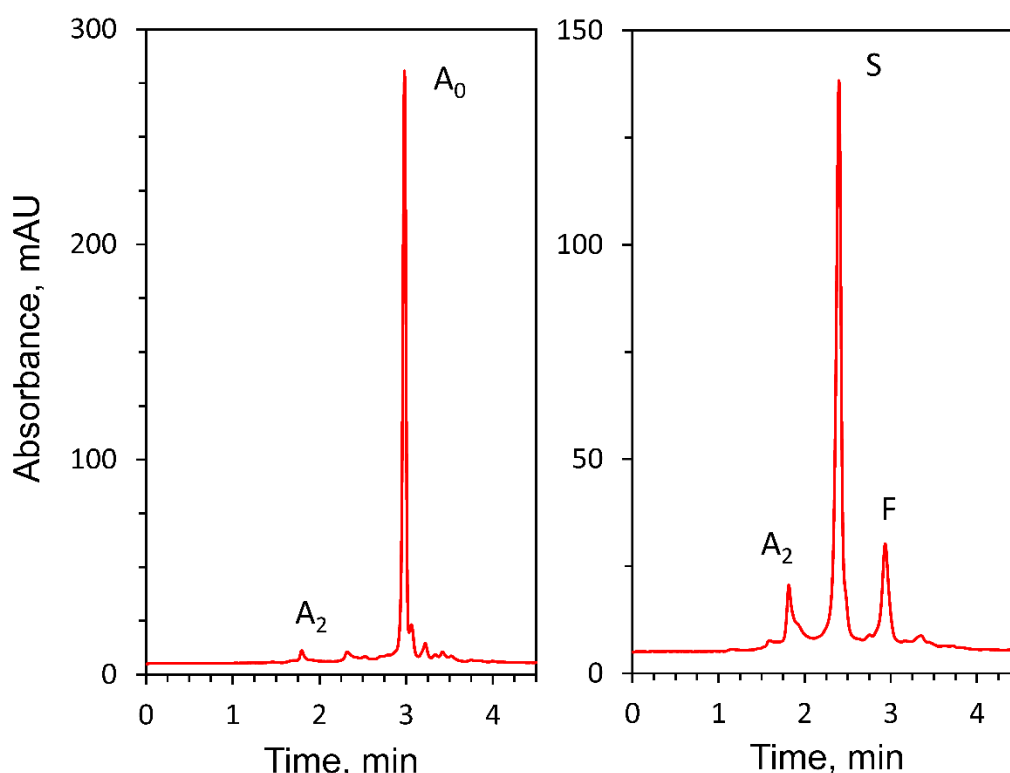


Figure 6. Hemoglobin constituents in normal blood (left), and blood from a patient with sickle cell disease by CIEF. Whole blood (10 μ L) was treated with 200 μ L of hemolyzing solution (EDTA, 5 mM; KCN, 10 mM), and then introduced into the capillary by low-pressure injection (30 mbar for 17 sec). Cathode and anode solutions were 20 mM NaOH and 100 mM H_3PO_4 in 0.375% methylcellulose, respectively. Prior to each assay, the capillary was flushed (5 min under 0.95 bar) with electrolyte containing 0.375% methylcellulose and 2.5% v/v Ampholyte mix solution (pH 6–8 / pH 3–10, 10/1 v/v). Sample constituents were focused for 4 min at 25 kV, then eluted past the detector (UV absorbance at 415 nm) under simultaneous low pressure (20 mbar) and same voltage. Capillary: Bis-crosslinked polyAAP coating, 50- μ m id and 35.0-cm length (26.5-cm effective length).

for Hb S and A_0 was about 5 and 23 %RSD for migration times and time-corrected peak areas respectively ($n = 260$). Regression analysis on the data (see Table S-2 of the Supplementary Material) reveals nearly zero slopes along with low correlation coefficients. The very small slope of, for instance, migration times *vs.* run number indicates that it would take an average of 15 and 45 runs to decrease by one second the migration times of Hb S and A_0 respectively. Figure S-11 of the Supplementary Material visually corroborates the fact that the Hb separation profile does not deteriorate significantly after many sample injections. Perhaps more important from a stability point of view, the hydrolytic deterioration of a CE coating ultimately leads to an increased exposure of ionizable silanol groups and, hence, greater EOM. The impact of a prolonged exposure of a coated capillary to the harsh conditions prevailing in CIEF should provide an “ageing” curve that assesses its hydrolytic stability. Indeed, the EOM appears to be more revealing regarding the effect of coating exposure to the harsh conditions

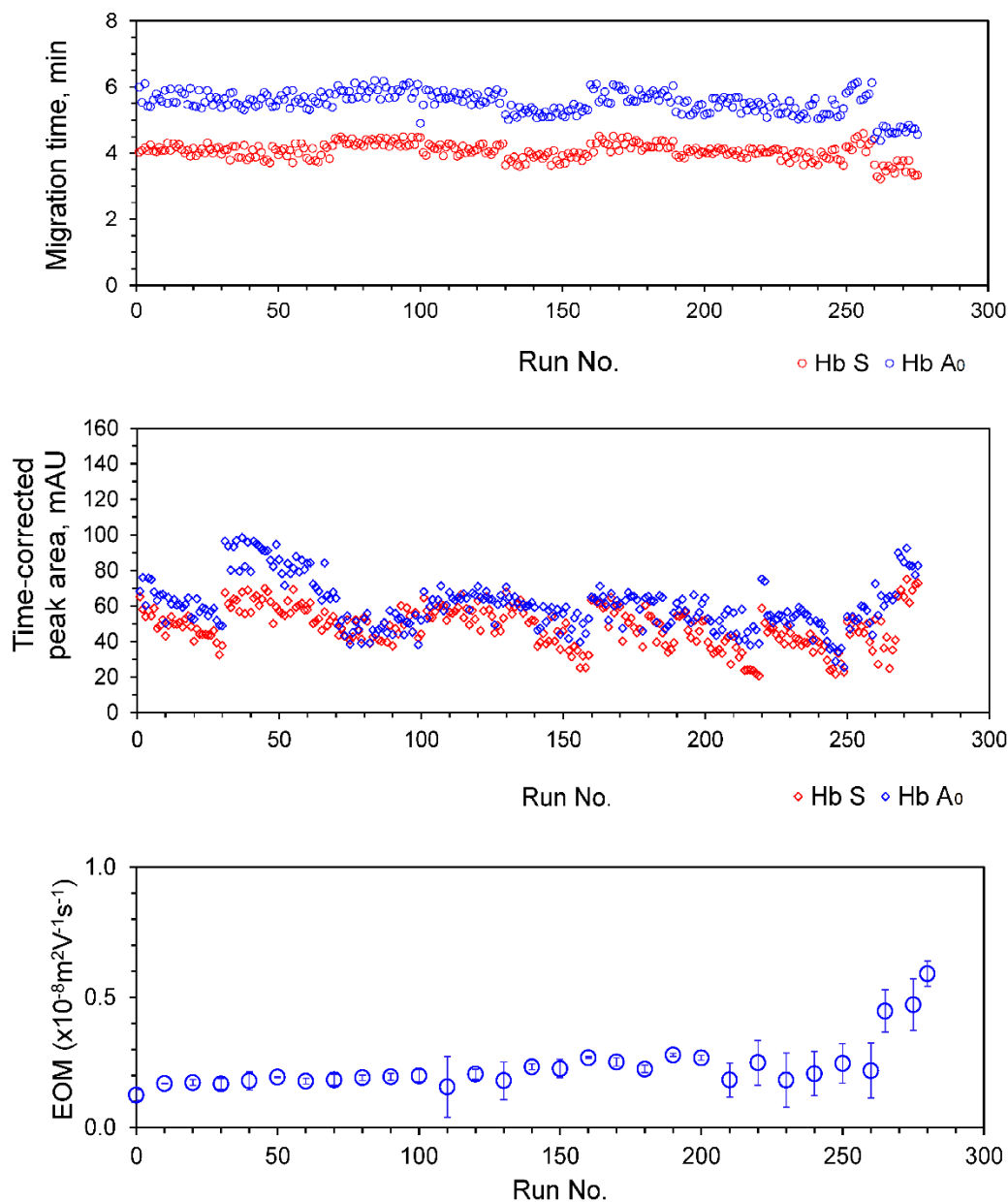


Figure 7. Long-term stability test under typical CIEF conditions for a Bis-crosslinked polyAAP coating immobilized on a 50- μm id capillary with 35.0-cm length (26.5-cm effective length). Experimental CIEF conditions were the same described in Figure 6, except that the sample was a 1:1 v/v mixture of hemolysates from same normal and abnormal blood samples. 10 consecutive injections per sample aliquot were carried out from the same hemolysis lot. Electroosmotic mobility (EOM) was measured after every 10 CIEF runs. CE conditions for EOM measurements: hydrodynamic injections (3 s at 50 mbar) of 10 mM DMSO (neutral marker) in the electrolyte solution (25-mM MOPS buffer, pH 7.0), 28.0 kV voltage applied (18 μA) for 5.0 min, hydrodynamic mobilization at 50 mbar, detection at 215 nm, 1 min electrolyte rinse between runs. Error bars represent ± 1 SD ($n = 3$).

prevailing in CIEF (see lower panel of Figure 7): precision of EOM starts declining after 200 runs and its value definitely increases above 260 runs. Yet, acceptable band profiles are obtained even after 270 runs, as depicted in Figure S-11 of the Supplementary Material. Unfortunately, comparable ageing experiments are not easy to find in the CIEF literature probably because these tests are expensive in terms of effort and time.

Conclusions and future perspectives

In this report, we describe surface-initiated and -confined ATRP methodology for chemically modifying silica surfaces with low-fouling polymer coatings. The results indicate that hydroxylated polyacrylamides and polyacrylates coatings of controlled thickness can be prepared by ATRP under very mild polymerization conditions (aqueous solvent, room temperature and short reaction times) and that the coated capillary tubes exhibit high performance for protein separations along with remarkable hydrolytic stability which affords favorable long-term reproducibility. Since polymerization is confined to the inner capillary surface, clogging of the narrow tube conduit becomes very unlikely if simple precautions are taken during the surface modification process. The controlled polymerization method can be easily extended not only to other hydrophilic monomers such as N-acryloylamino-ethoxyethanol (20, 43), but also to betain-related (*e.g.*, sulfobetaineacrylamide) monomers that are known to form zwitterionic polymer surfaces that exhibit high resistance to nonspecific protein adsorption (44). These families of N-substituted acrylamide monomers should provide a step ahead towards the solution to the need for non-adsorbing coatings that enable the use of CE at its full potential, particularly for protein separations.

Although capable of efficiently initiating a variety of polymer chains, immobilization of the α -bromoisobutyryl group did not occur with the high yields expected from hydrosilylation and esterification (see Figure 1). Clearly, further work is needed to achieve a better control of silica surface coverage. There is also a concern regarding the relatively poor stability of the ester functionality at the opposite end of the chain containing the α -bromoisobutyryl initiator group (see the final structure of the sequence depicted in Figure 1). Such labile ester group is in clear contrast with the highly stable Si–C bond (formed by hydrosilylation) which fastens the propyl chain to the inner capillary wall, and with the polymeric film itself –a stable N-substituted polyacrylamide. It is also very important to simplify the synthetic scheme where immobilization of the initiator α -bromoisobutyryl group takes two steps. Our early attempts to anchor initiator groups to the hydride silica via the direct hydrosilylation of 4-vinylbenzyl chloride as well as allyl α -bromoisobutyrate (both commercially available) were unsuccessful, as described in the last section of the Supplementary Material. We are currently working on a new procedure to anchor the α -bromoisobutyryl initiator by means of a single-step reaction, whose results will be the subject of another report.

Acknowledgments

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Supplementary material

Monomers and crosslinkers

Figure S-1 illustrates the chemical structures of monomers and corresponding crosslinkers used in this work.

NMR spectra

Figures S-2 to S-4 show ^{13}C CP-MAS NMR spectra that verify chemical bonding of, respectively, polyHEMA, polyAAE and polyAAP films to the silica surface.

Effects of monomer and cross-linker concentration

The effect of monomer concentration on the ability of the polymer film to reduce electroosmosis is shown in Figure S-5 for polyAAE, polyHEA and polyHEMA coatings. Generally speaking, electroosmosis was found to be quite insensitive to variations of monomer concentration.

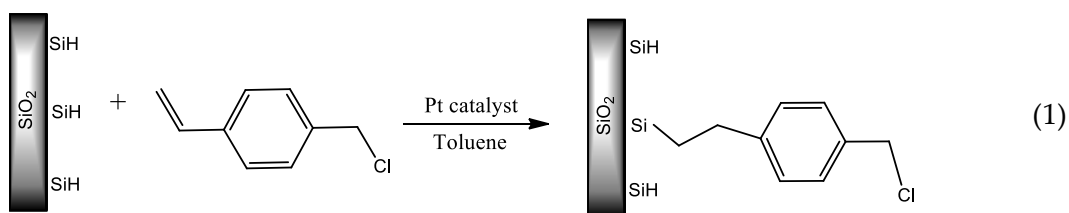
The effect of increasing monomer concentration on the electrophoretic performance of lysozyme is shown in Figure S-6, where it appears that 1-2 M levels appear to favor low asymmetry values and high plate count in the case of polyAAE, while it is essentially absent in the case of polyHEMA. In the case of polyHEA, a concentration ≥ 2 M appears to favor high plate counts.

The effect of cross-linker concentration on polymer film behavior is shown in Figure S-7. It is clear that, similarly to monomer concentration, there is no noticeable effect on EOM. Furthermore, as depicted by Figure S-8, low concentration of cross-linker (0.1% molar) appear to furnish lower asymmetry and higher plate count in the case of polyAAE, while there was very little or slightly detrimental effect for polyHEA and polyHEMA.

Preliminary assays for immobilization of polymer initiator groups

Two different molecular species, namely 4-vinylbenzyl chloride and allyl 2-bromo-2-methylpropionate, containing a halogen-terminated polymerization initiator group were initially evaluated. Given its chain's high hydrolytic stability (it possesses a totally hydrocarbonaceous backbone), its reasonable activity as an ATRP initiator (1, 2) and its commercial availability, 4-vinylbenzyl chloride

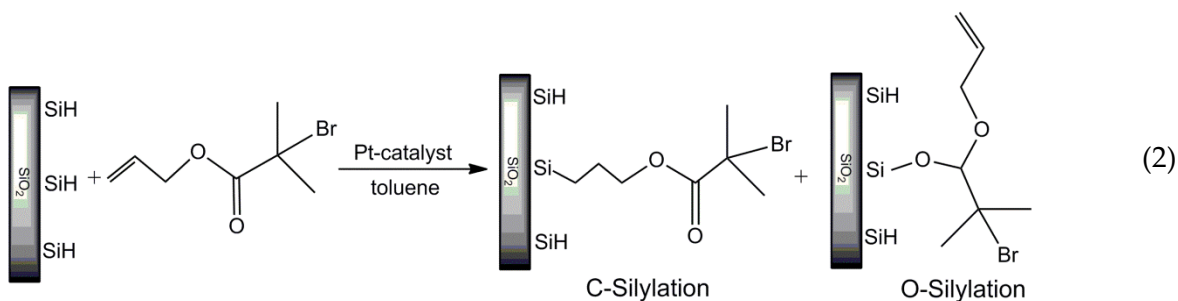
(CH₂=CHC₆H₄CH₂Cl) was used to anchor benzyl chloride groups on the silica surface via hydrosilylation:



Evidence for grafting on hydride silica micro-particles was obtained from DRIFT and ¹³C CP-MAS NMR spectra (not shown) and the carbon content of the bonded material. Progress of the reaction with time at 85 °C using different catalysts is shown in Figure S-1, where it is clear that the processes catalyzed with dichloro(dicyclopentadienyl) platinum(II) and the Karstedt's catalyst took place with a great tendency to polymerize, as indicated by surface coverage values which exceed the idealized figure for a full monolayer (~5 μmol m⁻²). On the other hand, the Speier's catalyst yields a low benzyl chloride coverage under similar conditions. Lowering the reaction temperature from 85 to 50 °C did not eliminate polymerization of the 4-vinylbenzyl chloride initiator during hydrosilylation (not shown). Furthermore, there was no evidence of significant ATRP reaction of N-acryloyl-aminoethanol on any of these benzyl chloride-modified silicas, as indicated by an almost negligible increase of carbon content after polymerization. To further substantiate this lack of film formation, when the same hydrosilylation/ATRP sequence was performed on hydride silica capillary columns, the coated surface showed no deactivation towards proteins as evidenced from the virtually complete adsorption of lysozyme. It seems likely that, during the unwanted polymerization occurring along with hydrosilylation, extensive termination reactions involving two surface neighboring benzyl radicals eliminate the halogen atom from the benzyl chloride functionality and hence prevented the subsequent ATRP process.

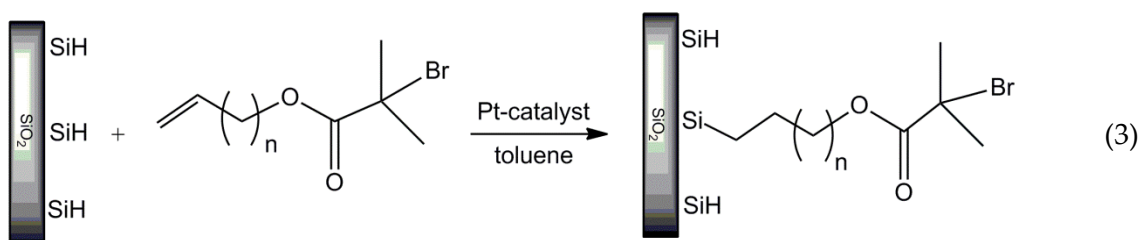
We next explored the use of allyl α-bromoisobutyrate (CH₂=CHCH₂OCOC (CH₃)BrCH₃), another terminal olefin bearing an initiator group at the opposite end of the molecule. The initiator group, α-bromoisobutyryl, is known to be about five hundred times more reactive than the benzyl chloride moiety (2) and its allyl ester is commercially available. Unfortunately, the hydrosilylation of allyl α-bromo isobutyrate on hydride silica yielded low surface initiator coverages, even after more than 50 h of reaction at 85 °C, as depicted by the coverage Γ_{BIB} vs. time plots for three different Pt-catalysts shown in Figure S-9. DRIFT spectra of the reaction products indicate that some initiator immobilization took place. For instance, as shown in Figure S-10 (final product corresponding to the upper curve of Figure S-9), characteristic C–H stretching at about 2853 and 2927 cm⁻¹ concomitant with a decreased Si–H stretching band at 2259 cm⁻¹ are clear evidence of bonding. Some bonding is also noticeable from the appearance of a very weak carbonyl peak at 1719 cm⁻¹ in the DRIFT spectrum. The ¹³C CP-MAS NMR spectrum for this product exhibited poor S/N values (even after 10 h of data collection, ~15,000 scans) and, although the low intensity of the NMR spectrum precludes unambiguous assignments of spectral

bands, some spectral features (not shown) suggest that unwanted O-silylation (at the carbonyl group) seemed to prevail over the expected C-silylation (at the terminal olefin group):



Compared to the benzyl chloride initiator described above, there was a somewhat more extended ATRP reaction for AAE on the α -bromoisobutyryl-modified silica with the Speier's catalyst, as indicated by a small increase in carbon content after polymerization. However, when the same hydrosilylation/ATRP synthetic sequence was carried out on hydride silica capillary columns, the coated surface exhibited significant adsorption of lysozyme, although to a much lesser extent compared to the benzyl chloride initiator. Most likely, the prevailing O-silylation, which eliminates the ester resonance stabilization of the original α -bromo isobutyryl radical, significantly hindered the subsequent ATRP reaction.

In an effort to address the issue of limited coverage of α -bromoisobutyryl species on the silica substrate, we explored the use of the same initiator whose ester group is farther away from the terminal double bond:



While the length of the spacer is simply determined by the number of methylene groups between the double bond and the α -bromoisobutyryl terminals, these esters are not commercially available. A longer spacer ($n = 2$ or 3) was obtained by replacing the allyl ($n = 1$) group of the olefin with a 1-butenyl or 1-pentenyl group, via an esterification process involving the reaction of the corresponding alcohol and acyl bromide (3-5). Unfortunately, no improvement over the former surface coverage obtained with allyl α -bromoisobutyrate resulted from using either of the longer spacers. Similarly, when the same hydrosilylation/ATRP synthetic sequence was applied to hydride silica capillaries, the modified surface exhibited comparable adsorption of lysozyme. Alike the allyl α -bromoisobutyrate, it appears

that O-silylation also prevails over C-silylation leaving only a small fraction of the anchored species (typically 1.2-1.5 $\mu\text{mol m}^{-2}$ coverages) available for ATRP.

Tables

Table S-1

Column separation efficiencies, expressed in number of theoretical plates per meter of capillary tube,^a as measured for several basic protein peaks.^b

protein	plates m ⁻¹ × 10 ⁶	SD	n
cytochrome C	0.244	0.007	6
lysozyme	0.24 ₆	0.02 ₀	9
trypsin	0.34 ₆	0.01 ₂	6
α-chymotrypsinogen A	0.65 ₆	0.02 ₇	12

^a Calculated from the width at half peak height.

^b CE experimental conditions as described in Figure 5 of main text. Measurements were carried out on 4 different sample solutions, each containing a mix of 3 or 4 solutes. Triplicate injections were performed for each sample.

Table S-2

Slopes and correlation coefficients for migration times and time-corrected peak areas of Hb S and A₀ as a function of run number.^a

feature	Hb	slope	slope SD	r
migration time, min	S	-0.0003 ₅	0.0001 ₇	0.124
	A ₀	-0.0011 ₁	0.0002 ₃	0.478
time-corrected peak area, mAU	S	-0.068 ₅	0.007 ₈	0.285
	A ₀	-0.09 ₀	0.01 ₀	0.497

^a CIEF experimental conditions as described in Figure 7 of main text.

Figures

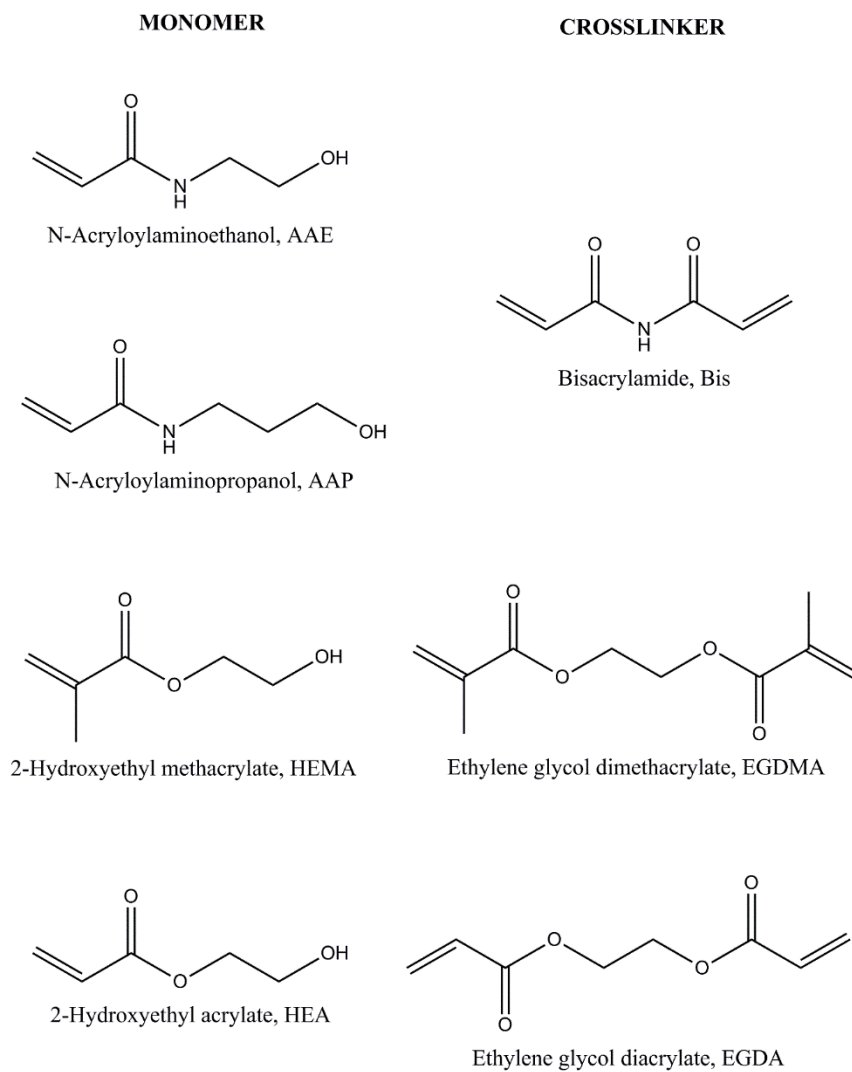


Figure S-1. Chemical structures of the monomers and corresponding crosslinkers used in this work.

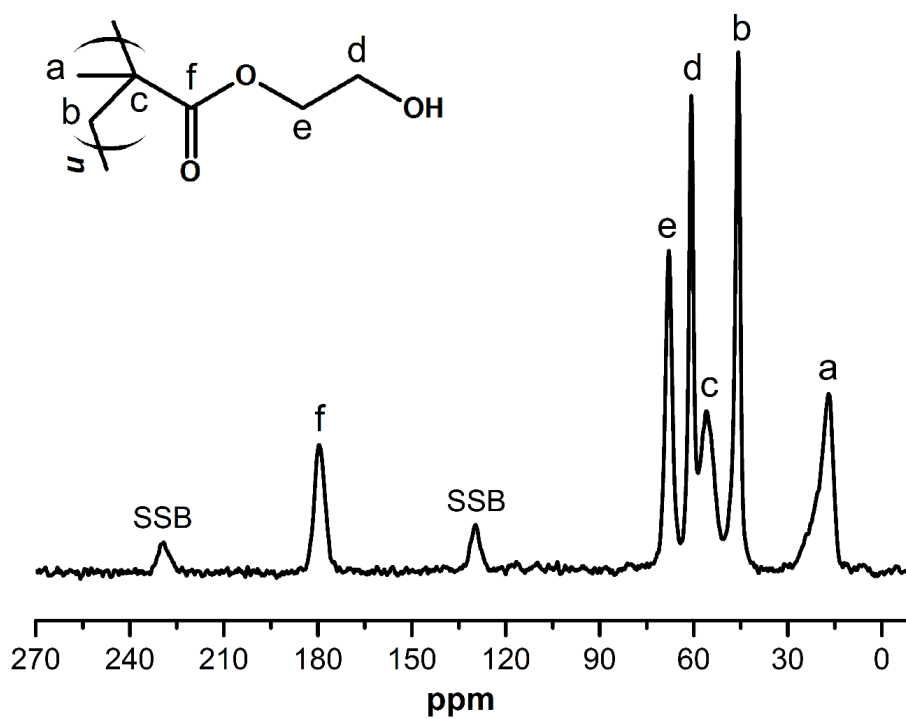


Figure S-2. ^{13}C CP-MAS RMN spectrum of polyHEMA coated porous silica. Substrate, Nucleosil silica; monomer, 1 M HEMA; catalyst, 20 mM $[\text{Cu}^{\text{I}}(\text{bpy})_2]^+$; solvent, 1:1 v/v methanol/water; temperature, 25°C; reaction time, 1 h. SSBs indicate spinning side bands.

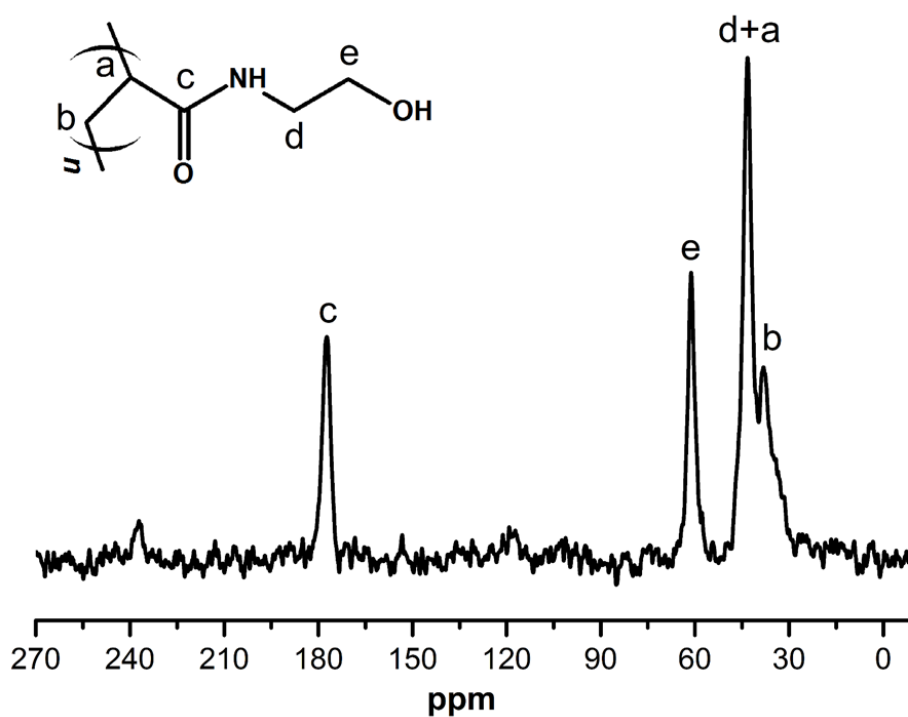


Figure S-3. ^{13}C CP-MAS RMN spectrum of polyAAE coated porous silica. Substrate, Nucleosil silica; monomer, 1 M AAE; catalyst, 20 mM $[\text{Cu}^{\text{I}}(\text{Me}_6\text{TREN})]^+$; solvent, 4:1 v/v ethanol/water; temperature, 25°C; reaction time, 1 h.

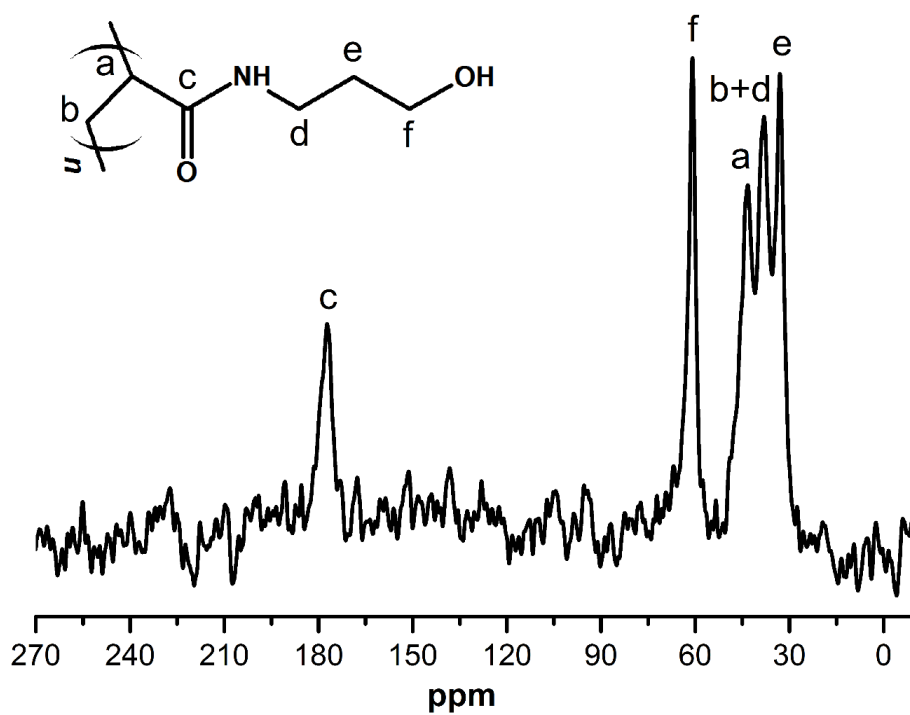


Figure S-4. ^{13}C CP-MAS RMN spectrum of polyAAP-coated porous silica. Substrate, Nucleosil silica; monomer, 1 M AAP; catalyst, 20 mM $[\text{Cu}^{\text{I}}(\text{Me}_6\text{TREN})]^+$; solvent, 4:1 v/v ethanol/water; temperature, 25°C; reaction time, 1 h.

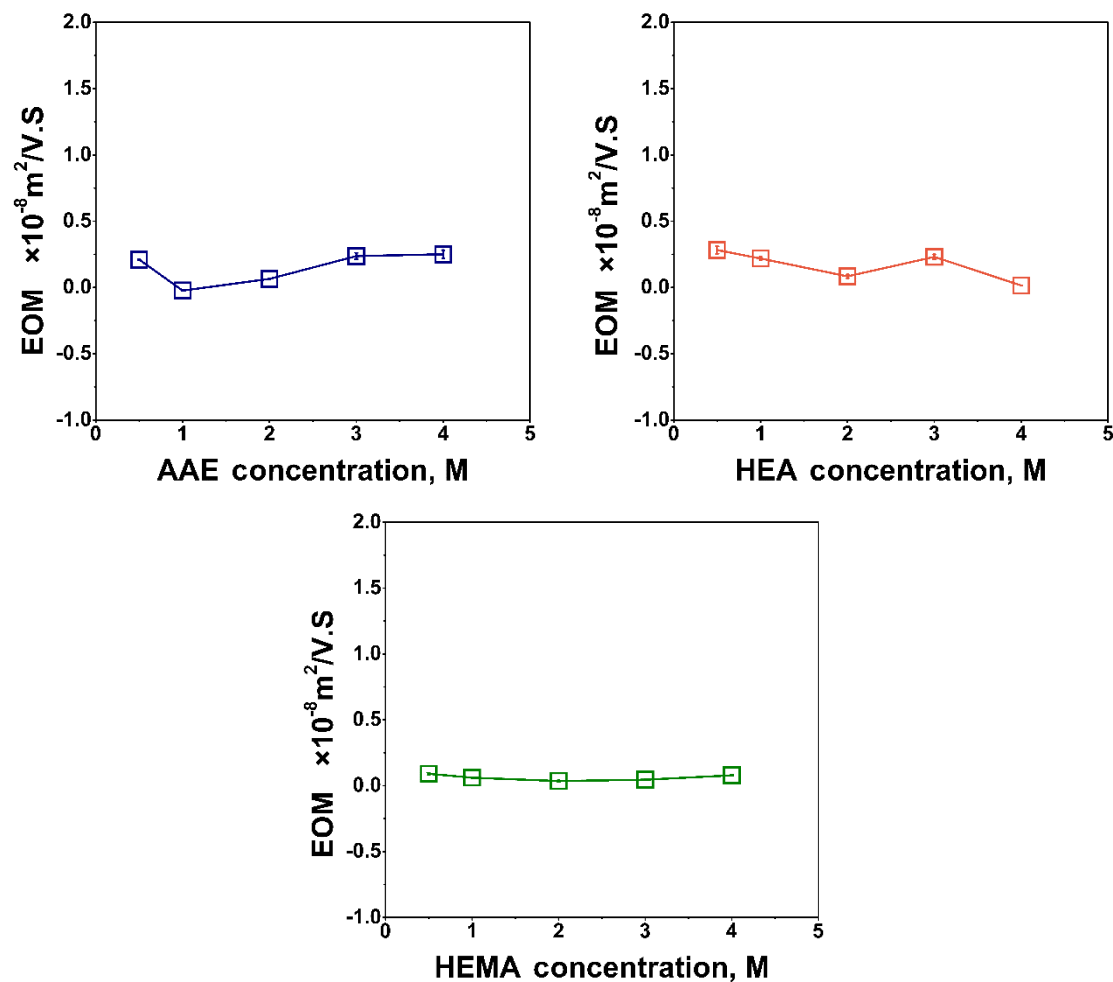


Figure S-5. Effect of monomer concentration on electroosmotic mobility. ATRP and CE conditions as described in Figure 4 of main text.

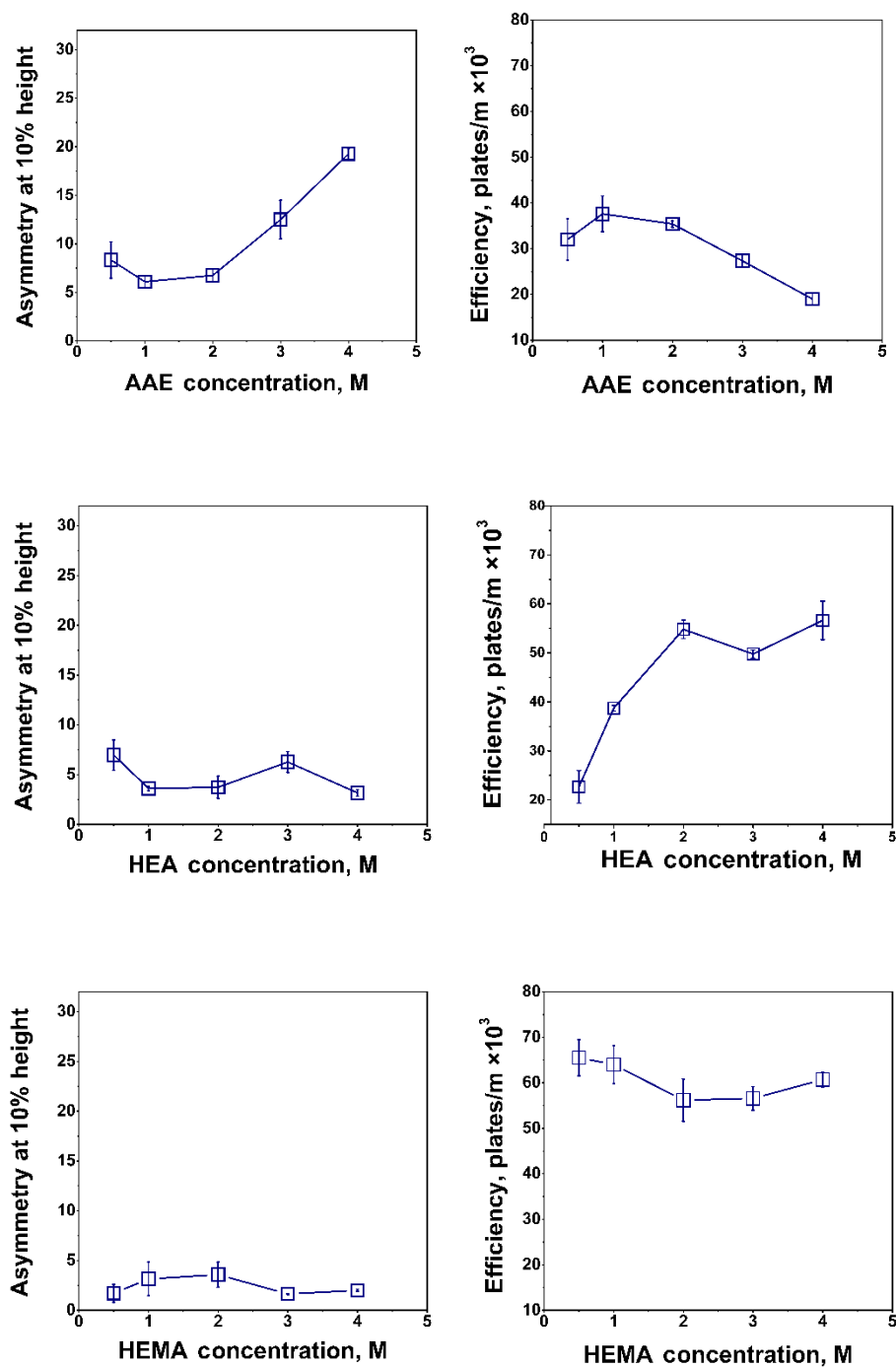


Figure S-6. Effect of monomer concentration on asymmetry and efficiency of lysozyme. ATRP and CE conditions as described in Figure 4 of main text.

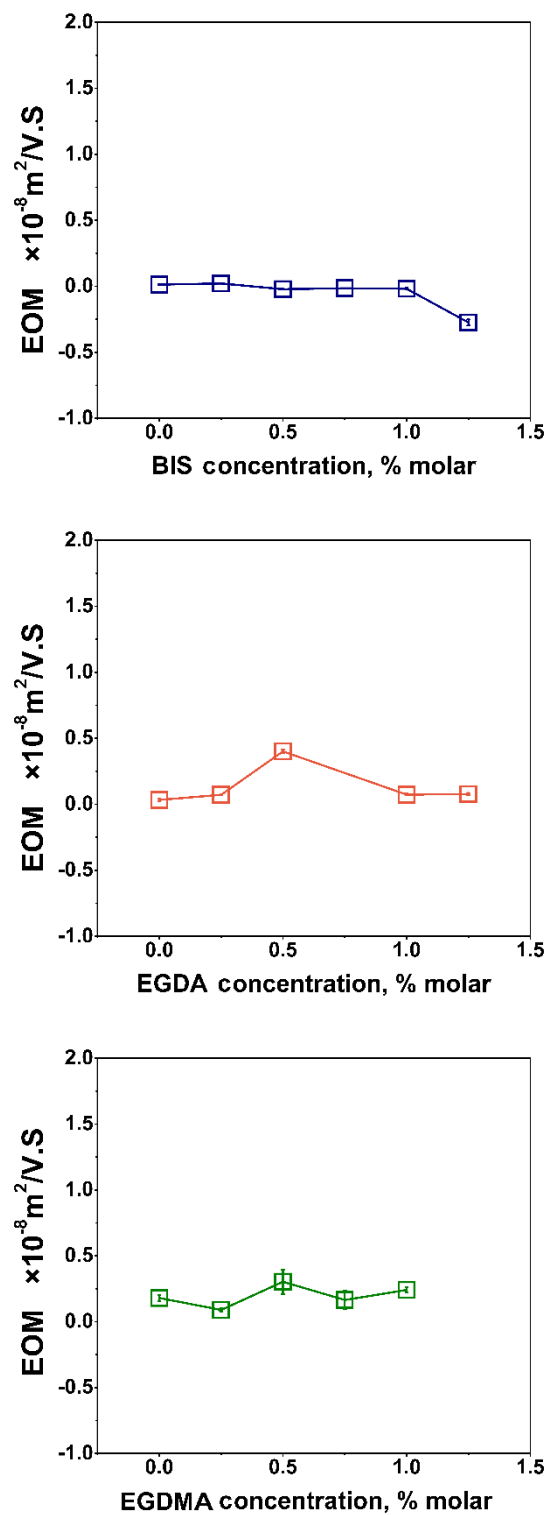


Figure S-7. Effect of cross-linker concentration on electroosmotic mobility. ATRP and CE conditions as in Figure 4 of main text.

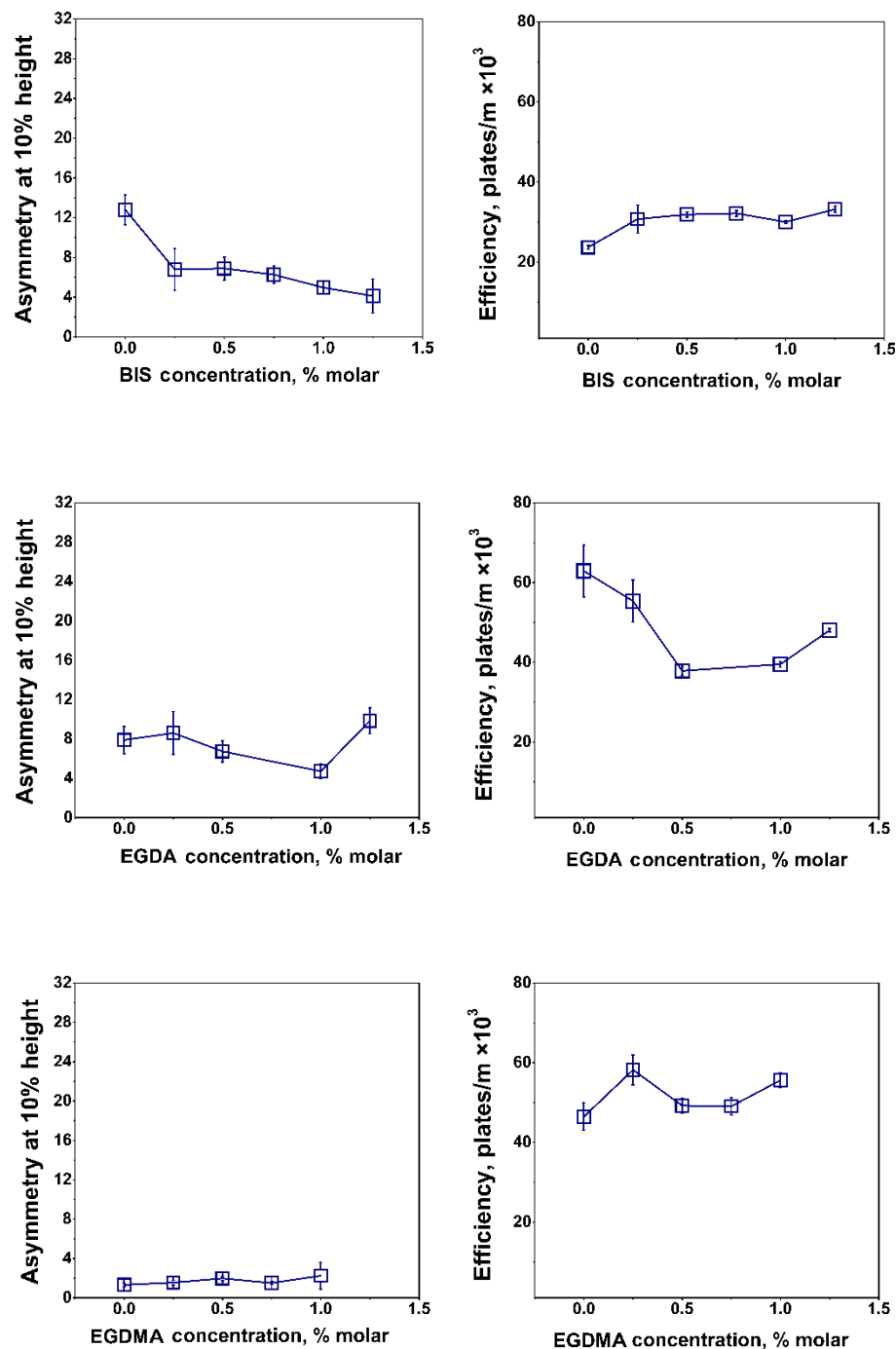


Figure S-8. Effect of cross-linker concentration on asymmetry and efficiency of lysozyme. ATRP and CE conditions as in Figure 4 of main text.

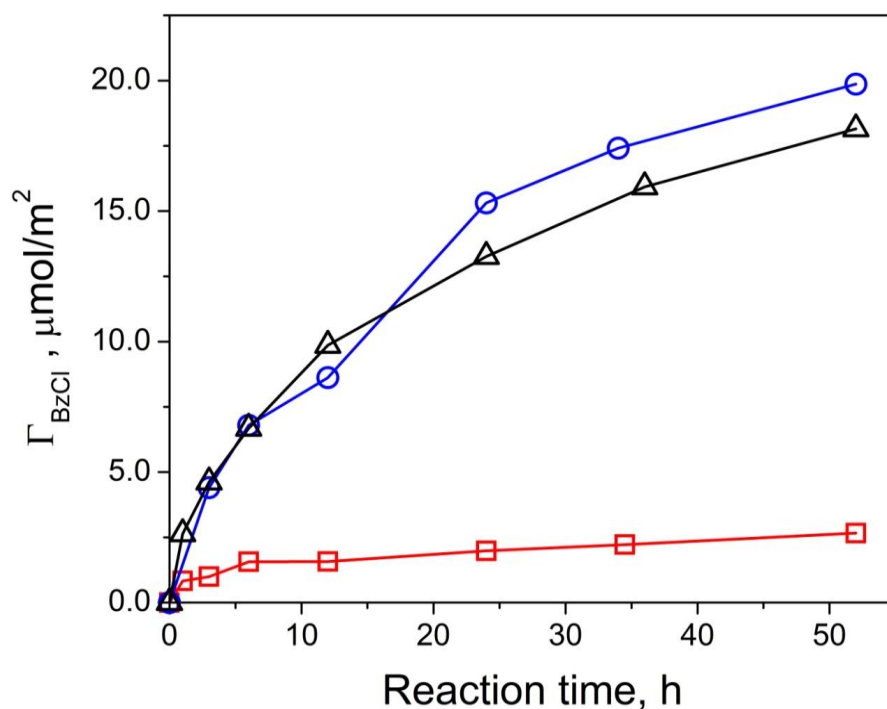


Figure S-9. Benzyl chloride surface coverage (Γ_{BzCl}) as a function of reaction time for the hydrosilylation of 4-vinylbenzyl chloride on hydride silica with several Pt-catalysts. Squares, Speier's (hexachloroplatinic acid in 2-propanol) catalyst; triangles, Karstedt's (Pt(0)-1,3-divinyl-1,1,3,3-tetra methylidisiloxane in toluene) catalyst; circles, dichloro (dicyclopentadienyl) Pt(II) in dichloromethane. Final Pt concentration was 0.25 mM in all cases. See Materials and Methods section of main text for further details.

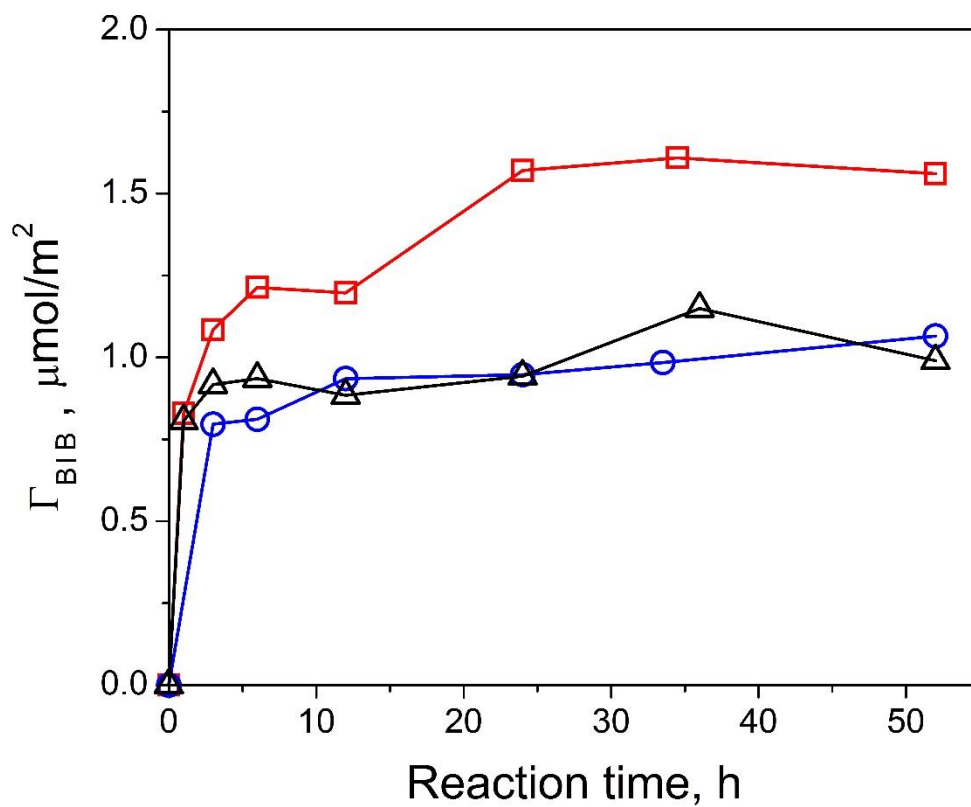


Figure S-10. α -bromoisobutyryl (BIB) surface coverage (Γ_{BIB}) as a function of reaction time for the hydrosilylation of allyl α -bromoisobutyrate on hydride silica with several Pt-catalysts. Catalyst curve keys and reaction conditions as described in Figure SI-9.

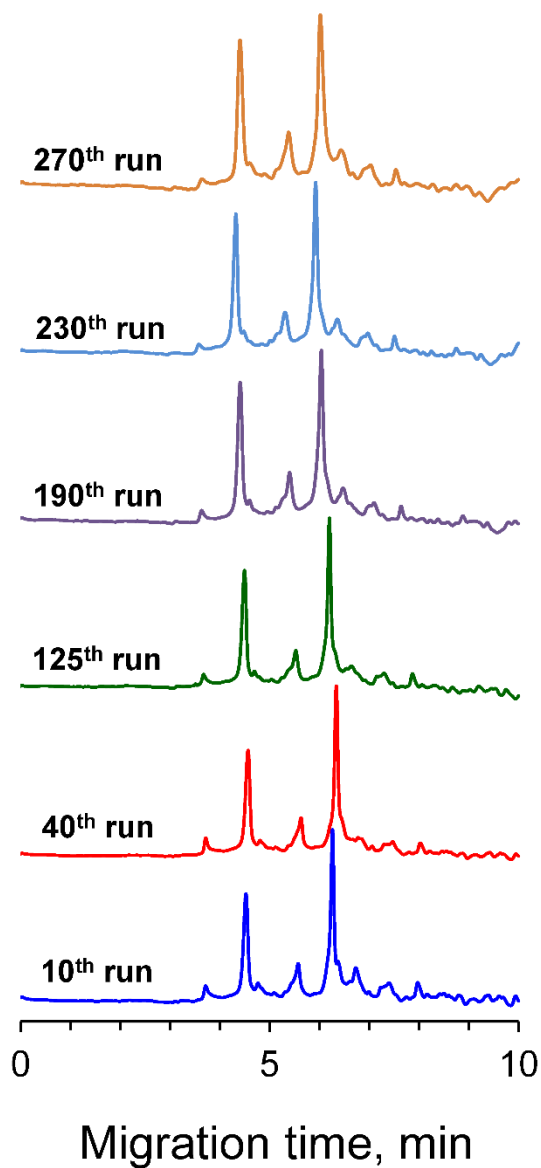


Figure S-11. Time evolution of the CIEF separation profile for a set of Hb solutes throughout a reiterating experiment. Sample and CIEF conditions as described in Figure 7 of main text.

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