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Characterization of polyethylene glycol modified proteins using charge-reversed capillary electrophoresis

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ABSTRACT

A capillary electrophoretic method employing a charge reversal technique [J. E. Wiktorowicz and J. C. Colburn, *Electrophoresis*, 11 (1990) 769] has been developed to characterize polyethylene glycol (PEG)–proteins. A removable coating is applied to a standard fused-silica capillary in order to change the negative charge of the capillary surface to a positive charge. This prevents the adsorption of basic and PEG–proteins. The positive electrode is positioned at the detector end of the capillary, orienting electroosmotic flow towards the detector. Automated reconditioning procedures prior to each analysis give relative standard deviations in migration time and area of less than 2%, with most analysis times under 20 min. As the number of PEGs conjugated to a protein increases, the net positive charge and migration time of the copolymer decrease. Resulting peak widths are broad, reflecting the broad molecular mass distribution of PEGs and the heterogeneous nature of the PEG conjugates. This method can be used to monitor process steps, optimize reaction conditions, determine extent of modification, or assess product quality and consistency. Examples of PEG derivatized molecules characterized in our laboratory by charge-reversed capillary electrophoresis are tryptophan, Lys–Trp–Lys, lysozyme, myoglobin, RNase and immunoglobulin G; the size of the attached monomethoxy-PEG molecules varied from 0.15 kDa to 10 kDa (10^3 dalton).

INTRODUCTION

Recombinant proteins are often produced in cells such as *Escherichia coli* which are not capable of glycosylation. Many unglycosylated proteins have limited solubility and a tendency to aggregate at neutral pH [1]. Polyethylene glycol (PEG) is a man-made polymer that can be covalently bound to a protein, typically via active ester reaction with the ε amino group of lysine or the protein's N-terminal primary amine. Covalent attachment of PEG to proteins results in conjugates that have: (a) increased pharmacokinetic half-life [1], (b) reduced immunogenicity [2,3] and (c) increased solubility [2]. Examples of such modified proteins are PEG–adenosine

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deaminase (Pegademase), recently approved by the Food and Drug Administration (FDA) for the treatment of severe combined immunodeficiency syndrome (SCIDS [4]) and PEG–asparaginase [5]. Characterization of PEG-proteins is a challenging analytical problem. By coupling a homogeneous polymer (the protein), to a heterogeneous polymer (the PEG) one creates an extremely heterogeneous mixture that must be characterized by class. For example, a protein such as myoglobin, with 20 reactive amino groups, derivatized with a 10 kDa (10^3 dalton) PEG that contains a mixture of 8 kDa sized PEG, may result in a mixture of greater than 10^{33} kinds of molecules. There are simply too many possible combinations to make an exhaustive analysis of each distinct PEG–protein conjugate feasible. Methods which have been used to characterize PEG–proteins include high-performance liquid chromatography (HPLC) [1,6], NMR [7], isoelectric focusing (IEF) [2] and sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). None of these methods is capable of exploiting both the charge and mass changes that are the result of PEG conjugation, in contrast to capillary electrophoresis (CE).

Previously reported CE methods employing an unmodified capillary and low pH buffers [8], buffers using a pH above the pI of the protein [9], or addition of organic modifiers to the sample matrix [10] are not applicable to the characterization of PEG conjugated proteins. Others describe coatings that slow or eliminate electroosmotic flow [11–14], which is not always desirable, as electroosmotic flow can be used to increase effective separation length and improve resolution. In this paper we describe an application of CE, using a charge-reversed, coated capillary, to the separation and characterization of six different PEG conjugated species. An amino acid, a tri-peptide, and four proteins of varying size were chosen in order to reflect a wide range of size and charge characteristics.

EXPERIMENTAL

Apparatus and materials

CE was performed on an Applied Biosystems Model 270A (ABI, Foster City, CA, USA). The capillary column consisted of fused-silica tubing (Polymicro Technologies, Phoenix, AZ, USA), 50 μm I.D., 360 μm O.D., with a total column length of 72 cm (52 cm to the detector). Approximately 0.5 cm of the polymide coating was burned off at a distance of about 20 cm from the detector end of the column to create a detector window. The UV data (215 nm) were analyzed using a Nelson Analytical data system (Perkin-Elmer/Nelson, Cupertino, CA, USA).

D-Tryptophan, Lys–Trp–Lys, myoglobin from horse heart, lysozyme from chicken egg-white, RNase from bovine pancreas and human immonoglobulin G (IgG) were purchased from Sigma (St. Louis, MO, USA); sodium phosphate (monobasic) was purchased from Fisher Scientific (Forest Lawn, NJ, USA); phosphoric acid, sodium phosphate (dibasic) and analytical grade ethylene glycol were purchased from Mallinckrodt (Paris, KY, USA). The 2 kDa and 10 kDa methoxy-PEGs (mPEGs) were obtained from Union Carbide; 2-(2-methoxy-ethoxy)ethanol was obtained from Aldrich (Milwaukee, WI, USA); Microcoat was obtained from Applied Biosystems (Foster City, CA, USA). All buffer solutions were filtered through 0.2-μm sterile polysulfone syringe filters (Gelman Scientific, Ann Arbor, MI, USA) and vacuum degassed before use. Water was HPLC grade.

Reagent synthesis

Both N-hydroxysuccinimide (NHS) and *p*-nitrophenyl (pNP) activated PEG esters were used for conjugation. The 6 kDa NHS active esters were obtained by using methoxy-PEG-glutarate prepared from mPEG and glutaric anhydride, followed by activation with dicyclohexylcarbodiimide and N-hydroxy-succinimide [15,16]. The 2 kDa and 10 kDa pNP active esters were synthesized as shown using a method described by Veronese *et al.* [17]:

$$\text{mPEG-OH} + \text{Cl-CO-O-C}_6\text{H}_4\text{-NO}_2 \rightarrow \text{mPEG-O-CO-O-C}_6\text{H}_4\text{-NO}_2$$

The 0.15 kDa PEG ester was synthesized as follows. In a well cooled flask containing 200 ml of dry diethyl ether, 20.2 g (0.1 mol) of *p*-nitrophenyl chloroformate was dissolved together with 12 ml (0.1 mol) of 2-(2-methoxy ethoxy)ethanol. Pyridine, 20 ml, was added slowly, causing a precipitate to form. The reaction was allowed to stand at ambient temperature for several hours. The precipitate was filtered and discarded. The mother liquor was diluted to 300 ml with diethyl ether, washed 3 times with water, 3 times with sodium chloride and dried over magnesium sulfate. The solvent was removed *in vacuo*; pale yellow oil resulted. Yield was 16.67 g. Analysis using a method described by Aldwin and Nitecki [18] indicated 87% active ester by weight. It should be noted that the molecular mass of the reagent (PEG ester) is 0.28 kDa but the resulting attached PEG residue is 0.15 kDa; with larger mPEG molecules the molecular weight change is negligible. To avoid confusion we will refer to both the PEG ester and its conjugate by the molecular mass of the PEG residue (*i.e.* 0.15 kDa).

Amino acid, peptide, and protein conjugation

The NHS ester reactions with ribonuclease, lysozyme, myoglobin and IgG were carried out in N-[2-hydroxyethyl]piperazine-N′-3-propane sulfonic acid (EPPS) buffer pH 8.5 at ambient temperature for 30 min; reactions were quenched by lowering the pH to 5.0 with acetic acid. In order to achieve various degrees of PEG conjugation, the PEG-ester was added to the protein solutions at different molar ratios ranging from 1:1 to 5:1, respectively. The activated pNP esters were mixed with tryptophan, Lys–Trp–Lys, myoglobin or IgG in 0.1 *M* 4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid (HEPES) buffer, pH 8.5. Typically, varying amounts of PEG ester calculated as a molar ratio of the PEG ester-to-target molecule were added to 5 mg/ml protein solutions and allowed to react overnight. Reactions times and activated ester-to-target molecule molar ratios were varied as noted in the Figures. Reactions were stopped by dilution (1:1) into pH 2.0 electrophoresis buffer (0.1 *M* phosphate, 10% ethylene glycol). The pNP esters are considerably slower reacting than the more commonly used NHS esters [19]. This feature allows analysis of the kinetics of the conjugation, as shown in the myoglobin time course electropherograms. The activity of the PEG–pNP esters was checked by following the appearance at 400 nm of the characteristic yellow color of *p*-nitrophenyl anion formed at pH above 8.0 [18]. In general, the percentage by weight of active esters was greater than 70%.

Procedures

Capillaries were first treated with 1.0 *M* NaOH for 20 min by drawing the solution into the capillary by vacuum, followed by 5 min with 0.1 *M* NaOH, according to the manufacturer's protocol. After a 5-min wash with water, the capillary was

coated for 20 min with Micro-coat in 2% ethylene glycol. In order to keep a consistent coating on the capillary surface, the capillary was washed between injections with 0.1 *M* NaOH for 1 min and reconditioned for two minutes with Micro-coat in 2% ethylene glycol. Samples were injected by application of vacuum for two seconds. This corresponded to an injection size of approximately 8 nl. Electrophoresis was performed at −30 kV (400 V/cm) for 15–40 min. The concentrations of the PEG conjugated species were on the order of 1–2.5 mg/ml.

RESULTS

Fig. 1 shows a schematic diagram of the capillary surface under both normal (Fig. 1A) and charge-reversed (Fig. 1B) conditions. Proteins and PEG–proteins below their isoelectric points are positively charged and can interact with the negatively charged capillary surface. After coating, the net charge on the capillary wall is positive, causing a reversal in electroosmotic flow (μ_{eo}). By reversing the polarity, the analytes can be swept by the detector as usual. The charged PEG–proteins are now repulsed from the capillary wall. Electroosmotic flow is fast enough to overcome the electrophoretic mobility (μ_{ep}) of the positively charged species. Since mobility is countered by electroosmotic flow, those species with greater net positive charge exhibit longer migration times, according to eqns. 1 and 2, where μ_{app} is the apparent electrophoretic mobility; μ_{ep} is the true electrophoretic mobility; vel_{app} is the peak velocity; V is the field strength; t_{app} is the peak migration time, and k is a constant reflecting the total length of the capillary, the length to the detector, and the applied voltage. In eqn. 2, q is the net positive charge, R_s is the Stokes radius, and n is the intrinsic viscosity.

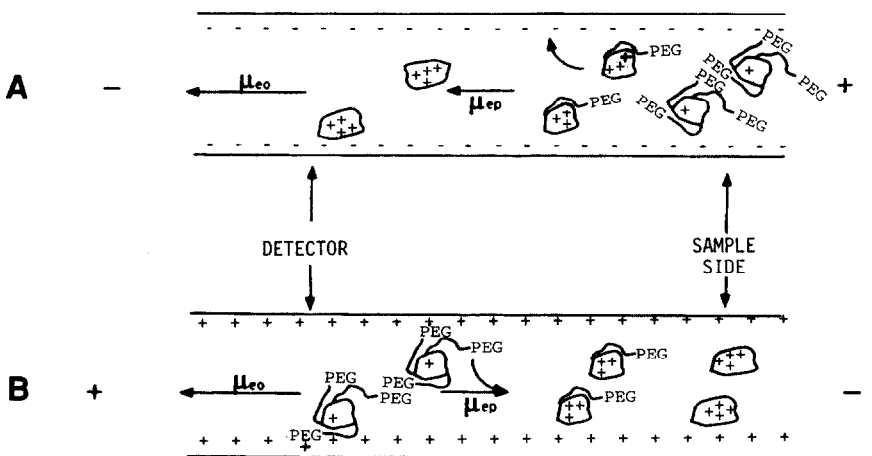

Fig. 1. Diagram of the capillary under (A) normal and (B) charge-reversed conditions. Note the electrode polarity, the direction of electroosmotic flow (μ_{eo}), the cationic PEG–protein electrophoretic mobility (μ_{ep}) and its attraction to the capillary surface.

$$\mu_{app} = \frac{vel_{app}}{V} = k\frac{1}{t_{app}} \quad (1)$$

$$\mu_{ep} = \frac{q}{6\pi R_s n} \quad (2)$$

CE of PEG esters, tryptophan and Lys–Trp–Lys

p-Nitrophenyl active PEG esters are stable and uncharged at pH 2.0 and adsorb strongly in the UV region. In the experiments described here, they migrated with the mesityl oxide neutral marker. The *p*-nitrophenol formed during conjugation, either by hydrolysis or as a by-product of the conjugation, served as a convenient neutral marker, being uncharged below pH 5.0. Uridine-5′-diphosphoglucose (UDPG) served as a negatively charged marker in the PEG ester electropherograms in order to compare relative migration rates of the PEG esters. Since the 0.15 kDa, 2 kDa and 10 kDa PEG esters all migrated with the neutral marker, we have concluded that the polyethylene glycol moiety was not interacting with the Micro-coat on the walls of the capillary. There was some peak broadening observed with the 10 kDa ester. This appeared to be the result of a greater mass of the larger PEG being injected in order to obtain the same number of moles (0.046 mol) on the capillary (*e.g.* 46 mg/ml for the 10 kDa PEG and 1 mg/ml for the 0.15 kDa PEG). When a minimum amount of the 10 kDa PEG was injected, the peak width more closely resembled that of the smaller PEG ester.

The amino acid tryptophan has one primary amino group available for modification. When a PEG is attached to this amine, the resulting conjugate is neutral at pH 2.0. The 0.15 kDa, 2 kDa and 10 kDa PEG–tryptophan conjugates all migrated with the neutral marker. Thus, similar to unreacted PEGs, in the case of uncharged conjugates, migration proved to be independent of size.

The Lys–Trp–Lys tripeptide has a molecular mass of 0.46 kDa and three amino groups available for conjugation. Upon PEG conjugation, three mono-PEG species, three di-PEG species and one tri-PEG species are expected. We found that as the molar ratio of the 0.15 kDa PEG ester-to-peptide was increased stepwise, the relative triplet height (Fig. 2) remained constant. Thus, the triplet migrating at 13–14 min most likely reflects the three possible mono-conjugates of Lys–Trp–Lys: the N-terminal α-amino, the N-terminal lysine ε-amino, and the C-terminal lysine ε-amino. At higher PEG ester-to-peptide ratios, the amount of di-PEG species (8.2 min) increased. The PEG-tripeptide was uncharged and migrated with the *p*-nitrophenol (6.0 min). The three possible di-PEG species apparently could not be resolved. When the Lys–Trp–Lys tripeptide was conjugated with 2 kDa and 10 kDa PEGs, the resulting products migrated closer to the neutral marker. The larger molecules migrated faster, consistent with eqn. 2.

CE of myoglobin

Myoglobin (17 kDa) showed essentially no change in mobility when conjugated with the 0.15 kDa PEG. When modified with the 2 kDa and 10 kDa PEGs, however, the myoglobin conjugates (which are larger) migrated faster (Fig. 3). A PEG ester-to-protein molar ratio of 5:1 was used in this reaction. By observing the appearance of the various PEG conjugated species of myoglobin as a function of reaction time, we assigned the number of PEGs attached to myoglobin for each peak (Fig. 4a). Since no

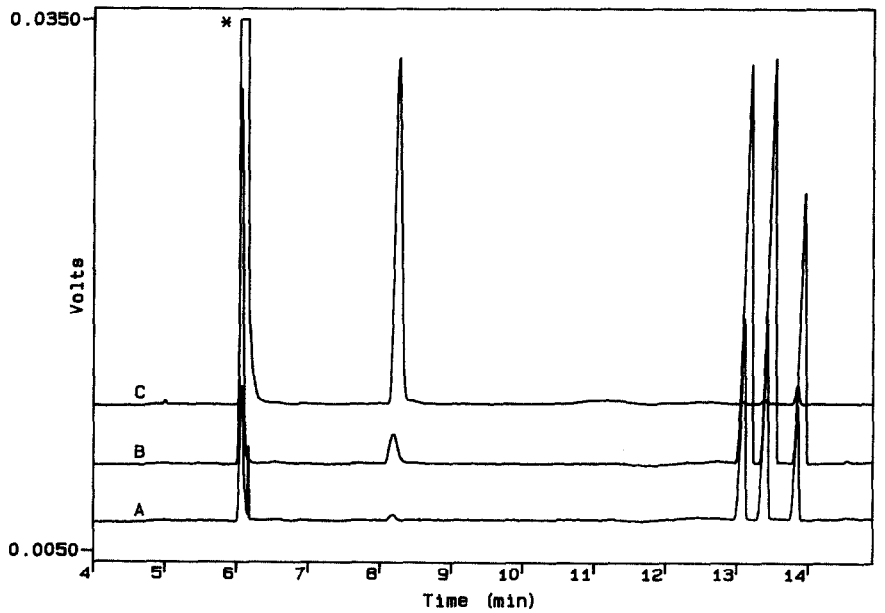

Fig. 2. High-performance capillary electrophoresis of Lys–Trp–Lys conjugated with 0.15 kDa PEG using pNP esters. The PEG ester-to-peptide molar ratio in these reactions was: (A) 1:1, (B) 3:1, (C) 10:1. The electropherograms were obtained using a 72 cm × 50 μm I.D. fused-silica capillary tube; 100 m*M* phosphate, 10% ethylene glycol, pH 2.0, −30 kV, 45 μA, UV detection at 215 nm. The hydrolyzed *p*-nitrophenol (*) was used as a neutral marker. Unmodified Lys–Trp–Lys migrated at 37.5 min.

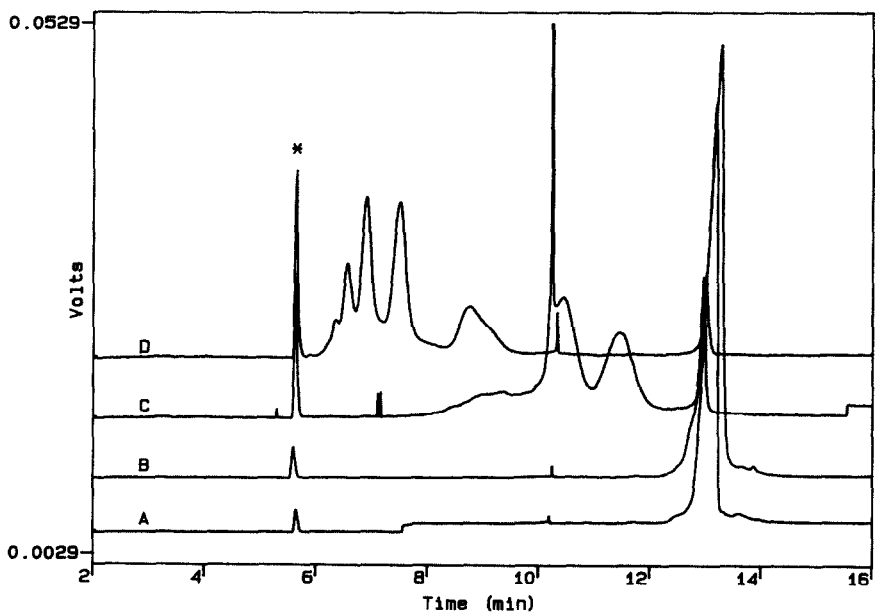

Fig. 3. High-performance capillary electrophoresis of myoglobin conjugated with pNP esters. The PEG ester-to-protein molar ratio in these reactions was 5:1. (A) Unmodified myoglobin, (B) 0.15 kDa PEG, (C) 2 kDa PEG, (D) 10 kDa PEG. Electrophoresis conditions are described in Fig. 2. Hydrolyzed *p*-nitrophenol (*) was used as a neutral marker. The peak at 10.1 min is not identified, and may be a system-related artifact.

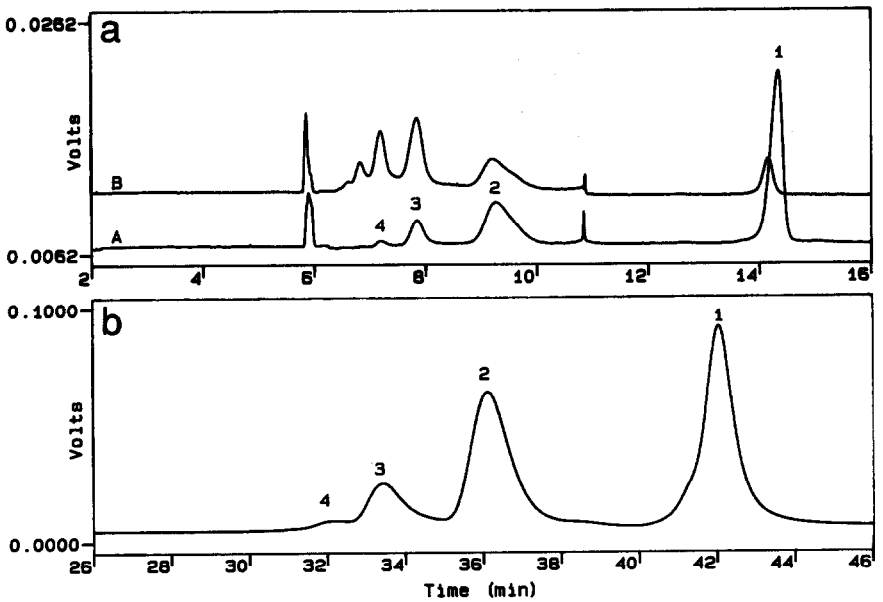

Fig. 4. (a) High-performance capillary electrophoresis of (A) myoglobin conjugated with 10 kDa PEG, 1 h reaction time, (B) myoglobin conjugated with 10 kDa PEG, 20 h reaction time. The PEG pNP ester-to-protein molar ratio in these reactions was 3:1. Reactions were stopped by the addition of pH 2.0 buffer. Electrophoresis conditions are described in Fig. 2. Peaks are identified as (1) unmodified myoglobin; (2) 1-PEG; (3) 2-PEG; (4) 3-PEG. (b) Size-exclusion chromatography of myoglobin conjugated with 6 kDa PEG using the NHS ester. The PEG ester-to-protein molar ratio in this reaction was 1:1. Chromatography was performed on two Dupont Zorbax GF-250 columns in series (25 cm × 9.4 mm I.D. each); 0.1 *M* sodium sulfate, 0.01 *M* sodium phosphate, pH 7.0; UV detection was at 280 nm. Peaks are identified as (1) unmodified myoglobin; (2) 1-PEG; (3) 2-PEG; (4) 3-PEG.

significant peaks appeared between 10 and 14 min of migration time, the peak that migrated at 9.4 min was assigned a mono-PEG identity. These experiments were done at a low pNP–PEG-to-myoglobin ratio (3:1) and stopped at various points by dilution into pH 2.0 buffer in order to favor a lower PEG-to-protein ratio.

When a similar experiment was performed using the NHS ester of 6 kDa PEG (ester-to-protein molar ratio 3:1), the same pattern of PEG conjugated species was observed. The electropherogram and sizing HPLC chromatogram of the myoglobin conjugates (Fig. 4b) were very similar. Using UV-RI size exclusion HPLC compositional analysis described by Kunitani *et al.* [6], weight composition of the PEG myoglobin conjugates formed was determined. Based on the found weight PEG per weight protein, the mole ratio in this sample was calculated to be 1.24. By using the PEG assignments shown in Fig. 4, we obtained a similar value by first correcting the areas of the myoglobin conjugate peaks for migration time, then summing the area percents of each peak multiplied by its conjugation number [*i.e.* 1(Area% 1-PEG)+ 2(Area% 2-PEG)...]. This gave a value of 1.27, which is consistent with the sizing HPLC results.

CE of IgG

IgG is a large (150 kDa) protein. Conjugation with 0.15 kDa PEG should

decrease the net positive charge on IgG but have little effect on the mass. Net positive charge at pH 2.0 was calculated using a program that sums the fractional charges from each charged amino acid at pH values from 1 to 14. The number of charged amino acids was estimated from sequence data found in the literature [20]. IgG conjugation with 0.15 kDa PEG changed the charge-to-mass less than 1%, while conjugation with the 10 kDa PEG changed it almost 10%. Thus, resolution of the 0.15 kDa PEG from unmodified IgG was poor. The PEG ester-to-IgG molar ratio in this reaction was 10:1.

Effects of pH

Based on the known amino acid composition of myoglobin, a theoretical charge-to-mass ratio was calculated at unit pH values from 1 to 13 for myoglobin conjugates with one to four 10 kDa PEG molecules (Fig. 5). Net positive charge and pI were calculated using a program that sums fractional charges, as described earlier. The largest difference in charge-to-mass ratio is observed at very low (1 to 3) and very high (>10) pH values. At extreme pH values charge interactions with the capillary wall or stripping of the coating phase may be a problem. Thus, the ideal working pH range appeared to be between pH 2 and 6. According to Fig. 5, as the pH of the separation buffer is increased, the difference in charge-to-mass between the myoglobin conjugates becomes smaller. The conjugates should migrate closer together, decreasing resolution. Data from a series of electropherograms of 10 kDa PEG–myoglobin at different pH values are summarized in Table I. At low pH, the net positive charge on the myoglobin is greatest, effecting the largest difference in charge-to-mass. This improved the separation between the various PEG conjugated species. The electroosmotic flow was also faster at lower pH, decreasing analysis time. Each PEG conjugate is uncharged at its p*I* (*e.g.* 4-PEG myoglobin p*I* is 6.9). As the pH of the separation buffer approached this p*I*, the 4-PEG myoglobin migrated closer to the neutral marker. Thus, as the pH of the separation buffer increased, the resolution between the PEG conjugates decreased, as predicted earlier. In general, as the net positive charge on a protein approaches 0, it should migrate with the neutral marker, thus providing a simple way to measure its p*I*. For the PEG-conjugated proteins discussed here, this is

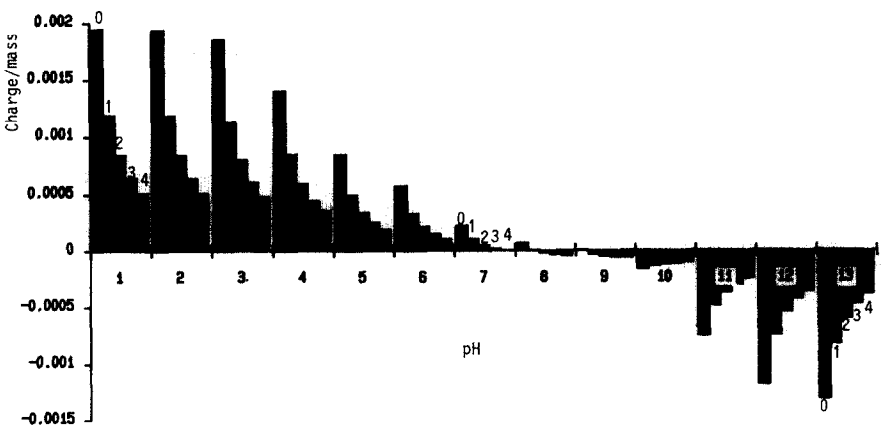

Fig. 5. Charge-to-mass *vs.* pH for myoglobin conjugated with 10 kDa PEG. Based on the assignments given in Fig. 4, data represents 0 to 4 PEGs per molecule myoglobin; 0 = 0 PEGS, 1 = 1 PEG, 2 = 2 PEGs, etc.

TABLE I

CE MIGRATION DATA OF 10 kDa PEG–MYOGLOBIN

72 cm × 50 μm I.D. capillary, 100 m*M* phosphate pH 2.0, 10% ethylene glycol, −30 kV, 45 μA, UV detection at 215 nm.

System pH	Migration time (min)					
	Neutral marker	Myoglobin +				
		0-PEG (p*I* 8.9)	1-PEG (p*I* 7.9)	2-PEG (p*I* 7.5)	3-PEG (p*I* 7.2)	4-PEG (p*I* 6.9)
2	5.87	14.17	9.23	7.85	7.20	6.84
4	7.35	15.48	9.93	8.80	8.35	8.11
5	9.38	13.27	10.95	10.44	10.36	9.79
6	11.66	15.33	14.82	14.37	14.37	14.37

not applicable in the case of very small PEG (0.15 kDa) attached to large (10 kDa or 150 kDa) proteins.

Reproducibility

Sequential injections of 10 kDa PEG–myoglobin were made in order to examine migration time and peak area reproducibility. Between each injection, the wash procedure described in the Experimental section was used to maintain a consistent surface on the capillary. The relative standard deviation (R.S.D.) of migration times for a series of 6 injections was 0.1%, while the R.S.D. of peak areas was 1.8%.

DISCUSSION

If the separation of PEG conjugates were based solely on differences in charge, then the size of the PEG used for conjugation would have no effect on electrophoretic mobility. This is not the case. If the separation were based only on size, then changes in pH would have little effect on relative migration times; this also is not the case. The separation mechanism appears to be governed by both size and charge. Eqn. 2 is consistent with this proposal. In order to test the validity of eqn. 2, the electrophoretic mobility of three PEG conjugated proteins and the PEG conjugated tripeptide were plotted in relation to their respective charge-to-Stokes radii ratios (Fig. 6). Electrophoretic mobility was corrected for capillary length and electroosmotic flow velocity by using eqn. 3, where μ_{ep} is the electrophoretic mobility; L and I are the capillary length and the length to the detector; t_0 and t are the migration times of the neutral marker and the sample; and V is the applied voltage.

$$\mu_{ep} = \frac{IL(1/t_0 - 1/t)}{V} \tag{3}$$

Protein Stokes radii were obtained from the literature; Stokes radii of the PEG were calculated from eqn. 4, using known values of PEG intrinsic viscosity [21], where R is the Stokes radius; M is the molecular weight; and $[n]$ is the intrinsic viscosity.

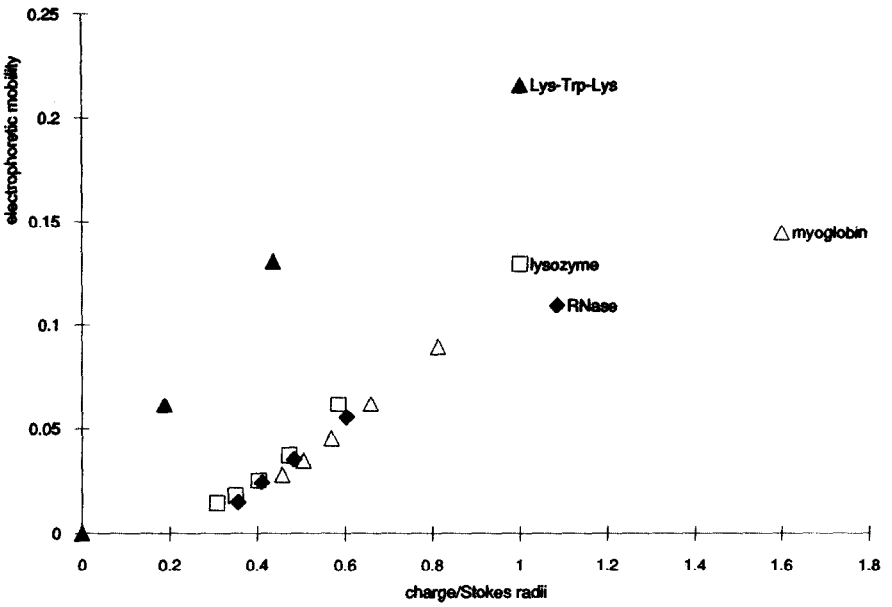

Fig. 6. Electrophoretic mobility (cm · s · kV^{-1}) *vs.* charge/Stokes radius. Data from lysozyme and RNase conjugated with 6 kDa PEG using the NHS ester. The PEG ester-to-protein molar ratios in these reactions were 1:1 and 3:1, myoglobin conjugated with 10 kDa PEG and Lys–Trp–Lys conjugated with 0.15 kDa PEG, using the pNP ester. The PEG ester-to-analyte molar ratio in these reactions was 3:1.

$$R = \left[\frac{30\,M[n]}{\pi(6.02 \cdot 10^{23})}\right]^{1/3} \tag{4}$$

Conjugate Stokes radii were calculated from eqn. 5, where R_s is the conjugate Stokes radii; $R_{protein}$ is the Stokes radii of the protein (or peptide); and R_{PEG} is the Stokes radii of the PEG.

$$R_s = \sqrt{(R_{protein})^2 + (R_{PEG})^2} \tag{5}$$

In theory, all the data should lie upon a single straight line, yet they do not. This may be a function of our ability to accurately estimates Stokes radii. The PEG conjugates exhibit a linear relationship, although the tripeptide conjugate relationship has a different slope than that of the protein conjugates. Other factors such as conformation can influence electrophoretic mobility (μ) and hence affect the relationship between μ, and size and charge. It is possible that this relationship may be improved by an independent measurement (*e.g.* dynamic light scattering) of the Stokes radii.

CONCLUSIONS

Capillary electrophoresis with charge-reversed capillaries provides a fast, simple and accurate method for determining the degree of PEG modification of amino acids,

peptides and proteins. The separation is governed by the size of the PEG and the net positive charge on the conjugate at the separation pH and is consistent with eqn. 2. The distribution of unmodified, mono-modified, di-modified and multi-modified PEGs can be determined by this method. This method can be used to determine the p*I* of a protein, to study reaction kinetics, monitor process steps, and to analyze a final product for purposes of quality control.

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