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Protein binding on polyelectrolyte-treated glass Effect of structure of adsorbed polyelectrolyte

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Abstract

Polyelectrolyte adsorption can be used to modify the surface of chromatographic packings in order to make them more suitable for protein separations. We studied the binding of proteins to controlled pore glass (CPG) on which the polycation poly(diallyldimethylammonium chloride) (PDADMAC) was noncovalently immobilized through electrostatic interaction. We found that the selectivity of PDADMAC for bovine serum albumin vs. β-lactoglobulin, identified in previous selective coacervation studies, is conserved after its immobilization on the CPG surface. Protein binding results showed that the pH, ionic strength, and mixing time for polyelectrolyte adsorption all affect subsequent protein binding, presumably via the molecular properties of the adsorbed polyelectrolyte layer. The polyelectrolyte adsorption layer thickness, for polyelectrolyte adsorbed at pH 9.0, ionic strength $I=0.001$, was measured with size-exclusion chromatography as $\delta_H=2.5\pm0.5$ nm. Quasielastic light scattering measurement of the polyelectrolyte hydrodynamic layer thickness (HLT) with a model system of PDADMAC and silica, supported a correlation between the structure of the adsorbed polyelectrolyte layer (e.g., loops and tails) and subsequent protein binding, although differences in magnitude between δ_H and HLT suggest that adsorption onto silica may not mimic adsorption on CPG.

Keywords: Stationary phases, LC; Proteins

1. Introduction

Protein purification is essential to many areas of scientific research, in that purified proteins are required for, e.g., sequencing, crystallography and therapeutic treatment [1]. Ultrafiltration, centrifugation, salt precipitation, extraction, size-exclusion chromatography (SEC), and other forms of HPLC are among the methods employed for protein separation. A relatively novel method for protein separation is selective polyelectrolyte coacervation [2], which occurs when proteins form complexes with oppositely charged polymers to yield a second condensed liquid phase [3]. While polyelectrolyte–protein coacervation has been studied for several decades [4], its use for protein separation is relatively new [5,6], and is motivated in part by its potential for large scale separation. Despite the advantages of polyelectrolyte coacervation, removal of the existing polyelectrolyte poses a problem. However, the principles of selective polyelectrolyte coacervation might be applied to chromatography, if the polyelectrolyte were immobilized on a chromatographic support phase.

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Interest in polymer modification of silica surface has recently been stimulated by potential applications to capillary electrophoresis [7–12]. Previously, polymer adsorption techniques had been developed mainly in the context of silica-based liquid chromatography stationary phases, e.g., silica gels [13–16]. In such studies it was demonstrated that polymers can be immobilized either through physisorption or through chemical modification. The first of these methods involves the adsorption of the polymer onto a silica surface without chemical reaction [10,13]. In this manner, both ionic and nonionic polymers can be directly deposited onto silica and may remain on the silica surface permanently. Physisorption may also be used to bind monomers or oligomers, followed by polymerization, with or without cross-linking, on the silica surface [7,17]. In the second class of immobilization, polymers are covalently bound to the silica surface by reaction with silanol groups [8,11,13,18–20] or with other functional groups introduced onto the silica surface in order to allow the polymer to be chemically attached [21]. Although chemical modifcation appears to yield a relatively stable polymer stationary phase, the cost and inflexibility of this method impede further application, and recent attention has been focused on physisorption [9,10]. Polyelectrolytes are readily physisorbed onto oppositely charged surfaces through electrostatic interactions. Such polyelectrolyte modification could dramatically alter the chromatographic properties [10].

Two approaches toward the examination of polymer-modified silica can be visualized: either by examination of chromatographic properties or by elucidation of the structure of the adsorbed polymers. Chromatographic properties include column efficiency, resolution, and relative retention; by ''structure of adsorbed polymers'', we refer to phenomena at the molecular level such as the dimensions, configuration, and local environment of the immobilized polymer chains. These two approaches should complement each other in the interpretation of chromatographic results.

While extensive chromatographic studies have been carried out on polymer-modified HPLC columns, few have been focused on the effect of the structure of adsorbed polymers on chromatographic separations. A similar problem is evident in the field of capillary electrophoresis, despite the current research in polymer modification of CE capillaries. The absence of such studies may point to a need for a more complete understanding of how polymers, especially polyelectrolytes, adsorb on HPLC substrates and the concomitant relationship between the configuration of the adsorbed polymers and resultant chromatographic properties.

Recently, polyelectrolyte adsorption has received considerable attention, both from theoretical and experimental perspectives [22–33]. The configuration of adsorbed polyelectrolytes has been described in terms of trains, loops and tails [22]. These configurations are determined by adsorption conditions, such as pH, ionic strength (I), polymer molecular mass, polymer concentration, adsorption time, surface geometry, and substrate chemical heterogeneity. It would be expected that the nature and number of loops and tails of the adsorbed polymers in the stationary phase should strongly influence the subsequent retention of solute molecules, especially proteins.

The separation of proteins on stationary phases based on adsorbed polyelectrolytes should resemble the separations of proteins by polyelectrolyte coacervation [2], since both methods involve the same electrostatic interactions. We have attempted to establish some empirical relations for the efficiency and selectivity of protein separation via polyelectrolyte coacervation [34]. If the structure of the polyelectrolyte is partially conserved after immobilization on silica surface, similar efficiency and selectivity should be observed in chromatographic separations.

The current work has several purposes. The primary question is whether the selectivity of polyelectrolyte for protein separation is retained after it is adsorbed onto a siliceous surface. A more general goal is to establish the relation between the polyelectrolyte adsorption process and the subsequent chromatographic properties of the modified silica surface. To accomplish this, polyelectrolyte-modifed glass, prepared under different adsorption conditions, was used to bind proteins. Both size exclusion chromatography and dynamic light scattering were employed to study the structure of the adsorbed polymer layer, and the results were used to interpret the protein binding experiments.

2. Materials and methods

2.1. Materials

Poly(diallyldimethylammonium chloride) (PDADMAC) (Merquat 100, Calgon, Pittsburgh, PA, USA) with a nominal of M_r of $2 \cdot 10^5$ and $M_W/M_n > 5$ was dialyzed (molecular weight cut-off = 12 000–14 000) and freeze-dried before use. PDADMAC L-120 (M_n = 35 000) was a gift from Dr. W. Jaeger (Fraunhofer-Institut, Teltow, Germany). Monodisperse silica particles, with Stokes' radius R_s = 40 ± 0.5 nm, (KE-E10, Nippon Shokubai, Osaka) were kindly supplied by Dr. Y. Morishima (Macromolecular Chemistry Department, Osaka University, Japan). Bovine serum albumin (BSA) was purchased from Boehringer Mannheim (Indianapolis, IN, USA) (Lot 100062), and β-lactoglobulin was obtained from Sigma (Lot T1126). Proteins were used without further purification. Controlled pore glass (CPG) (BioRan-CPG; 30–60 μm grain size, pore diameter 29.4 nm, and 136 m^2/g specific surface area) was obtained from Schott Gerate (Mainz, Germany). Pullulan standards (Shodex standard, P-82, Lot 50501) were from Showa Denko K.K. (Tokyo, Japan).

3. Methods

3.1. Preparation of PDADMAC–CPG

Prior to polyelectrolyte adsorption, CPG was first cleaned using the procedure recomended by the manufacturer, namely washing with pH 9.0, 1% sodium dodecyl sulfate (SDS) at room temperature for 2 h, then with deionized (DI) water until no foaming was observed, and drying at 89°C for more than 12 h.

PDADMAC was adsorbed onto cleaned CPG at different pH, ionic strength, and adsorption times. CPG was added slowly into the PDADMAC solution with stirring for 24 h to reach equilibrium. The solution was centrifuged and the solid was washed 5 times with DI water before drying at 89°C for more than 24 h. The pH, ionic strength, and adsorption time for polyelectrolyte adsorption are designated as pH_0, I_0 and t_0.

3.2. Protein binding

All protein binding experiments were performed at $pH_1 = 9$ and $I_1 = 0.1$. About 100 mg of PDADMAC–CPG was added into 0.1 g/l protein solution with stirring for 1 h. The solution was centrifuged and the protein concentration in the supernatant was measured by UV at 278 nm. Fig. 1 schematically illustrates the procedure for polyelectrolyte adsorption and subsequent protein binding.

Polyelectrolyte desorption was not monitored during protein binding experiments due to lack of applicable detection methods. However, we found that pullulan elution volumes from PDADMAC–CPG packed columns, measured with the chromatographic procedures described in the next section, did not change during 48 h of chromatographic elution. Since pullulan elution volumes were significantly different for PDADMAC-treated vs. nontreated columns, this result strongly suggests the absence of polyelectrolyte leaching for PDADMAC–CPG. Similar results were also found in capillary electrophoresis experiments where the electrophoretic flow on a polyelectrolyte-coated capillary was stable during multiple runs [9,10].

3.3. Chromatography

The chromatographic system included a Minipump (Milton Roy, St. Petersburg, FL, USA), a 100 μl sample loop, a R401 differential refractometer (Waters, Milford, MA, USA), and a Kipp and Zonen Recorder (Model BD 112, Delft, Netherlands). A stainless-steel column (25 cm × 0.5 cm I.D.) was dry packed with PDADMAC–CPG ($pH_0 = 9$, $t_0 = 12$h, $C_p = 20$ g/l and $I_0 = 0$, 0.10, 0.50, 1.00, respectively). The column efficiency measured with ^2H_2O was $2.4 \cdot 10^3$ plates/m. Boric acid–NaOH buffer at selected pH and ionic strength was used as the mobile phase. The flow-rate was maintained between 0.5 and 0.6 ml/min.

The SEC partition coefficient of SEC is defined as:

$$K_{sec} = \frac{(V_e - V_0)}{(V_t - V_0)} \quad (1)$$

where V_e is the retention volume of the solute, V_o is the void volume of the column, and V_t is the total

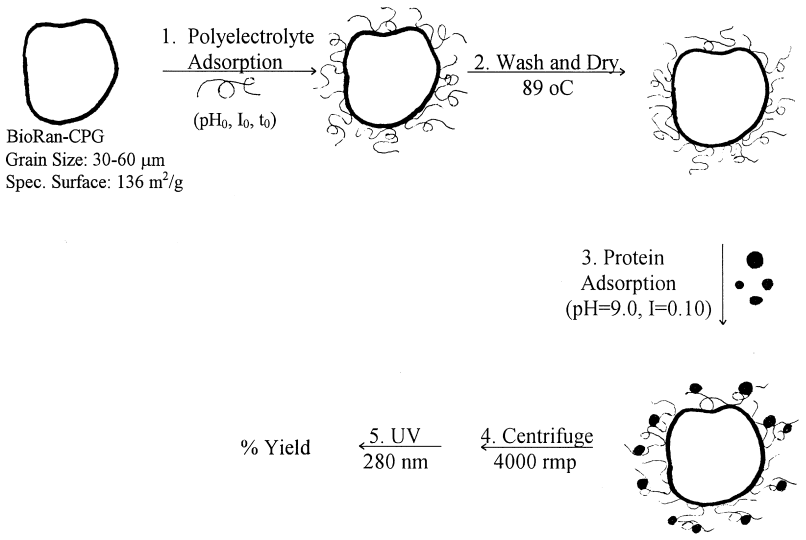

Fig. 1. Schematic depiction of batch adsorption experiment.

volume of the column. V_0 and V_t were measured with P-1600 (Pullulan, $M_w = 1.6 \cdot 10^6$) and ^2H_2O, respectively.

3.4. Quasielastic light scattering (QELS)

PDADMAC was adsorbed on silica particles by slowly adding 0.002% w/w silica into a solution of 0.2% w/w PDADMAC at preadjusted pH and ionic strength. The solution was filtered through 0.45 μm Whatman flters before light scattering measurement.

The apparent Stokes' radius R_s^{app} of PDADMAC-silica was determined by quasielastic light scattering (QELS) with a Brookhaven (Holtsville, NY, USA) 72 channel BI-2030 AT digital correlator, using a 100 mW Argon ion laser. For a polydisperse sample, the field correlation function $g(q,t)$ is often given by:

$$g(q,t) = \int_0^\infty C(\Gamma)\exp(-\Gamma\tau)\mathrm{d}\Gamma \qquad (2)$$

where $C(\Gamma)$ represents the distribution of decay rate. The cumulant method was chosen to analyze the correlation functions. This treatment involves Laplace inversion of Eq. (2), and expansion about an average decay rate $\bar{\Gamma}$. This results in:

$$\ln G(\tau) = \ln B^{1/2} - \bar{\Gamma}\tau + \frac{K_2\tau^2}{2} - \frac{K_3\tau^3}{6} + \ldots \qquad (3)$$

In practice, $\ln G(\tau)$ is fitted to a polynomial in τ, and the second cumulant is used to assess the average particle dimensions.

4. Results

4.1. Protein binding

The degree of protein binding (yield) on polyelectrolyte-treated glass was studied as a function of pH_0, I_0, and t_0. Fig. 2 shows the yield upon batch mixing of BSA with polyelectrolyte-treated glass at $pH_1 = 9.0$ ($pH_0 = 9.0$ and 10.0), and $I = 0.1$. It is clear that protein binding on polyelectrolyte-treated glass is affected by the initial conditions of polyelectrolyte adsorption. According to Fleer [35], the amount of adsorbed polyelectrolyte increases with the substrate surface charge density. The pH titration curve of CPG [36] shows that its charge density is doubled from pH 9.0 to 10.0. Consequently, more polyelectrolyte is adsorbed at pH 10, and the resultant PDADMAC–CPG binds more protein. The very modest effect of ionic strength on subsequent protein adsorption was unexpected and is discussed in more detail later.

Fig. 3 shows the effect of polyelectrolyte adsorption time (t_0) on subsequent protein binding:

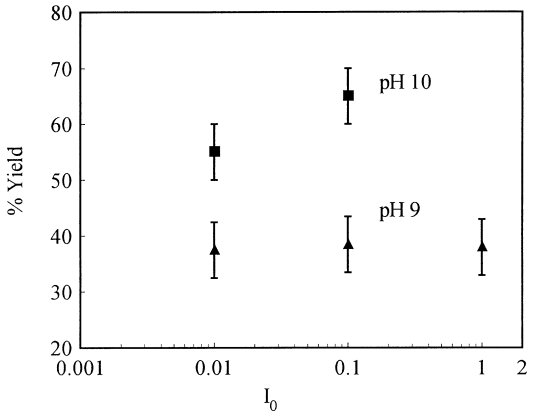

Fig. 2. Effect of preparation pH (pH_0) and ionic strength (I_0) on subsequent BSA binding. PDADMAC adsorption conditions: $pH_0 = 9.0$ or 10.0, $C_p = 0.10$ g/l. BSA binding conditions: $pH_1 = 9.0$, $I_1 = 0.10$, $C_{pr} = 0.10$ g/l, 10 g/l PDADMAC–CPG.

longer PDADMAC/CPG mixing time clearly enhances the protein binding process. de Laat et al. [32] found that amount of PAA adsorbed on $BaTiO_3$ changed rapidly at first, especially for short polymer chains, but then gradually over three weeks. Such adsorption processes of variable rates are attributed to the following phenomena. First, polyelectrolyte adsorption is diffusion controlled near the solid surface [32]. For polydispersed sample as the one used here, the low-molecular-mass polymer is initially adsorbed due to its fast diffusion, then replaced by the energetically more favored high-molecular-mass polymer [32]. Second, the initial configurational entropy of the adsorbed polymer may be increased by rearrangement of configuration after adsorption [28]. Both of these two processes are slow and may lead to additional adsorption. It is likely that an increase in the amount of polymer adsorption with mixing time accounts for the results in Fig. 3.

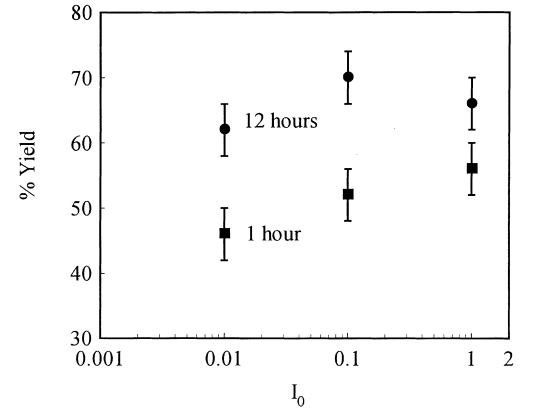

Fig. 3. Effect of preparation time (t_0) and ionic strength (I_0) on subsequent BSA binding. PDADMAC adsorption conditions: $pH_0 = 8.5$, $C_p = 1.0$ g/l. BSA binding conditions: conditions same as for Fig. 2.

Fig. 4 shows selective protein binding by PDADMAC–CPG. β-Lactoglobulin is more favorably bound to PDADMAC–CPG than BSA, which is consistent with the coacervation selectivity of PDADMAC for these proteins [34]. Thus, the selective protein binding properties of PDADMAC in solution are retained after it is immobilized on the CPG surface.

4.2. Polyelectrolyte adsorption layer

The amount of adsorbed polyelectrolyte is a key parameter in the interpretation of protein binding results. Commonly used techniques for the quantitation of adsorbed polymer include adsorption isotherms, ellipsometry, and reflectometry [37]. However, these techniques are not applicable to the PDADMAC–CPG system for several reasons. First, a relatively concentrated polymer solution was used in the current experiments to ensure adsorption leading to loop- and tail-type configuration. The amount of polymer adsorbed was therefore insignificant compared to the bulk polymer concentration. Attempts to generate adsorption isotherms through

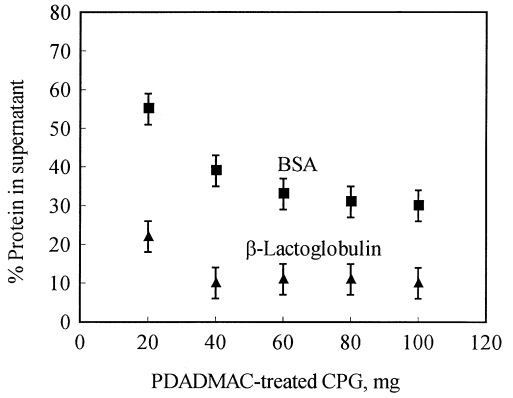

Fig. 4. Binding of BSA and β-lactoglobulin to PDADMAC–CPG. PDADMAC adsorption conditions: $pH_0 = 9.0$, no added salt, $C_p = 1.0$ g/l. Protein binding condition same as for Fig. 2.

elemental analysis failed. Other techniques, such as ellipsometry and reflectometry are normally applied only to planar surfaces. Recently, the absorption of polymers tagged via fluorescence [38] or radiolabels [39] has been effectively used to monitor polymer adsorption. However, the lack of such labeled polymers impeded the use of these two powerful techniques. Therefore, we employed two semiqualtitative methods to determine polymer adsorption layer thickness on PDADMAC–CPG, and the results were used to interpret protein binding experiments.

4.3. Chromatography

SEC was employed to measure the pore radius of polyelectrolyte-treated and native CPG using the cylindrical pore model [40]. According to this model:

$$K_{SEC} = \left(1 - \frac{R}{r_p}\right)^2 \quad (4)$$

where K_{SEC} is the measured partition coefficient, R is the solute radius, and r_p is the pore radius. Although Eq. (4) rests on an unrealistically well-defined pore geometry, it has been empirically verified by several groups [41,42], who have observed that plots of $K_{SEC}^{1/2}$ vs. R yield straight lines with slopes of $1/r_p$. With this method, the pore radius of CPG was obtained both before and after polyelectrolyte adsorption, and the difference between these two values (Δr_p) was used to estimate polyelectrolyte adsorption layer thickness (δ_H).

Fig. 5 Shows a plot of $K_{SEC}^{1/2}$ vs. R for pullulan standards eluted at $I_1 = 0.001$ on both native and polyelectrolyte-treated CPG ($pH_0 = 9.0$, $C_p = 20$ g/l, no salt added), giving $r_p = 11.4 \pm 0.4$ and 8.9 ± 0.3 nm, for native and polyelectrolyte-treated CPG, respectively. Therefore, $\delta_H = \Delta r_p = (11.4 \pm 0.4) - (8.9 \pm 0.3) = 2.5 \pm 0.5$ nm. The error limits shown here are obtained from the standard deviation of the slope. Additional measurements at $I_1 = 0.01$ and 0.10 both gave $\delta_H = 1.7 \pm 1.5$ nm. The relatively large error in δ_H led us to seek other methods to quantitate δ_H.

4.4. QELS

QELS was chosen as an alternative method to examine the structure of the adsorbed polyelectrolyte. Since CPG particles were too large to be characterized by QELS, we used small silica particles (KE-E10) and low-molecular-mass polyelectrolyte (L-120) to model PDADMAC adsorption on CPG. The system was chosen to minimize sedimentation and bridging flocculation. It is expected that the influence of pH_0, I_0, and t_0 on polyelectrolyte adsorption thickness should be similar to that of CPG/PDADMAC.

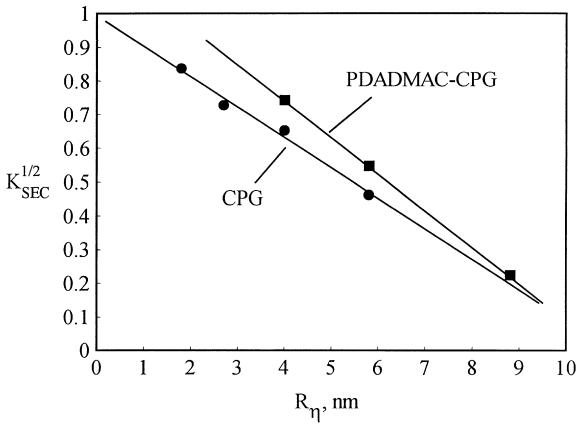

Fig. 5. Dependence of SEC chromatographic partition coefficient on pullulan Stokes radius, used to determine CPG effective pore size. SEC mobile phase: pH 9.0, 0.001 *M* Tris buffer. PDADMAC–CPG prepared at pH = 9.0, $I = 0$, $C_p = 20$ g/l.

The hydrodynamic radius of silica was measured both before and after polyelectrolyte adsorption, and the difference between the two ΔR_s^{app} was taken as the hydrodynamic polyelectrolyte adsorption layer thickness (HLT), with the results shown in Fig. 6. The numbers in the parentheses are the conditions used for the QELS measurement, and thus the filled circles represent results obtained at pH other than adsorption conditions ($pH \neq pH_0$). HLT is seen to increase with pH_0 and to be virtually independent of the measurement pH. HLT for weakly adsorbed chains would be expected to change with measurement pH. Therefore, this initial pH dependence suggests a relative strong adsorption of PDADMAC on silica surface.

Fig. 7 shows the effect of polyelectrolyte adsorption mixing time on HLT. At least 4 h are required for the adsorption process to reach equilibrium. This observation demonstrates that polyelec-

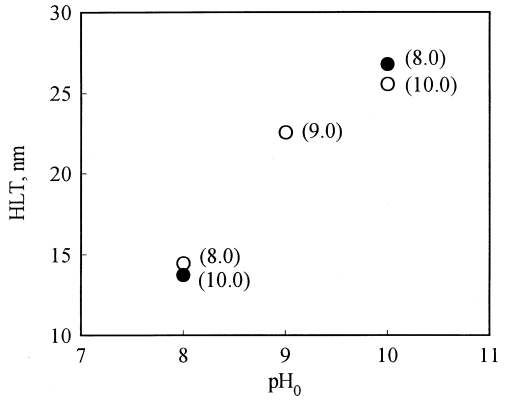

Fig. 6. Effect of adsorption pH on hydrodynamic layer thickness on silica (by QELS). Silica concentration = 0.02 g/l, C_p = 2 g/l, I = 0.01. The numbers in parentheses indicate the measurement pH.

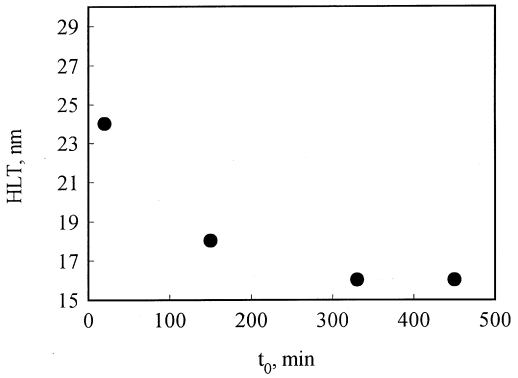

Fig. 7. Effect of adsorption time on hydrodynamic layer thickness.

trolyte adsorption may include a slow process, consistent with the results of de Laat et al. [32].

The effect of ionic strength on HLT is summarized in Table 1. HLT was first measured at I_0 after 6 h mixing time, as shown in column 2. After the initial measurement, the ionic strength was abruptly changed to I_1 = 0.1 by adding NaCl, with the corresponding QELS results shown in column 3. Lastly, a repeat measurement after 23 h is shown in column 4. Dahlgren et al. [43], who measured the adsorption of poly{[2-(propionyloxy)ethyl]trimethylammonium chloride} (PCMA) on mica surface with an interferometric surface force technique, showed that the structure of the adsorbed layer of polyelectrolyte is strongly dependent on the ionic strength of the solution from which the initial adsorption takes place. It was believed that the major contribution for the surface force measured by the interferometric surface force technique was from the loops. In other words, the structure of adsorbed loops were determined by initial ionic strength. However, Van de Beek and Cohen Stuart [44] reported that HLT for PEO adsorption on silica particles as measured by QELS was dominated by free chain ends (tails). As shown in column 3, the decrease of HLT upon increasing the measurement ionic strength suggests that tails are partially collapsed (since we assume that loops cannot collapse), which is similar to the ionic strength effect on polyelectrolyte in solution. The measurement after 23 h shows that a stable HLT was achieved soon after ionic strength adjustment, which is consistent with the solution properties of tails.

Table 1
Adsorption layer thickness (HLT, nm) of PDADMAC on silica

I_0	HLT_0[a]	HLT_1[b]	HLT_1, 23 h[c]
0	33±2	20±3	20±2
0.001	30±2	13±3	–
0.010	17±2	16±2	–

[a] Measured at $I = I_0$.
[b] Measured at I = 0.1, immediately after adsorption.
[c] Measured at I = 0.1, 23 h after adsorption.

The dependence of protein binding to polyelectrolyte-treated CPG on pH_0, I_0, and t_0 suggests that the structure of the adsorbed polyelectrolyte, namely the arrangement of loops and tails, affects subsequent protein binding. Therefore, it should be possible to relate protein binding to PDADMAC–CPG to δ_H and HLT obtained from SEC and QELS, respectively.

An explanation for the effect of pH on polyelectrolyte adsorption layer thickness and subsequent protein binding is illustrated schematically in Fig. 8. Let us assume that n binding sites per polymer chain require polyelectrolyte retention on the CPG surface. At low pH, where the surface charge density of CPG is low, a relatively flat configuration is needed to produce the requisite number of ionic contacts, and fewer polyelectrolyte molecules are adsorbed. On the other hand, an increase in surface charge density at high pH means that a shorter length of adsorbed chain can provide energetic stabilization of the

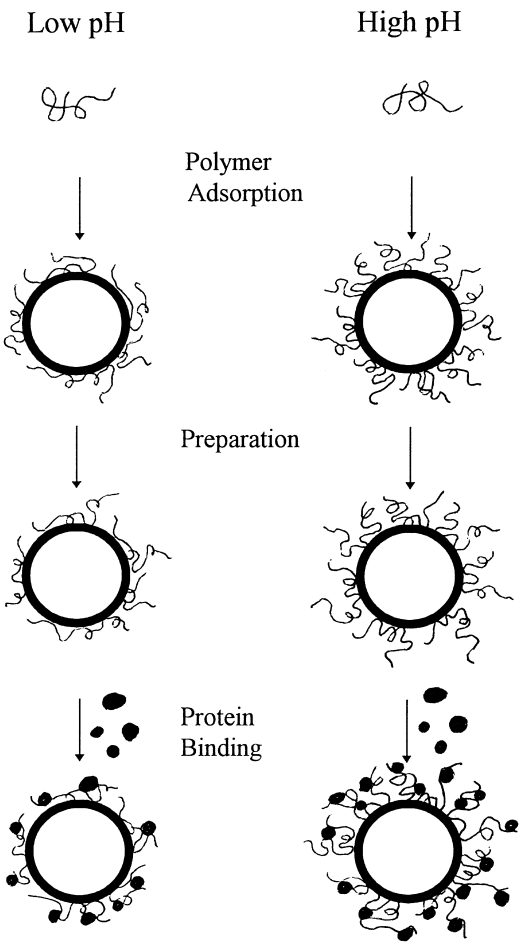

Fig. 8. Schematic depiction of effect of polyelectrolyte adsorption pH on subsequent BSA binding.

bound state. This results in an increase of adsorption amount. Furthermore, repulsion between adsorbed chains yields a thicker δ_H. QELS results show that HLT increases with pH_0 (Fig. 6); therefore more sites are available for subsequent protein binding, as verifed by the protein binding results in Fig. 2.

It is difficult to reconcile the effect of ionic strength I_0 on protein binding with the effect of I_0 on HLT measured by QELS, since the former is almost negligible (Fig. 2), while a large effect was observed for HLT. According to Claesson [45], a more extended (flatter) conformation of the adsorbed layer can be achieved when the ionic strength is larger than 1 mM. Therefore, we suggest that polyelectrolytes adsorbed at low ionic strength adopt a loose structure of loops and tails due to electrostatic repulsion among adsorbed chains, and each polymer chain may adsorb several protein molecules, as shown in Fig. 9. On the other hand, repulsion among polyelectrolytes is effectively screened at higher ionic strength, leading to a denser adsorbed polyelectrolyte layer. Although more polyelectrolyte is adsorbed, proteins can only bind at the periphery. These various effects may cancel each other, and reduce the influence of ionic strength.

Although Figs. 8 and 9 rationalize the effect of pH_0 and I_0 on protein binding with QELS results, it should be noticed that a significant discrepancy exists between the silica model system and polyelec-

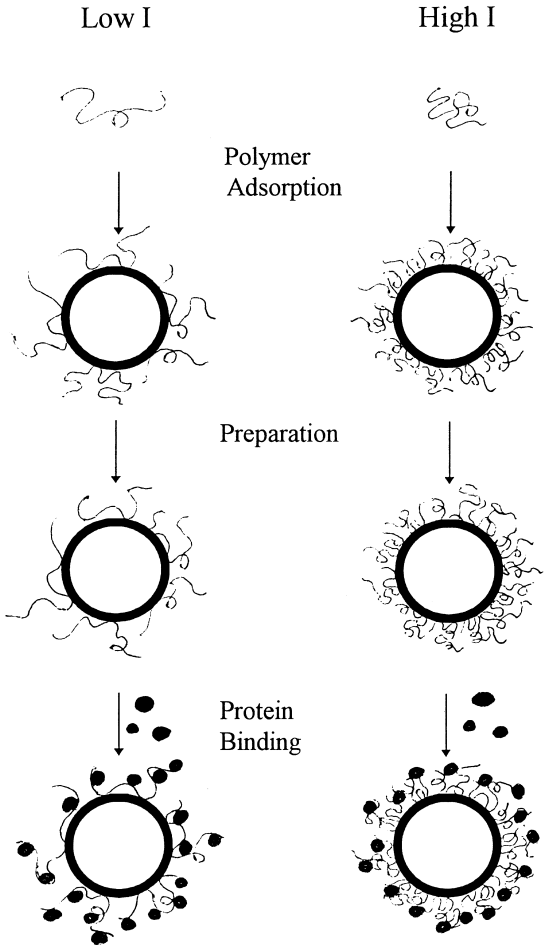

Fig. 9. Schematic depiction of effect of polyelectrolyte adsorption ionic strength on subsequent BSA binding.

trolyte-treated CPG. First, the discrepancy between δ_H (2.5 nm) and HLT ($\geq$17 nm) suggests that the model system does not represent the true polyelectrolyte adsorption process on CPG. Silica is more highly curved than CPG, so that the configurational entropy of train-adsorbed polyelectrolyte on silica is more unfavorable than on CPG. If loops and especially tails are more predominant for PDADMAC on silica, HLT could be much higher than δ_H. Second, the surface charge density of silica is expected to be higher than that of CPG, which is likely to affect the polyelectrolyte adsorption layer thickness. Third, CPG has a porous surface while silica is relatively smooth. An over-simplified picture of polyelectrolyte adsorption within the pore used to obtain δ_H from SEC might contribute to the discrepancy as well. Finally, QELS is primarily sensitive to the tails of adsorbed polyelectrolytes, while both loops and tails contribute to the protein binding. All of these problems remain as challenges for future research.

5. Conclusions

Protein binding to polyelectrolyte-treated CPG depends on the configuration of the adsorbed polyelectrolyte, which is determined in the polyelectrolyte adsorption stage. After adsorption, the protein-binding selectivity of the polyelectrolyte is maintained. Therefore, a properly designed polyelectrolyte-treated glass could be widely used in chromatographic separation of proteins.

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