Capillary Electrophoresis of PEGylated Proteins

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Introduction

Capillary electrophoresis has become a well-established technique for evaluating the composition and concentrations of a wide range of protein samples[1-4]. In addition, capillary electrophoresis can be used to study a variety of protein properties including the net protein charge[5], the extent of ion binding[6], and the presence of specific biomolecular interactions[7-9]. This wide range of applications is possible, at least in part, because of the strong theoretical foundation for describing the electrophoretic mobility of globular proteins. Smoluchowski's initial analysis of the electrophoretic mobility is valid for arbitrary particle shape under conditions where the Debye length (double layer thickness) is much smaller than the characteristic particle size. Subsequent studies have accounted for the finite size[10] and relaxation of the electrical double layer[11], the effects of non-spherical particle geometry[12], and the influence of particle porosity[13-16]. The resulting theoretical expressions for the electrophoretic mobility are in good agreement with experimental results. In contrast, much less is known about the electrophoretic mobility of more complex biomolecules. For example, there is considerable interest in the use of PEGylated therapeutic proteins, formed by the covalent attachment of one or more polyethylene glycol chains to the native protein. PEGylation can significantly increase the biological half-life by reducing the rate of kidney clearance[17], and it can also minimize adverse immunological reactions[18], dramatically improving the overall efficacy of the therapeutic protein.

Fundamental studies of PEGylated proteins have clearly demonstrated that the attachment of the PEG chain(s) increases the effective protein size as determined by size exclusion chromatography[19, 20] and membrane sieving[20]. PEGylation also increases protein hydrophobicity, as determined by reverse phase chromatography[21], and it significantly reduces dynamic binding capacity in ion exchange chromatography [22], although no detailed models have been presented for either of these effects. PEGylated proteins do retain their electrical charge, with the resulting electrostatic interactions leading to a significant increase in retention during ultrafiltration through electrically-charged membranes with like polarity[23]. In addition, there is evidence that the polyethylene glycol chains on PEGylated proteins can elongate under flow, reducing the effective protein size at high flow rates[20].

Several investigators have used capillary electrophoresis for analysis of PEGylated protein samples. Semi-aqueous capillary electrophoresis has been used to resolve the different PEGylated forms of ribonuclease A[24], lysozyme[25], and recombinant TNF-α. Li et al.[24] used capillary electrophoresis to measure the extent of aggregation of PEGylated ribonuclease A under different storage conditions. More recently, Na et al.[27] used SDS capillary gel electrophoresis to check the purity of PEGylated α-interferon with both single and multiple PEG chains. In addition, Sharma and Carbeck[28] used capillary electrophoresis to calculate the effective size of PEGylated lysozyme, implicitly assuming that the electrical charge on the native protein is displaced to the outer radius of a PEGylated sphere. The calculated values of this effective radius were in good agreement with results from partitioning in size exclusion chromatography, although the authors provided no physical basis for the use of this model.

The objective of this study was to obtain quantitative data for the electrophoretic mobility and resolution of PEGylated α-lactalbumin using capillary electrophoresis. Data were also obtained with an acetylated form of α-lactalbumin, which was generated by covalent modification of the lysine amino groups but without any significant change in the protein molecular weight due to the small size of the

ligand. Data were analyzed using available theoretical models for the electrophoretic mobility of composite objects to obtain additional insights into the underlying structure of the PEGylated proteins.

Materials and Methods

All experiments were performed using α-lactalbumin as a model protein (Sigma Chemical, St. Louis, MO). The protein was PEGylated with mPEG-SPA (activated with succinimidyl propionic acid) or mPEG-SMB (activated with succinimidyl α-methylbutanoate), each having nominal weight-average molecular weight of 2, 5, 10, 20, or 30 kDa. PEGylation was performed by slowly adding the activated PEG to a 10 g/L solution of α-lactalbumin in 10 mM Bis-Tris buffer at pH 7. Acetylated proteins were synthesized using the general procedure described by Gao and Whitesides[5], which converts the free amine groups on one or more lysines to the corresponding (uncharged) amide.

The electrophoretic mobility of the PEGylated and acetylated proteins were determined using an Agilent G1600A high performance capillary electrophoresis system equipped with a dual-polarity variable high voltage DC supply (0-30 kV) and a diode array UV/visible absorbance detector (214 nm wavelength for detection). Experiments were performed with negatively-charged fused-silica capillaries (inner diameter of 0.5 μm) with a total length of 80.5 cm and an effective length (from injection to the detection window) of 70.2 cm. The capillary and solution reservoirs were filled with buffered electrolyte, most commonly either 10 mM Tris/Glycine (pH 8.1) or 10 mM Bis Tris (pH 7.0). Protein samples (approximately 40 - 140 nL) containing 5 mM of mesityl oxide as a neutral marker were injected for 4 to 25 s at a pressure of 4 kPa. Data were obtained at a constant applied voltage of 25 kV, with the field direction chosen so that the bulk electroosmotic flow was toward the detector (and cathode). The electrophoretic mobility was calculated from the migration times as:

$$\mu_e = \frac{L_d}{E_z}\left(\frac{1}{t} - \frac{1}{t_o}\right) \qquad (1)$$

where L_d is the effective capillary length of capillary to the detector, E_z is the applied electric field, and t and t_o are the times for the protein and neutral marker to reach the detector.

Results and Discussion

Several different running buffers were examined to obtain good resolution and sharp symmetric peaks. Previous studies have shown that the buffer type and composition can strongly influence the overall resolution and separation[29]. Initial experiments used the semi-aqueous (water + acetonitrile) buffer employed by Li et al.[30] for analysis of PEGylated RNA-ase. Results were obtained between 10 and 80% acetonitrile, but peak resolution was poor and decreased at high acetonitrile concentrations. Typical results using 10 mM Bis Tris buffer (the buffer used in the PEGylation reaction) are shown in Figure 1 (lower electropherogram). The first peak is the neutral marker (mesityl oxide) that was added to the PEGylated sample. The second distinct peak is the PEGylated α-lactalbumin with one 20 kDa branch. The third peak is the native α-lactalbumin. Although it was possible to resolve peaks corresponding to the PEGylated proteins with different numbers of PEG chains and the native protein, there was significant peak asymmetry and the overall resolution was poor using the Bis Tris buffer. The upper electropherogram in Figure 1 shows typical results using a 10 mM Bis Tris with 0.1 M glycine added to the buffer. The resolution between α-lactalbumin and the PEGylated α-lactalbumin with a single 20 kDa PEG chain increased from 2.1 to 4.7 upon addition of the glycine, consistent with results obtained previously using other zwitterionic additives[2]. In addition, the symmetry factor of the α-lactalbumin peak decreased from 13.6 to 3.6 in the presence of glycine. The improved resolution is

likely due to competition of the glycine for the silanol groups on the capillary, which reduces the extent of protein adsorption, along with the suppression of joule heating associated with zwitterions[31].

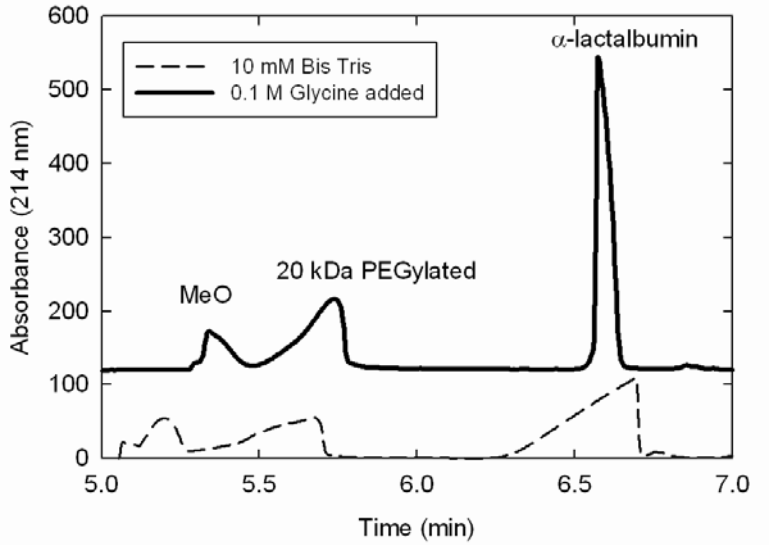

Figure 1. Electropherogram of a 20 kDa PEGylated protein with running buffers of Bis Tris (pH 7.0, 10 mM) (lower curve), and Bis Tris with 0.1 M glycine (pH 7.0, 11mM) (upper curve).

Figure 2 shows electropherograms for both the acetylated (lower curve) and PEGylated (upper curve) α-lactalbumin using a 10 mM pH 8.1 Tris-Glycine running buffer at 25 kV. In both cases, the first peak is the neutral marker (mesityl oxide), which is carried to the detector solely by the electroosmotic flow generated within the negatively charged capillary. The second peak with the acetylated sample corresponds to the native α-lactalbumin, which migrates back against the electroosmotic flow and thus exits at a later time than the neutral marker. Each subsequent peak in the lower curve represents an acetylated α-lactalbumin with one more lysine group capped by reaction with acetic anhydride, resulting in a more negatively charged species. Twelve distinct peaks were easily resolved, with only the first four shown in Figure 2. Note that even the most heavily acetylated species in this system has a molecular weight that is only 3.6% larger than the unmodified α-lactalbumin. The upper curve in Figure 2 shows the electropherogram for the PEGylated α-lactalbumin formed by reaction with 5 kDa mPEG-SPA. In contrast to the data for the acetylated α-lactalbumin, the peaks for the PEGylated species lie between those for the neutral marker and the unmodified α-lactalbumin due to the reduction in the electrophoretic mobility associated with the addition of the large neutral polyethylene glycol chain(s). Three distinct peaks, each corresponding to a PEGylated species with one more PEG chain, are easily observable in the electropherogram. A fourth peak, corresponding to α-lactalbumin PEGylated with four PEG chains, appears as a shoulder at t = 6.22 min. The peaks for the PEGylated species are much broader than those for the acetylated α-lactalbumin, due primarily to the polydispersity in the mPEG-SPA.

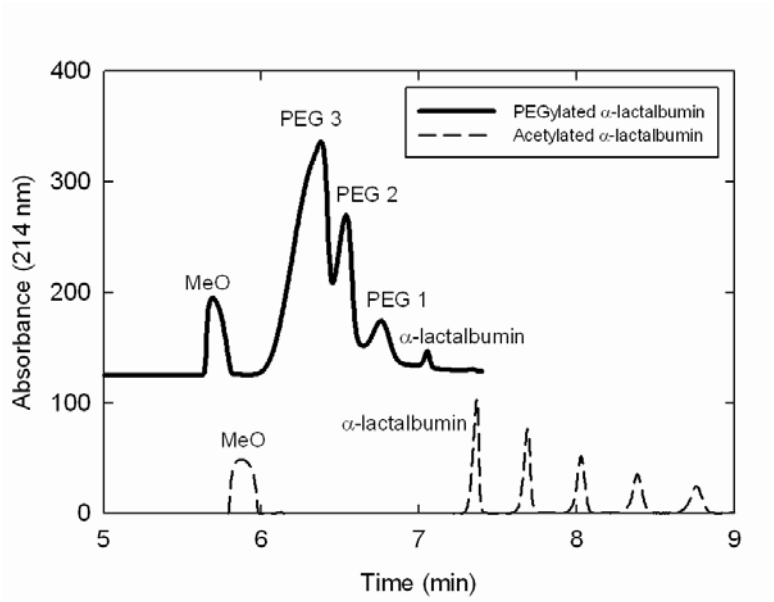

Figure 2. Electropherograms of acetylated α-lactalbumin (lower curve) and α-lactalbumin PEGylated with 5 kDa mPEG-SPA (upper curve).

The data in Figure 2 were used to calculate the electrophoretic mobility of the acetylated and PEGylated species using Equation 1. Results are shown in Figure 3 for experiments performed with PEGylated α-lactalbumin formed by attachment of PEG with molecular weight of 2, 10, 20, and 30 kDa PEG along with the corresponding results for the acetylated α-lactalbumin. In each case, the data are plotted as a function of the number of modifications to the native protein; thus, the species with $n = 1$ represents α-lactalbumin with either a single PEG group or a single acetylated lysine. The electrophoretic mobility of the acetylated species increases with increasing number of acetyl groups due to the increase in net negative charge associated with the conversion of the protonated amino group to an amide. In contrast, the electrophoretic mobility of the PEGylated α-lactalbumin decreases with increasing number of attached PEG groups even though PEGylation eliminates multiple amine groups (analogous to the acetylation reaction). The electrophoretic mobility of the PEGylated α-lactalbumin with one 20 kDa PEG chain is slightly smaller than that of the PEGylated protein with two 10 kDa PEG chains which is turn smaller than that for the PEGylated protein with four 5 kDa PEG chains even though these species all have basically the same molecular weight (and effective size as determined by size exclusion chromatography[19]). These differences are a direct result of the change in molecular charge, in addition to size, due to PEGylation.

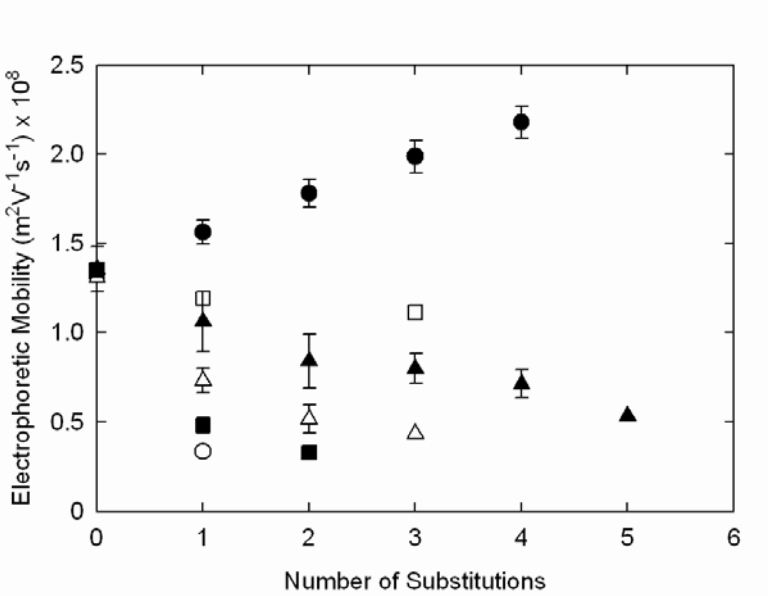

Figure 3. Electrophoretic mobility of the acetylated α-lactalbumin (•) and PEGylated α-lactalbumin with 2 kDa branches (□), 5 kDa branches (▲), 10 kDa branches (Δ), 20 kDa branches (■) and 30 kDa branches (○) as a function of the number of substituted lysine groups.

In order to account for the change in both size and net charge of the PEGylated proteins, the experimental data in Figure 3 have been analyzed using the drag ratio, K_d, which is equal to the electrophoretic mobility of the PEGylated α-lactalbumin divided by the mobility of the corresponding acetylated species with the same number of chemical substitutions. Thus, the drag ratio for the PEGylated α-lactalbumin with a single 10 kDa PEG group was evaluated by normalizing the mobility using data for the singly-acetylated protein while the drag ratio for the PEGylated α-lactalbumin with two 5 kDa PEG groups was evaluated by normalizing with data for the doubly-acetylated protein. This method effectively eliminates the contribution of charge since the PEGylated and acetylated proteins have the same number and distribution of charged amino acid residues. Sharma and Carbeck[28] used a similar approach to calculate the effective size of PEGylated proteins using capillary electrophoresis.

Experimental data for the drag ratio for the different PEGylated species are shown in Figure 4 as a function of the effective protein radius, which was calculated using the correlation presented by Fee and Van Alstine[32] based on partitioning data in size exclusion chromatography:

$$a_{PEGylatd} = \frac{C}{6} + \frac{2}{3C} a_{PEG}^2 + \frac{1}{3} a_{PEG} \qquad (2)$$

$$C = \left[108 a_{pro}^3 + 8 a_{PEG}^3 + 12 \left(81 a_{pro}^6 + 12 a_{pro}^3 a_{PEG}^3 \right)^{1/2} \right]^{1/3} \qquad (3)$$

where a_{pro} and a_{PEG} are the radii of the unmodified protein and the free PEG chain, respectively. The radius of a free PEG molecule was calculated as:

$$a_{PEG} = 0.01912 \times MW^{0.559} \qquad (4)$$

where a_{PEG} is in nm and the molecular weight (MW) is in Da[33]. The data in Figure 4 collapse to a single curve when plotted in this manner. For example, the drag ratio for the PEGylated α-lactalbumin with a single 10 kDa PEG chain is 0.44, which is within 10 % of the value for the PEGylated α-lactalbumin having two 5 kDa PEG chains (which is within the standard deviation of the measurements). This is in

sharp contrast to the 40% difference in the electrophoretic mobility of these species, which is directly due to the difference in electrical charge associated with the different number of modified lysine groups.

The solid curve in Figure 4 represents the predicted drag ratio calculated using Henry's equation:

$$\frac{\mu_e}{\mu_{e,o}} = \left[\frac{f(\kappa a_{PEGylated})}{f(\kappa a_{\Pr o})}\right]\left(\frac{a_{\Pr o}}{a_{PEGylated}}\right)\left(\frac{1+\kappa a_{\Pr o}}{1+\kappa a_{PEGylated}}\right) \qquad (5)$$

Equation (5) assumes that the PEGylated protein can be treated as a hard-sphere with net electrical charge equal to that of the acetylated species having the same number of modifications. The radius of the unmodified α-lactalbumin is a_{pro} = 1.98 nm while the radius of the PEGylated protein was evaluated using Equations (2) – (4). Henry's function, $f(\kappa a)$, was evaluated using the following expansion in κa[10]:

$$f(\kappa a) = 1 + \frac{1}{16}(\kappa a)^2 - \frac{5}{48}(\kappa a)^3 - \frac{1}{96}(\kappa a)^4 + \frac{1}{96}(\kappa a)^5 + \frac{1}{8}(\kappa a)^4 \exp[\kappa a]\left(1 - \frac{(\kappa a)^2}{12}\right)\int_{\kappa a}^{\infty}\frac{\exp[-t]}{t}dt \qquad (6)$$

where κ is the inverse Debye length. The model is in very good agreement with the experimental data, properly capturing the reduction in electrophoretic mobility caused by the increase in drag arising from the polyethylene glycol chains, with the change in protein charge associated with the PEGylation reaction incorporated through the normalization of the data using the electrophoretic mobility of the acetylated α-lactalbumin having the same number of substitutions.

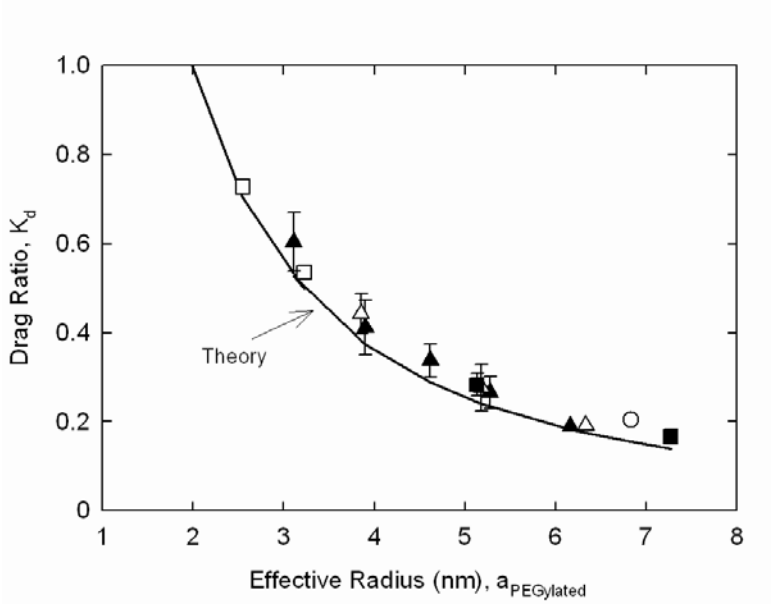

Figure 4. Drag ratio for PEGylated proteins as a function of the effective protein radius as shown with branches of 2 kDa PEG (□), 5 kDa branches (▲), 10 kDa branches (Δ), 20 kDa branches (■) and 30 kDa branches (○).

The good agreement between the experimental data and this simple model is in some ways surprising. Henry's analysis implicitly assumes that the electrical charge is distributed uniformly over the outer surface of a hard sphere, while the charge groups on the PEGylated proteins are distributed over the surface of the protein, which should be at least partially "buried" beneath a layer of polyethylene glycol. One possible physical explanation for this behavior is that the polyethylene glycol forms a shell that has a substantially lower ionic strength than in the bulk solution. The thermodynamics of PEG-salt systems have been studied quite extensively[34-37]. These systems tend to phase separate due

to the strong "negative" interactions between the salts and the PEG. The salt concentration in the PEG phase can be as much as seven times smaller than the salt concentration in the non-PEG phase[34]. Although it is difficult to directly extrapolate these data for PEG-salt systems to the behavior of PEGylated proteins, the results clearly indicate that there may be a significant exclusion of buffer salts from the polyethylene glycol layer in the PEGylated protein. Alternatively, the polyethylene glycol chain(s) may form a "tail" behind the moving protein, with the electrical force governed by the charge on the actual surface of the protein while the drag is determined by the sum of the drag forces on the protein and polyethylene glycol "tail". Theoretical calculations using a model developed by Long and Ajdari[38] based on this physical situation gave values of the drag ratio that were much smaller than those determined experimentally, although some of the key assumptions in this model are unlikely to be valid in our experimental system .

Conclusions

The addition of glycine significantly improved the peak symmetry and resolution for the capillary electrophoresis of PEGylated proteins with different numbers of attached PEG chains. PEGylation alters the electrophoretic mobility by increasing the hydrodynamic drag (due to the presence of the PEG chains) and by shifting the net protein charge (through reaction with the $-NH_2$ of the lysine). The experimental data for the electrophoretic mobility of the different PEGylated species were collapsed to a single curve by plotting the drag ratio, equal to the electrophoretic mobility of the PEGylated α-lactalbumin divided by the mobility of the acetylated protein having the same number of covalently linked lysines, as a function of the effective radius of the PEGylated protein. In addition, the drag ratio data were in very good agreement with model predictions based on Henry's equation assuming that the net charge on the surface of the PEGylated protein is the same as that on the surface of the acetylated protein, despite the presence of the polyethylene glycol layer. This unusual behavior may reflect the exclusion of charged ions from the polyethylene glycol layer, a phenomenon that could have important implications for understanding the transport and separation characteristics of PEGylated proteins.

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