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Measurement of the Binding of Proteins to Polyelectrolytes by Frontal Analysis Continuous Capillary Electrophoresis

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We have developed a novel technique, frontal analysis continuous capillary electrophoresis (FACCE), to study the binding of proteins to polyelectrolytes. Compared with existing electrophoresis methods such as conventional frontal analysis and the Hummel−Dreyer method, FACCE offers enhanced lower detection limits and is free from effects due to slow binding kinetics, thus making it suitable for studying equilibrium systems. In addition, with a single calibration, FACCE provides for efficient quantitative analysis. Here we report results obtained with *β*-lactoglobulin as the ligand and sodium poly(styrenesulfonate) (NaPSS) as the ligand-binding substrate. For this model system, FACCE yields reproducible calibration curves and binding isotherms. The binding parameters so determined are compared with previous results for other protein−polyelectrolyte systems.

Interactions between proteins and polyelectrolytes give rise to a number of important phenomena. Biologically, such interactions are critical to DNA replication and gene regulation.[1] Technologically, the association of proteins and polyelectrolytes can be applied to protein purification[2,3] and separation,[4,5] enzyme immobilization and activity control,[6] and polymer-mediated drug delivery.[7,8] Record et al.[9] have reviewed studies on protein complexation with DNA, and Dubin et al.[10] have described the literature on the interactions between proteins and synthetic polyelectrolytes. Some of the experimental methods employed to study protein−polyelectrolyte complexes include sedimentation,[11] size exclusion chromatography,[12] colloid titration,[13] light scattering,[14,15] electrophoretic light scattering,[16,17] gel electrophoresis,[18] fluorescence,[14,19] filter binding assays,[20] and measurements with ion-selective electrodes.[21] More recently, capillary electrophoresis (CE) has been increasingly used for substrate−ligand binding studies, such as protein association with drugs,[22−25] inorganic ions,[26,27] sugars,[28] and micelles.[29,30] These CE methods have involved either mobility measurements[25] or the determination of concentrations of free or bound ligand.[22,23,31] In this paper, we describe a novel method, frontal analysis continuous capillary electrophoresis (FACCE), for the study of protein−polyelectrolyte binding.

In contrast to conventional frontal chromatography (CFC) or its counterpart in capillary electrophoresis, FACCE involves continuous sampling. Figure 1 illustrates the differences between CFC and FACCE. Conventional frontal chromatography employs regular chromatography methods but with a sample volume relatively large compared to the capacity of the separation column. Careful selection of the amount of sample injected, the flow rate of the mobile phase, and the length of the separation column leads to elution of analytes as continuous but distinct plateaus, followed by resumption of detector baseline response (Figure 1a). In FACCE, the capillary is filled and equilibrated with the run buffer prior to sample introduction. The inlet end of the capillary is then immersed in the sample vial, and a voltage is applied across the capillary to initiate the sample introduction and separation process. Note that, in this particular frontal capillary electrophoresis

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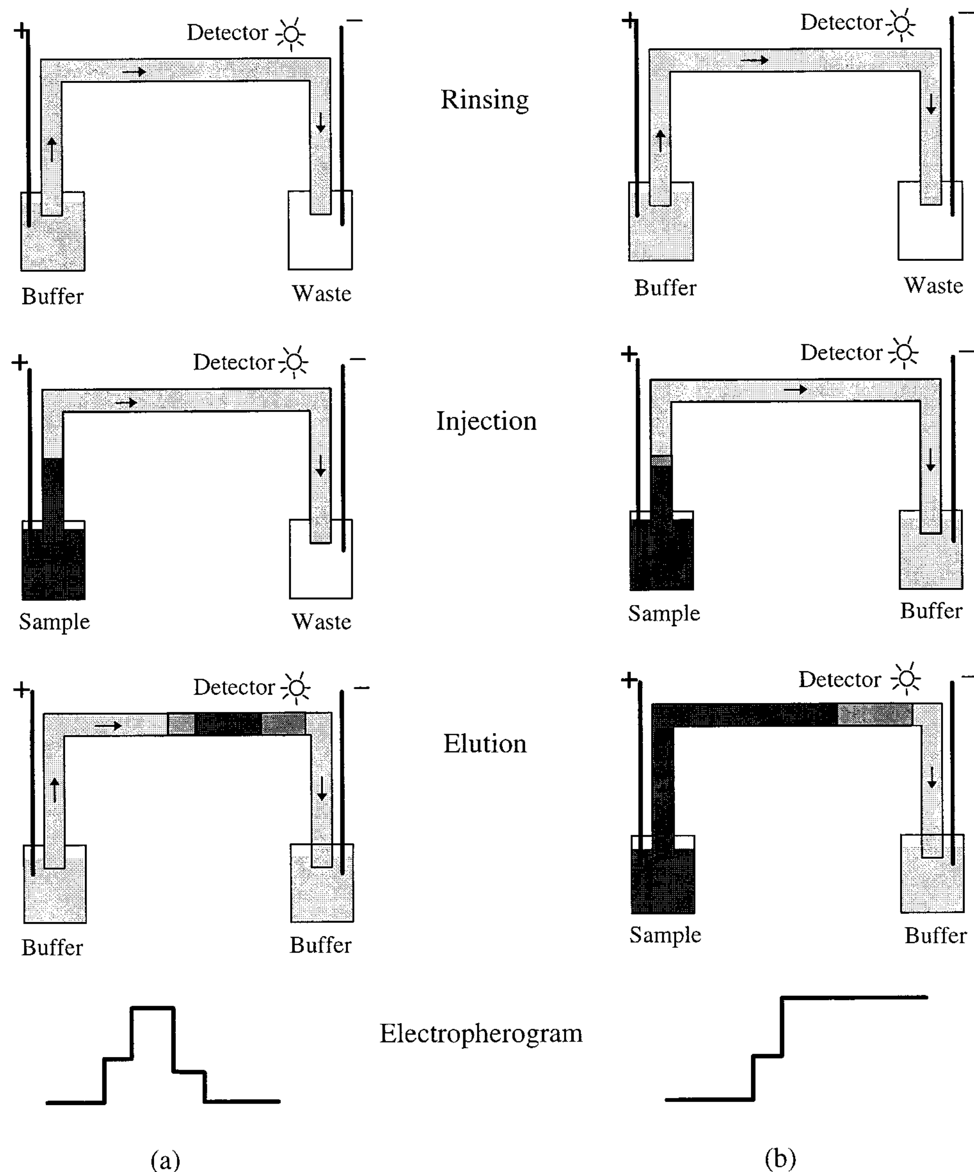


Figure 1. Schematics of CFC in CE (a) and FACCE (b).

method, sample introduction and sample separation are integrated into one process. Species separated by electrophoresis appear as discrete and progressive plateaus in the electropherograms, similar to those of isotachophoresis using a conductivity detector, except that only the first plateau in the electropherogram contains a single analyte (Figure 1b). The separation profile of FACCE is particularly suitable for binding studies, in which the determination of the concentrations of free or bound ligands without complete separation avoids perturbation of the binding equilibrium.

The accuracy and the reproducibility of FACCE rely on the correct selection of the type of capillary and the proper conditioning of the capillary surface. The walls of the capillaries used should have minimum solute interactions in order to avoid adsorption and the concomitant deterioration of reproducibility. In addition, the capillary must be conditioned to ensure that the surface conditions are identical at the start of each run. The linearity of the calibration curve is one indication of the suitability of the capillary used and the effectiveness of the capillary conditioning process.

The principal system in this work consists of β-lactoglobulin (isoelectric point pI = 5.2) and a polyanion, sodium poly(styrenesulfonate) (NaPSS), in phosphate buffer. Here, NaPSS is the substrate and β-lactoglobulin is the ligand, since each NaPSS can bind a large number of protein molecules. As is typical of protein−polyelectrolyte systems, complexation of β-lactoglobulin with NaPSS is highly dependent upon solution conditions, especially pH and ionic strength.[4,9,13] A decrease in pH increases the net positive charge on β-lactoglobulin and, hence, its electrostatic interactions with NaPSS. A decrease in ionic strength weakens the shielding of charges on both the protein and the polymer, thus increasing the binding. Various degrees of complexation between proteins and polyelectrolytes under different conditions lead to a range of effects, from the formation of soluble complexes[3,16] to bulk phase separation.[4,13] The goal of this research is to measure the binding parameters when soluble complexes are formed in homogeneous solution. Interestingly, soluble complexes are formed at pHs above the pI of β-lactoglobulin, i.e., at conditions when the net charge on β-lactoglobulin is negative and thus of the same sign as the polyanion. It has been proposed that such complexation is a consequence of protein surface charge heterogeneity.[16] Such systems are particularly suitable for CE since the net negative charge on both binding

species minimizes surface adsorption on the uncoated fused-silica capillary. FACCE may then be used to determine the concentrations of free and bound β-lactoglobulin in a series of experiments, from which binding isotherms can be obtained.

EXPERIMENTAL SECTION

a. Reagents and Solutions. β-Lactoglobulin A and B (Catalog No. L-2506) was purchased from Sigma (St. Louis, MO). Sodium poly(styrenesulfonate) (NaPSS) of $M_w = 465$ kDa with $M_w/M_n < 1.4$ was obtained from Polymer Standard Service (Germany). High-purity water with $R \approx 18$ MΩ generated by a Milli-Q water purification system was used to make all solutions. Phosphate buffers were prepared from monobasic and dibasic salts of sodium phosphate obtained from J. T. Baker Chem. Co. (Phillipsburg, NJ). Mesityl oxide was purchased from Aldrich Chemical Co. (Milwaukee, WI). Samples of different protein and polymer concentrations based on volume were made from the freshly prepared stock solutions of β-lactoglobulin and NaPSS based on weight in run buffer. The concentration ranges of protein and polyelectrolyte were 0.32–2.4 and 0.06–0.6 g/L, respectively. Sample pH was adjusted with 0.1 N NaOH if necessary to be within 0.01 pH unit of the pH of the run buffer.

b. Apparatus, Operation Parameters, and Experimental Procedures. Capillary electrophoresis was carried out on a Beckman P/ACE 5500 system with a UV detector. A bare fused-silica capillary with i.d. = 50 μm from Restek (Bellefonte, PA) was used, and the capillary cartridge temperature was controlled at 25.0 ± 0.1 °C.

Short capillaries are preferred to enhance the efficiency of the experiments and to minimize problems related to solute adsorption on the capillary surface. We used the shortest capillary allowed by the instrument, with a total length of 27 cm and an effective separation length (from inlet to detection window) of 20 cm.

The use of high voltages can lead to faster separation with higher efficiencies and resolution, but high voltages also result in more Joule heating of the sample. If the heat is too great to be dissipated, the temperature inside the capillary will increase, leading to broader peaks with lower peak heights, and possibly to protein denaturation. To determine the optimum separation voltage, a neutral marker of 0.2% (w/w) mesityl oxide in 0.05 M phosphate buffer was repeatedly injected hydrodynamically for 3 s under different separation voltages. The dependence of peak height upon separation voltage was used to determine an optimum voltage range of 5–12 kV.

Although this paper focuses on the use of FACCE, other techniques in CE were included for comparison. Each method was carried out according to the respective procedures described below.

(1) Hummel–Dreyer Method.[32] The capillary was rinsed with 0.1 M NaOH for 2 min and then with the run buffer containing a certain concentration of β-lactoglobulin for 6 min under pressure. Peak height calibration was done by collecting electropherograms of a series of known β-lactoglobulin concentrations. Finally, samples with a fixed amount of NaPSS were injected and studied by electrophoresis. The above procedures were repeated for each buffer containing different concentrations of β-lactoglobulin. To facilitate the analysis of the protein peaks in the electropherograms, the detector was set in the indirect UV mode to convert the negative protein peaks into positive ones.

(2) Conventional Frontal Analysis. The capillary was rinsed with 0.1 M NaOH for 2 min and then phosphate buffer for 6 min, then a long sample plug of a mixture of β-lactoglobulin and NaPSS was injected into the capillary under pressure, followed by electrophoretic separation.

(3) FACCE. The capillary was rinsed with concentrated phosphate buffer (1.0 M, pH = 6.7) for 6 min, followed by run buffer for 6 min. Since the plateau heights are not affected by a slight drift in electroosmotic flow, concentrated buffer instead of base was used to rinse the capillary to maintain the equilibrium of the phosphate buffer with the capillary. The capillary inlet end was then transferred to the sample vial with voltage on, to initiate sample introduction and separation. Plateau height calibration for each series of experiments was done with known concentrations of β-lactoglobulin either immediately before or immediately after sample runs. Free β-lactoglobulin concentrations in samples were determined directly from the electropherograms on the basis of plateau height calibration. Bound β-lactoglobulin concentrations were calculated by subtracting the free from the total concentrations in each sample.

RESULTS AND DISCUSSIONS

Figure 2 shows typical electropherograms for a β-lactoglobulin–NaPSS sample obtained by different methods in 0.05 M phosphate buffer at pH = 6.7 with the same capillary. Electropherograms obtained with the Hummel–Dreyer method using indirect UV detection show positive peaks for the protein and negative peaks for the complexes (Figure 2a). The Hummel–Dreyer method makes it possible to control the free β-lactoglobulin concentration as a truly independent variable. However, we were not able to get reproducible binding data with this method, even though peak height calibrations show good linearity ($R^2 > 0.99$). Initially, we thought this difficulty might result from extrapolation of the calibration curve to determine the free protein concentrations in the samples. To avoid this problem, samples that showed vacancy peak heights out of the calibration range were supplemented with additional protein and then rerun. However, we found that the bound and the free β-lactoglobulin concentrations so determined are dependent upon the initial amount of protein present in the injected sample. Suspecting that slow binding kinetics of formation of soluble complexes may affect the amount of free or bound protein, we studied the complex sample, the protein sample, and the polymer sample separately in regular electrophoresis by injecting each of these samples in the phosphate run buffer. The broader peak of the NaPSS for the complex sample in Figure 3 indicates that the binding kinetics is indeed slow compared with the separation time in the capillary. Problems associated with a slow binding process are intrinsic to the Hummel–Dreyer method since samples have to equilibrate during separation. Unless the separation time is significantly lengthened, the Hummel–Dreyer method is not appropriate for the current study. For similar reasons, affinity capillary electrophoresis would not be expected to be useful for the systems of this study.

(32) When the ligands are in the run buffer and the substrate is the injected sample, CE techniques have been referred to as either Hummel–Dreyer method[22] or affinity capillary electrophoresis (ACE).[25,31] The former, by analogy to the H–D method in size-exclusion chromatography, involves the quantitation of a ligand vacancy peak. ACE also involves the same process but commonly entails the measurement of mobility shift arising from ligand binding. The terminology for ACE arises from affinity chromatography since interaction between substrate and ligand is typically similar to the binding of protein to affinity columns. In our case, the interaction between substrate (polyelectrolyte) and ligand (protein) bears little resemblance to "protein–ligand" interactions, so we refer to this process as a Hummel–Dreyer approach.

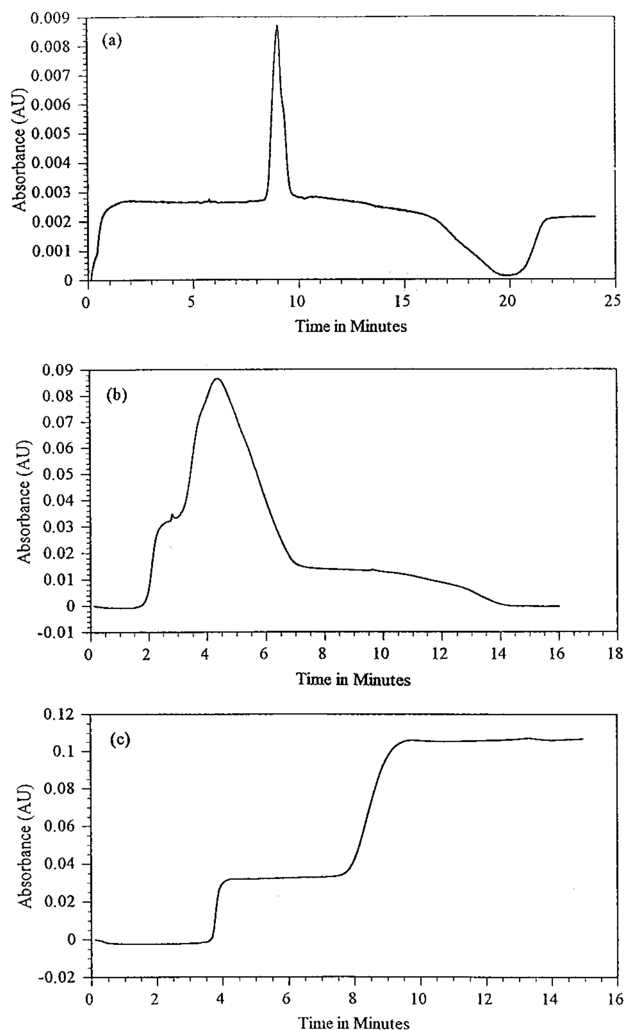


Figure 2. Typical electropherograms obtained with (a) the Hummel–Dreyer method with indirect UV detection, (b) CFC in CE, and (c) FACCE. Sample: (a) 0.2 g/L NaPSS; (b) and (c) 1.0 g/L β-Lactoglobulin + 0.2 g/L NaPSS. Run buffer: (a) 0.5 g/L β-lactoglobulin in 0.05 M phosphate at pH = 6.7; (b) and (c) 0.05 M phosphate at pH = 6.7. Sample injection: (a) 3.0 s, pressure; (b) 95 s, pressure. Detection: UV, 200 nm. Separation voltage: 7 kV.

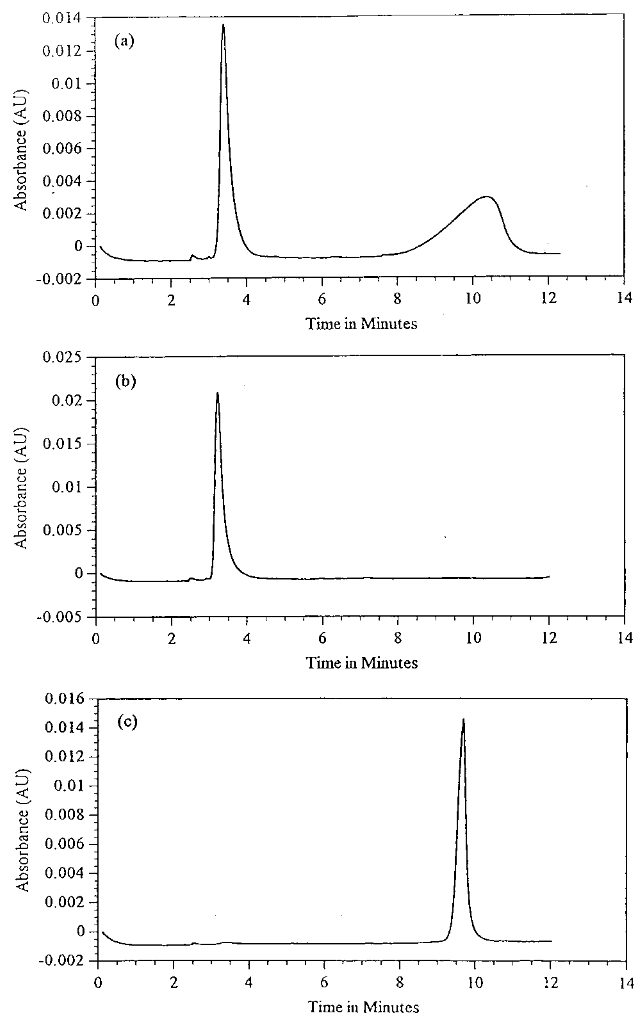


Figure 3. Electropherograms obtained for (a) 1.0 g/L β-lactoglobulin + 0.2 g/L NaPSS, (b) 1.0 g/L β-lactoglobulin, and (c) 0.2 g/L NaPSS. Run buffer: 0.05 M phosphate at pH = 6.7. Sample injection: 3.0 s, pressure. Detection: UV, 200 nm. Separation voltage: 7 kV.

The electropherogram obtained by the conventional frontal CE method (Figure 2b) reveals the separation of several species. The first shoulder peak arising from free β-lactoglobulin is followed by a large, complex peak with a long tail, resulting from the dissociation of the complex in the region where the equilibrium was perturbed by depletion of free protein. Although this method is not adversely affected by slow binding kinetics, the free β-lactoglobulin peak is difficult to quantitate. Any quantitative use of this method would require extensive development of methodologies for calibration.

In comparison to the electropherograms obtained by Hummel–Dreyer and conventional CE methods, the one obtained by FACCE (Figure 2c) is much simpler. The first plateau in the electropherogram of β-lactoglobulin–NaPSS is due to the elution of free protein, and the second plateau is due to free β-lactoglobulin and complex. The level plateaus, which allow accurate determination of heights, indicate negligible adsorption. Duplicate calibrations of plateau heights against protein concentrations agreed within 2% and generated straight lines with $R^2 > 0.99$, showing that the rinsing procedures were effective in conditioning and preserving the capillary surface. Samples with constant NaPSS concentration but different β-lactoglobulin concentrations were used to generate the binding isotherm. For every sample, the free β-lactoglobulin concentration was determined from the plateau height of the electropherogram; the bound β-lactoglobulin concentration was then determined by subtracting the free from the total β-lactoglobulin concentration. Assuming there is no free NaPSS in the samples, the average number of bound β-lactoglobulin molecules per NaPSS molecule, Y, can be calculated. A plot of Y versus the free protein concentration is the binding isotherm. Duplicate experiments indicate that the individual data in the binding isotherms are reproducible to within 10% (Figure 4).

The data for the first set of experiments in Figure 4 were analyzed with a Scatchard plot in order to establish an appropriate binding model. The appearance of two slopes in the Scatchard plot (Figure 5a) suggested that the corresponding binding isotherm in Figure 4 could be fitted with a two-class binding site model expressed in eq 1, where Y is the average number of

$$Y = \frac{n_1 k_1 [\mathrm{L}]}{1 + k_1[\mathrm{L}]} + \frac{n_2 k_2 [\mathrm{L}]}{1 + k_2[\mathrm{L}]} \qquad (1)$$

β-lactoglobulin molecules bound per NaPSS molecule, the sub-

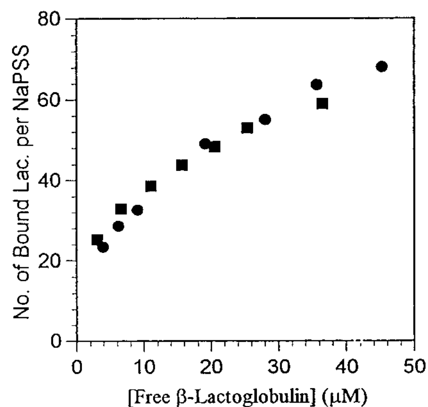
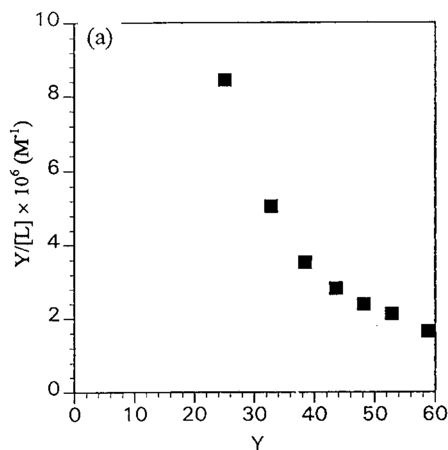


Figure 4. Reproducibility of the binding isotherm for various concentrations of β-lactoglobulin mixed with 0.2 g/L NaPSS (■, experiment 1; ●, experiment 2). Run buffer: 0.05 M phosphate at pH = 6.7.



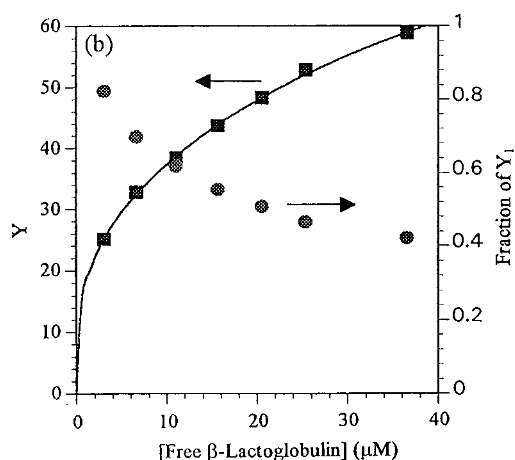
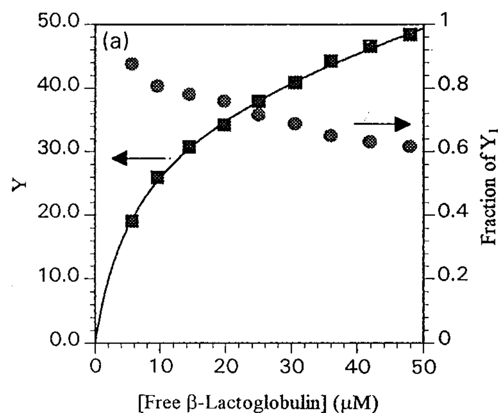


Figure 5. (a) Scatchard plot for data of experiment 1 in Figure 4. (b) Nonlinear curve-fitting (—) for the data of experiment 1 in Figure 4 (■, left axis) and the fraction of the binding contributions from the class 1 site (●, right axis).

scripts 1 and 2 represent the two classes of binding sites, n is the total number of binding sites in each class, k is the binding constant for each class, and $[L]$ is the concentration of free β-lactoglobulin. The nonlinear fitting shown in Figure 5b was done with PC-based DeltaGraph software employing a user-defined function to iterate the best-fit parameters. The binding isotherm and corresponding Scatchard plot were also obtained for β-lactoglobulin and NaPSS samples in 0.05 M phosphate buffer at pH = 6.9 using similar techniques for electrophoresis and data fitting (Figure 6). Table 1 shows the best-fit values of each parameter in eq 1. Since the contributions from both classes of binding sites to the total binding at each data point in Figures 5b and 6a are significant, the fitting is mathematically appropriate.



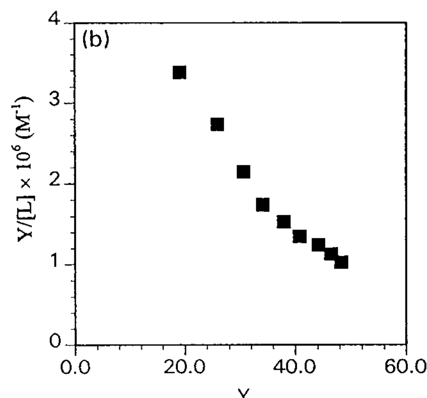


Figure 6. Results for β-lactoglobulin + 0.2 g/L NaPSS in 0.05 M phosphate buffer at pH = 6.9. (a) Best-fit binding isotherm (—) for the data (■, left axis) and the fraction of the binding contributions from the class 1 site (●, right axis). (b) Scatchard plot.

Table 1. Binding Parameters Derived from Two-Class Binding Sites Model

exptl params	pH		
	6.7[a]	6.7[a]	6.9
n_1	25	16	33
k_1 (M^{-1})	1.5×10^6	1.5×10^6	1.8×10^5
n_2	76	94	96
k_2 (M^{-1})	2.3×10^4	2.8×10^4	5.1×10^3

[a] Duplicate experiments, separate samples and calibrations.

As seen in Table 1, an increase in the buffer pH from 6.7 to 6.9 leads to nearly an order of magnitude decrease in the binding constants extracted from the best-fit equation, i.e., from 1.5×10^6 to 1.8×10^5 M^{-1} for k_1 and from 2.5×10^4 to 5.1×10^3 M^{-1} for k_2. On the other hand, the change in the number of binding sites for each class is relatively small. This change is consistent with a decrease in binding when the protein charge becomes more negative, as expected, but is remarkable in magnitude, given that the protein charge is changed by only ~10% (from −11.6 to −12.7).[33,34] While the current measurements appear to be the only data for a protein binding to a synthetic polyelectrolyte, the

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effect of ionic strength and ligand charge on the binding constant of oligo- and macroionic ligands to DNA has been the subject of both theoretical calculations[9,35-37] and experimental observation.[9,36] The large effect of protein charge on binding constant observed in our study is consistent with current models dealing with ligand binding to polyelectrolytes.[37,38]

The excellent fit of our experimental data to a two-class binding site model must be tempered by the recognition that four adjustable parameters are used in this process. Among the many possibilities that could give rise to the same binding behavior are cooperative binding or multiple binding affinities due to multiple classes of binding sites. In the present case, it is also necessary to appreciate the potential consequence of the mixture of β-lactoglobulin A and B or, in principle, the possible presence of protein oligomers in equilibrium with each other.[19,39-41] More extensive experimental data would be required to confirm such complicated models. The Hill equation may also be applied to cooperatively binding systems, but only if the bound ligand concentration at saturation has been determined, which was not possible in the concentration range of the present study. The possible influence of protein association on protein–polyelectrolyte binding could be tested with multiple binding isotherms obtained at several different protein and polyelectrolyte concentrations.[40]

The binding of proteins to polyelectrolytes is highly sensitive to solution conditions such as pH and ionic strength, as well as to the macromolecular solutes. Thus, comparisons with previous work are difficult. Nevertheless, we can compare the magnitude of the binding constants obtained in this study with previous measurements for a variety of protein–polyelectrolyte systems. By monitoring the quenching of tryptophan fluorescence in SSB-1, a protein mutant of *E. coli*, Bujalowski and Lohman[42] determined the intrinsic binding constants of tetramer and monomer of the protein to dT_{16}, a short DNA chain, to be 8×10^6 and 7×10^5 M^{-1}, respectively. With double-filtering techniques, Wong et al.[20] obtained multiple binding constants for the binding between dT_{16} and the *E. coli* Rep protein, ranging from 2×10^6 to 8×10^6 M^{-1}, depending on the models used to fit the binding isotherms. Yun et al.[21] used a heparin-responsive polymeric membrane electrode to obtain binding constants ranging from 6×10^5 to 9×10^7 M^{-1} for heparin binding to various proteins and polypeptides. Lohman et al.[11] used sedimentation techniques to obtain binding constants ranging from 10^2 to 10^5 M^{-1} for the binding of RNase to T7 DNA in solutions with different salt concentrations. Thus, our values of $5 \times 10^3 - 2 \times 10^6$ M^{-1} are well within the range of binding constants reported previously.

Under various experimental conditions, FACCE electropherograms can exhibit more features than multiple plateaus. Just as a sample peak in normal electrophoresis can be skewed in the form of fronting and tailing due to the mobility difference in the co-ions,[43] FACCE electropherograms may exhibit a gradual transition between plateaus or a hump at the beginning of a

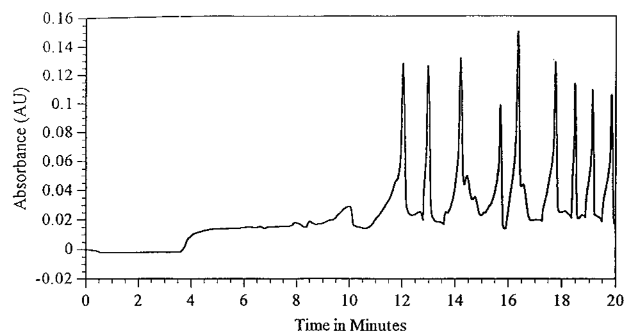

Figure 7. Electropherograms obtained for 1.0 g/L β-lactoglobulin + 0.2 g/L NaPSS. Run buffer: 0.02 M phosphate at pH = 6.9. Detection: UV, 200 nm. Separation voltage: 7 kV.

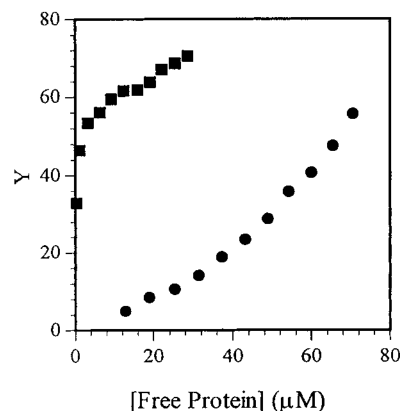

Figure 8. Binding of BSA (■) and β-lactoglobulin (●) to 0.1 g/L NaPSS in 0.1 M phosphate buffer at pH = 7.5.

plateau. For samples prepared in dilute buffer while using a more concentrated run buffer, local sample stacking at the sample/run buffer interface may occur due to the conductivity difference and create a hump at the beginning of the sample plateau. In general, these extra features do not interfere with the determination of the plateau heights, which contain the quantitative binding information. In low ionic strength buffers, severe distortion of plateau shape in FACCE electropherograms may be seen with an uncoated capillary. In 0.02 M phosphate buffer, for example, repeated experiments on several samples produced electropherograms similar to the one shown in Figure 7, in which multiple peak patterns replaced the second plateau for the complexes. Since the exact peak patterns are irreproducible and capillary dependent, we think that the phenomenon was due to adsorption of the complex on the capillary in low ionic strength solution when the shielding of its electrostatic interaction with the capillary wall is decreased. Regardless of the complicated pattern in this electropherogram, the desired binding data can still be extracted since the first plateau is due to the elution of free β-lactoglobulin.

To illustrate the application of FACCE in an additional system, we carried out limited studies with BSA and NaPSS. This protein, a fatty acid scavenger in blood serum, is commonly described as a hydrophobic protein, so comparisons of these two proteins might yield some information about hydrophobic contributions to the polyelectrolyte–protein interaction. As shown in Figure 8, BSA (pI = 4.9) does, indeed, bind more strongly to NaPSS than β-lactoglobulin. Considerations of molecular structure suggest that the polymeric sulfonate groups would preclude contact

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between styrene repeat units and the hydrophobic region of BSA, and we therefore consider it more likely that such contacts involve occasional sequences of unsulfonated styrene (the degree of sulfonation of conventional NaPSS is only ~90%). However, comparison of BSA to β-lactoglobulin is also complicated by the possibility that differences in charge distribution could favor the binding of the former.

Under the right experimental conditions, binding studies employing FACCE can be applied to various samples in which the mobilities of the free and the bound ligands are different. Capillary selection and separation mode must be optimized for the particular system under study. However, for protein–polyanion systems, with the normal separation voltage polarity and an uncoated capillary, unbound protein always elutes before bound protein and polyanion. To optimize separation and to minimize solute adsorption on capillary surface, it may be advantageous to use a deactivated capillary without reversing the electroosmotic flow. For protein–polycation systems, a polyimine-coated capillary may be used to alleviate solute adsorption on the capillary surface, and reversed separation voltage polarity would ensure that the free protein elutes first.

As a continuous electrophoresis method, FACCE can also be employed to monitor the time dependence of such complex equilibrium systems in a manner similar to previous studies in which continuous CE was used as a sample introduction step.[44,45] Such studies can be carried out either for samples kept under static conditions or subject to change, e.g., pH adjustment. If the experimental conditions are optimized so that only the free ligands elute in the capillary, FACCE can be configured to study the dependence of binding on a specific parameter such as pH, ionic strength, or concentrations of substrates/ligands in a continuous and uninterrupted fashion. It can be noted that the amount of ligand eluting in the capillary is negligible compared with the bulk volume of a typical sample vial. Focusing on one of the above-mentioned variables could greatly facilitate the study and the understanding of the binding equilibria in many macromolecular and biological systems.

In summary, FACCE has been used to obtain reproducible binding parameters for the interaction of β-lactoglobulin with NaPSS. FACCE shows several advantages over existing capillary electrophoresis methods. First, the problem of inadequate lower detection limit, which is intrinsic to the capillary electrophoresis method, is effectively minimized by the much longer sample introduction time (essentially the same as the sample separation time). Second, since FACCE measures a static equilibrium in the sample vial, not a dynamic equilibrium in the capillary as is the case in the Hummel–Dreyer method, it is thus free from the reproducibility problems arising from slow protein–polyelectrolyte complexation/dissociation kinetics. Finally, FACCE requires only a single calibration curve to quantify the dependence of plateau height on free ligand concentration, in direct contrast to the need for multiple calibrations for each run buffer with the CE Hummel–Dreyer method.[24] The last feature of the method allows data for a Scatchard plot to be collected in 1 day instead of weeks as required by chromatographic methods.[46] While FACCE may consume more sample than other CE-based methods, the calibration process conserves ligands (i.e., protein). All CE methods, including FACCE, require minute sample volumes, e.g., 0.5 μL for each analysis.

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