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Characterization of polyethylene glycol-modified proteins by semi-aqueous capillary electrophoresis

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Abstract

A new program to characterize polyethylene glycol-modified (PEGylated) proteins is outlined using capillary zone electrophoresis (CZE). PEGylated ribonuclease A and lysozyme were selected as examples. Five separation procedures were compared to select out the mixed buffer of acetonitrile–water (1:1, v/v) at pH 2.5 as the best to characterize the PEGylated proteins without sample pretreatment. Polyethylene oxide (PEO) with a high molecular mass of 8×10^6 was applied to rinse the capillary to form a dynamic coating which would decrease the undesirable proteins adsorbed to the inner wall of the silica. The electroosmotic flow (EOF) mobility of the five procedures was determined, respectively. It is found that acetonitrile is mainly responsible for the good resolution of PEGylated proteins with the help of PEO coating in the semi-aqueous system. The low EOF mobility and current in the semi-aqueous system might also have some responsibility for the high resolution. The semi-aqueous procedure described in this paper also demonstrates higher resolution of natural proteins than aqueous ones.

Keywords: Semi-aqueous capillary electrophoresis; Capillary electrophoresis; Buffer composition; Proteins; Poly(ethylene glycol)

1. Introduction

As a selection of the site-directed modification, polyethylene glycol (PEG) has been widely used to modify the surface of proteins to enhance their physiological properties, including low immunogenicity and/or prolonged blood retention time for therapeutic use [1]. It might be the most widely used polymer for drug conjugation today [2]. The US Food and Drugs Administration (FDA) has approved PEG modified (PEGylated) proteins, adenosine deaminase [3] and asparaginase [4], as therapy drugs. More than 20 PEG–protein conjugates are under research or clinic investigation.

Analysis of PEGylated product is important in research and drug quality control, but the following properties of PEG modification (PEGylation) make it difficult. Firstly, PEGylation generates a heterogeneous mixture of proteins with different number of PEG attached and different amino acid residues modified. Secondly, the linear polymer of PEG attached to proteins has a certain range of molecular mass. Thirdly, the hydrodynamic radius of PEG is equal to those of the globular proteins whose molecular weights are 4–5 times of it [5], and it curls

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randomly in liquid phase, which would interfere the chromatographic behavior of PEGylated proteins. Some investigators explored several analytical methods of PEG–proteins with various degree of success. For example, the average degree of PEG modification could be determined by the spectrometry of 2,4,6-trinitrobenzenesulfonic acid (TNBS) or the fluorometry of fluorescamine based on the numerical difference of the un-PEGylated amino groups between PEGylated proteins and natural proteins [6,7]. But apparent overestimation and drawback might be given and the heterogeneity information of the PEGylated protein could not be obtained by these methods [8–10]. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [11], high-performance liquid chromatography (HPLC) [12], nuclear magnetic resonance (NMR) [13] and matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) [14] have been used to characterize PEGylated proteins, too. None of these was accepted as an accurate and simple method that could be used to give the composition of the PEGylated molecules directly.

Soon after the introduction by Jorgenson and Lukacs [15], high-performance capillary electrophoresis (HPCE or CE) was developed to analyze complicated biopolymers due to its high resolution, unique selectivity and small sample requirement. However, owing to the polyionic property of large biomacromolecules, proteins may be irreversibly adsorbed to the inner surface of the silica capillary, which lead to band broadening and low separation efficiency [16]. Cunico et al. was the first to introduce CE to analyze PEGylated myoglobin [17]. In their paper, negative charge of the inner surface of the silica was reversed to positive by Micro-coat (ABI, Foster City, CA, USA) to eliminate the protein adsorption. At the same time, the high mobility of the reversed electroosmotic flow (EOF) resulted in short real migration time and low resolution of the protein components of high degree of PEGylation. Some authors used new CZE procedures to analyze PEGylated superoxide dismutase (SOD) [18] and PEGylated lysozyme [8]. The uncharged polymer, polyethylene oxide (PEO) with molecular mass of 1.1×10^5 to 6.0×10^5, was added to both the rinse and the running buffer to maintain the effect of dynamic coating to lessen the protein adsorption.

In this study, the procedure of dynamic coating was further simplified: PEO with high molecular mass of 8×10^6 was only used to rinse the capillary column before CE separation. A semi-aqueous buffer made by acetonitrile–water (1:1, v/v) was adapted to improve the resolution of PEGylated proteins. It is well known that a large proportion of organic solvent, such as acetonitrile, is often used as a kind of flow phase in reversed-phase HPLC, but in CE separation of proteins, it is not accepted as a routine solvent although some researchers use it as an additive. Our results indicate that large proportional acetonitrile is efficient to enhance the resolution of PEGylated proteins, while the total analysis time is reduced compared to the reported methods.

In our experiment, the binary mixture of acetonitrile–water (1:1, v/v) was used to make the sodium phosphate buffer. Pure acetonitrile was not applied as the solvent of the buffer because NaH_2PO_4 did not resolve in it.

Ribonuclease A (RNase) [19,20] and lysozyme [21,22] were selected to be PEGylated for they were not only model proteins but also potential therapeutic agents after PEGylation.

2. Experimental

2.1. Materials

Bovine pancreatic ribonuclease A (RNase) (EC 3.1.27.5), chicken egg white lysozyme (EC 3.2.1.17) and bovine pancreatic insulin were purchased from Sigma (St. Louis, MO, USA). Methoxypolyethylene glycol (MPEG) of M_v (viscosity-average molecular mass) 5000 ± 250, was obtained from Union Carbide (Danbury, CT, USA). PEO of M_v 8×10^6 and 6×10^5, were from Aldrich (Milwaukee, WI, USA). *N*-Hydroxysuccinimide (NHS) was provided by Sigma. Succinic anhydride was from Juneng Chemical (Tianjing, China). Dicyclohexylcarbodiimide (DCCI) was from Sheshan Chemical Factory (Shanghai, China). Acetonitrile (HPLC grade) was from Fisher (Fair Lawn, NJ, USA). Dimethyl sulfoxide (DMSO) was from Merck (Whitehouse Station, NJ, USA).

All other reagents were of analytical grade. Water

used in the experiments was prepared with RiOs ultra-pure water system (Millipore, Bedford, MA, USA).

2.2. Preparation of PEG–proteins

Firstly, the MPEG with one hydroxyl group should be activated to methoxypolyethylene glycolyl *N*-succinimidyl succinate (MPEG-SS) before reaction with protein. MPEG-SS was prepared according to the following procedure [23,24].

MPEG, 3 g, and succinic anhydride, 0.18 g, were dissolved in 6 ml *N*,*N*-dimethylformamide. The mixture was stirred for 3 h at 100°C. Then 30 ml cold diethyl ether were added to precipitate MPEG. The precipitation was filtered and washed 3–5 times by cold diethyl ether. The solid product was stored in the drier overnight.

The dried product, 2.5 g, DCCI, 0.23 g, and NHS, 0.13 g, were dissolved in 6 ml *N*,*N*-dimethylformamide. The mixture was filtered after being stirred for 12 h at 30°C. Then the filter liquor was precipitated, filtered and washed by diethyl ether with the same procedure described above. The final dried solid was MPEG-SS.

To attach MPEG to the ϵ-amino groups of lysine residues, MPEG-SS was added to a 2 mg/ml protein solution. The background borate buffer was 0.05 *M*, pH 8.4. The mixture was vortexed for 30 min under room temperature. The molar ratio of MPEG-SS to RNase was 5:1, and lysozyme was 1:1, 3:1, and 5:1.

2.3. Capillary electrophoresis

A P/ACE 5000 capillary electrophoresis system (Beckman, Fullerton, CA, USA) was equipped with a fused-silica capillary (50 μm inner diameter; 30 cm effective length; and 37 cm total length, Yongnian Optical Fiber Factory, Hebei, China). UV detection was set at 200 nm. The temperature of the capillary cartridge was maintained at 25±0.1°C using the thermostated coolant from Beckman. The rinse pressure was 20 p.s.i. (1 p.s.i.=6894.74 Pa). A plug of the sample solution was injected hydrodynamically (0.5 p.s.i. for 8 s). The running voltage was set at +30 kV.

2.4. Buffer and solution

In the experiments, we used a similar procedure to that described by Iki et al. [25] to make a solution of PEO 8×10^6 as the agent for dynamic coating. PEO 8×10^6 was dissolved in 0.1 *M* HCl until its concentration was 2 mg/ml. To avoid PEO decomposition by acid over a long time, 10 mg of PEO 8×10^6 was added to 4.5 ml water and stored for 2 days under room temperature, then 0.5 ml of 1 *M* HCl was added to the PEO solution just before use. The sodium phosphate buffer (0.025 *M*, pH 2.5) prepared by water or binary solvent of acetonitrile–water (1:1, v/v) was used as separation buffer. The buffer pH was tested by a ϕ72 pH meter (Beckman). All the buffers should be filtered through a 0.45-μm membrane and ultrasonicated before use.

Electroosmotic mobilities were calculated from the migration time of 0.1% DMSO, an electrically neutral marker substance, applied as sample.

3. Results and discussion

In capillary zone electrophoresis (CZE), the electrophoretic mobility (μ_{ep}) of protein is proportional to its charge (q) and is inversely proportional to its Stoke's radius (r) or molecular mass (M). There are many theoretical relationships suggested to predict the μ_{ep} of a protein, and they can be unified as the following equation [26]:

$$\mu_{ep} = \frac{Z_c}{KF_z M^s} \quad (1)$$

where s varies from 1/3 to 2/3 and K is a constant according to particular CE conditions. F_z is a pH-dependent proportionality factor defined as Z_c/Z_a, with Z_a being actual protein valence and Z_c being calculated protein valence according to the amino acid composition of the protein.

When MPEG is added to the protein surface, the values of M and r of the PEGylated proteins would increase and the value of μ_{ep} would decrease (Eq. (1)). So the PEGylated proteins move towards the cathode in following order: the unmodified protein, proteins with one MPEG attached, proteins with two MPEG attached, and so on (Fig. 1). The mobilities

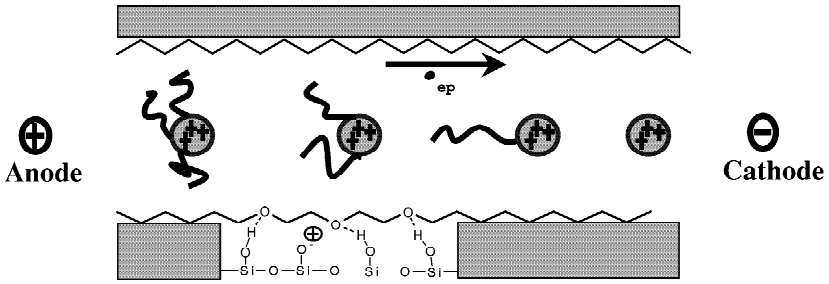

Fig. 1. Schematic diagram of the migration of PEGylated proteins in CE separation. PEO chain is bound to the inner surface of the capillary through silanol hydrogen bonding. The cationic PEGylated proteins move towards the cathode/UV detector in the acidic buffer.

of PEGylated proteins in CZE mode primarily depend on the number of MPEG chains attached. The acidic buffer (pH 2.5) in CE separation enables the proteins protonated completely and minimizes the charge difference of the PEGylated proteins [18].

3.1. Comparison of five separation procedures

The electropherograms of PEGylated RNase obtained from a series of rinse and separation procedures, which are shown in Table 1, are compared in Fig. 2. When a bare capillary was used to separate the sample of PEGylated RNase, it gave out two peaks (Fig. 2A). It needs to note herein that MPEG itself has no absorbance at 200 nm in the electropherograms. The peak eluting earlier could be identified as unmodified RNase by internal standard method, so the broaden peak behind the unmodified RNase was the unresolved MPEG–RNase adducts (Fig. 2A). The protein adsorption to the inner wall of the bare capillary was predicted to be the main reason responsible for the low resolution. PEO of molecular mass 1.1×10^5 to 6.0×10^5 were reported to be efficient coating agents to help analyze some PEGylated proteins [18,8], and the similar conditions was used in procedure E (Fig. 2E). Another PEO coating procedure recommended by Iki et al. to separate basic proteins [25] was employed in procedure B (Fig. 2B). The difference between procedures B and E was that, in B, PEO with rather high molecular mass of 8×10^6 was only involved in the rinse buffer, but in E, PEO of 6×10^5 was used in

Table 1
The rinse and separation procedures used in CE experiments

Procedure	Rinse and separation condition[a,b]	Electropherogram
A	Rinse, solution 1 for 3 min; sample inject, pressure for 8 s; separation buffer, solution 1; voltage, 30 kV	Figs. 2A, 6A
B	Rinse, solution 2 for 4 min, solution 1 for 3 min; sample inject, pressure for 8 s; separation buffer, solution 1; voltage, 30 kV	Figs. 2B, 6B
C	Rinse, solution 2 for 4 min, solution 3 for 3 min; sample inject, pressure for 8 s; separation buffer, solution 3; voltage, 30 kV	Figs. 2C, 3, 4, 6C
D	Rinse, solution 3 for 3 min; sample inject, pressure for 8 s; separation buffer, solution 3; voltage, 30 kV	Figs. 2D, 6D
E	Rinse, solution 4 for 20 min; sample inject, pressure for 8 s; separation buffer, solution 4; voltage, 30 kV	Figs. 2E, 6E

[a] There are four solutions used in the experiments: solution 1, 0.025 *M* sodium phosphate buffer (pH 2.5, 100% water); solution 2, 2 mg/ml PEO 8×10^6 in 0.1 *M* HCl; solution 3, 0.025 *M* sodium phosphate buffer (pH 2.5, 50% acetonitrile and 50% water); solution 4, 0.1 mg/ml PEO 6×10^5 in 0.025 *M* sodium phosphate buffer (pH 2.5, 100% water).

[b] Before every run, a rinse of 0.1 *M* NaOH for 4 min and a rinse of water for 4 min were scheduled to maintain the repeatability, which was not included in the procedures.

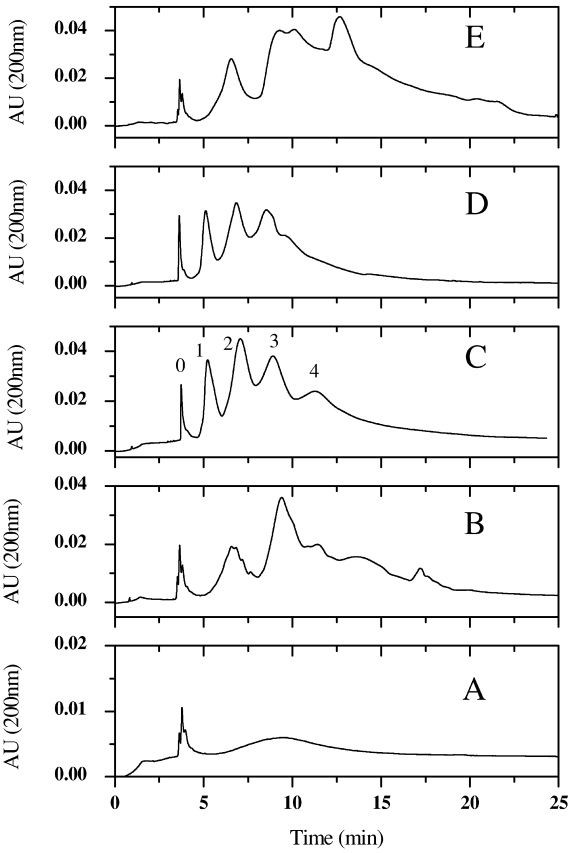

Fig. 2. Comparison of five procedures for CE separation of PEGylated RNase. The rinse and separation procedures are described in Table 1. More details could be found in Section 2. Before every run, a rinse of 0.1 *M* NaOH for 4 min and a rinse of water for 4 min were scheduled to maintain the repeatability. The sample of PEGylated RNase (2 mg/ml) was injected hydrodynamically for 8 s. Numbers over peaks correspond to the number of MPEG chains per protein molecule.

both the rinse and separation buffer. However, neither of these coating procedures achieved satisfactory resolution of PEGylated RNase.

Procedure C preserved PEO coating from procedure B, but the separation buffer's composition was quite different. In procedure C, a semi-aqueous phosphate buffer of acetonitrile–water (1:1, v/v) was used to separate the sample. The result was showed in Fig. 2C. A rather good resolution was given compared to Fig. 2B,E. It seemed that acetonitrile had important effects on the resolution. If the PEO coating in procedure C was omitted, then we got procedure D (Fig. 2D). But the resolution in D did not decrease much and was even better than the aqueous systems (Fig. 2B,E). It is suggested that acetonitrile might also have some effects to lessen the adsorption of proteins to the silica wall though PEO coating was absence (Fig. 2D).

In the experiments, all the procedures applied the same voltage ($U=30$ kV). The comparatively low current of procedure C might also contribute to the good resolution. When PEO coating was applied in the aqueous system, the current decreased from 80 μA (procedure A) to 54 μA (procedure B) and 51 μA (procedure E). But in the semi-aqueous systems, the currents were only around 22 μA (procedures C and D). It is known that Joule's heat is an important factor inducing band broadening, and it has direct proportion to electric current in CE. That is to say, the lower current, the sharper peaks, the higher resolution; and this possibility was partly proved in non-aqueous CE nowadays [27].

It is worth noting that in order to attach MPEG to the surface of proteins, an excessive amount of activated MPEG was added to the protein solution, which might increase the solution viscosity of the PEGylated sample and disturb the electrophoretic behaviors of PEGylated proteins in CE separation. This negative influence was observed in SDS–PAGE analysis of PEGylated proteins [12,28]. But to avoid the condition's change of PEGylation and the protein loss, the crude reaction solutions were used as samples directly without further treatments in the experiments. This might be one cause for the low resolution in the aqueous systems (Fig. 2B,E). However, in the semi-aqueous systems of CE, acetonitrile might improve the solubility of PEGylated proteins in the running buffer because of the lipophilicity of PEG.

To investigate the separation reproducibility of procedure C, the semi-aqueous system with PEO coating, two groups of consecutive injections were carried out with the same sample of PEGylated RNase on separate days (Fig. 3). The separations were reproduced well, with run-to-run RSD for the migration time of the five peaks (peaks 0–4 in Fig. 3) averaging 1.2% and day-to-day RSD averaging 2.8%. The resolution of peak 4 decreased with the same sample injected after 5 days (b_1, b_2 versus a_1, a_2 in Fig. 3), which was due to the slow hydrolytic

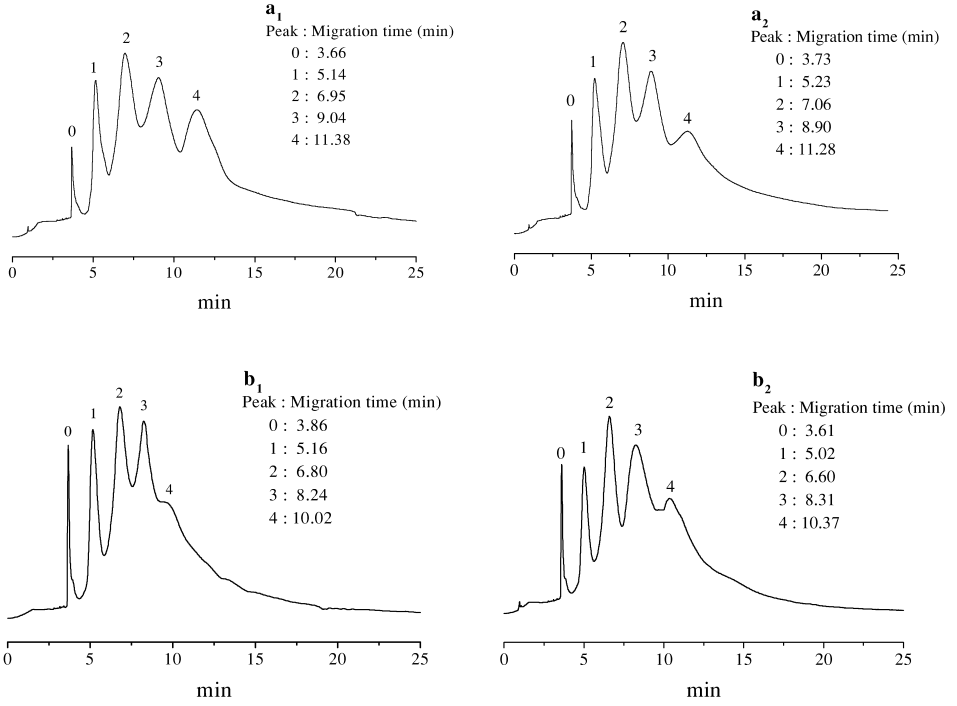

Fig. 3. A series of electropherograms of PEGylated RNase resolved by procedure C (Table 1). The subjacent consecutive injections (b_1, b_2) were replicates of the upper ones (a_1, a_2) after 5 days with the same sample. All other conditions are as presented in Fig. 2. Numbers over peaks correspond to the number of MPEG chains per protein molecule.

cleavage of the ester linkage between the MPEG chain and the amino group of PEGylated proteins [29].

3.2. Separation of PEGylated samples with different reaction ratio

Another model protein, PEGylated lysozyme, was selected to investigate the separation ability of the semi-aqueous CE system and the result is satisfactory, too (Fig. 4).

It was found that the semi-aqueous CE system was convenient not only to resolve the PEGylated product, but also to evaluate the reaction conditions, such as reaction molar ratio. As we know, the composition of PEGylated proteins can be controlled by adjusting the molar ratio of activated MPEG to proteins in reaction solution. In Fig. 4, lysozyme reacted with MPEG-SS under different molar ratios of 1:1, 1:3 and 1:5. More MPEG-SS was added, more the amount of highly modified PEG–proteins increased in final product. The percent of each component in a PEGylated mixture can be calculated from the peak area resolved. The corrected peak area by dividing the peak area by the migration time might be suitable for the quantitative analysis of PEGylated protein [18]. Relative works would be reported in our other papers.

Another fact should be mentioned here. With the increase of the modification degree, the product's resolution decreased in the same separation system (Fig. 4). This could be explained by the reaction selectivity of PEGylation. When the concentration of MPEG-SS was high, almost all the amino groups of the protein, including high sensitive and low sensitive ones, could react with the modifier. So the product's composition was heterogeneous. We could find more peaks in CE electropherogram and the composition of a particular peak was more complex in the meantime (Fig. 4c). That was why the resolution decreased apparently. However, when the concentration of MPEG-SS was low, only those high

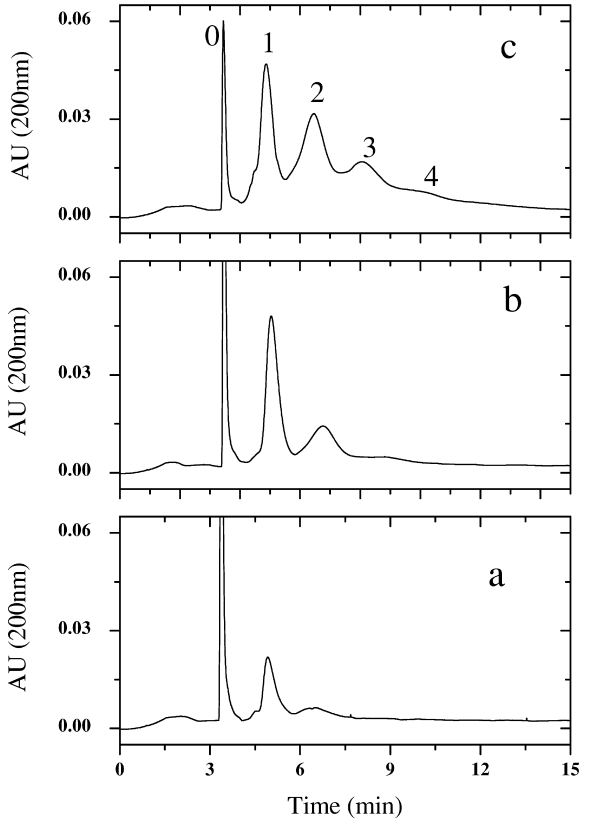

Fig. 4. Electropherograms of PEGylated lysozyme with various reaction molar ratios. The molar ratio of MPEG-SS to lysozyme was: (a) 1:1, (b) 1:3, (c) 1:5. Electrophoretic conditions are the same as procedure C in Table 1. All other conditions are described in Fig. 2. Numbers over peaks correspond to the number of MPEG chains per protein molecule.

sensitive amino groups could react with the modifier, so the product composition was relatively simple. At the same time, the composition of a particular peak was simpler, too (Fig. 4a,b).

3.3. Effects of PEO and acetonitrile

Fig. 5 shows the difference of EOF mobilities (μ_{eo}) of the five procedures from A to E. The value of μ_{eo} was obtained by determining the mobility of 0.1% DMSO as a neutral marker. From Fig. 5, we can see that the two systems without PEO coating had the largest value of μ_{eo} (procedures A and D), while the systems with PEO coating had apparently

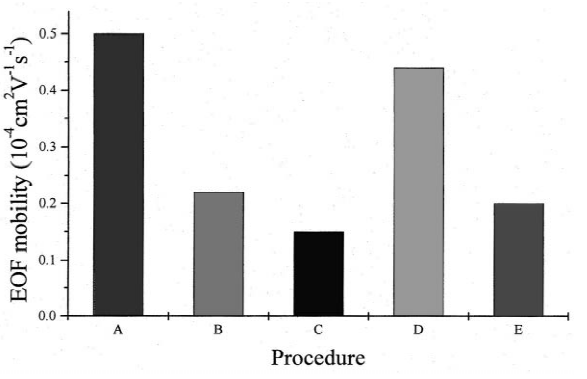

Fig. 5. EOF mobilities of the five procedures. A sample of 0.1% DMSO was loaded hydraulically for 8 s to determine the EOF mobilities. Procedures A to E are as presented in Table 1. All other conditions are described in Fig. 2.

lower value (procedures B, C and E). The result indicated that PEO coating was the main factor, which decreased the EOF mobility in either aqueous or semi-aqueous systems. Low μ_{eo} meant that PEO had a good coating effect on the inner wall of the silica. That is to say, PEO could reduce the protein adsorption through its coating effects. Considering that μ_{eo} in procedure D was lower than in procedure A, we found that acetonitrile also had some effect to reduce the EOF mobility independently. This result agrees with Schwer and Kenndler's conclusion that the EOF mobility decreases with the increase of organic solvents in the separation buffer [30].

It was found that organic solvent, such as acetonitrile could decrease zeta potential of the capillary wall due to the increase in the pK_a (and thus, decrease in the charge generation) of silanol group that composed the capillary wall [30,31]. The decrease of zeta potential and surface charge would reduce the charge attraction between the silica wall and the analyzed protein. For this reason, acetonitrile could increase the resolution of PEGylated proteins by decreasing the undesirable protein adsorption. In our research, it was found that acetonitrile had more important influence on the resolution than PEO coating. The systems with acetonitrile (procedures C and D) had the best resolution in all the procedures, either there was PEO or not (Fig. 2). But this does not mean that PEO is unimportant. It was clear that with PEO's help, the peak symmetry was improved.

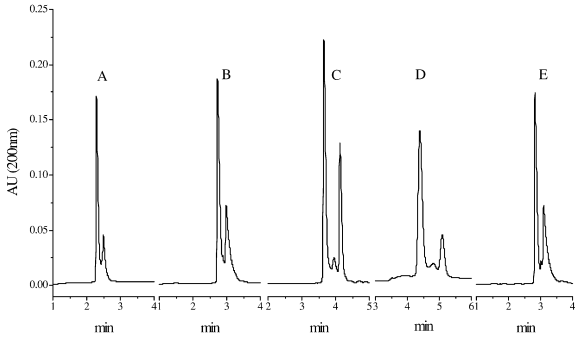

Fig. 6. Electropherograms of natural proteins resolved by the five procedures. Left peaks, RNase; right peaks, insulin. The sample was 1.0 mg/ml of RNase and 1.0 mg/ml of insulin in water. Procedures A–E are as presented in Table 1. All other conditions are described in Fig. 2.

3.4. Separation of natural proteins

The semi-aqueous CE system can be used to separate not only PEGylated proteins, but also natural ones, which was proved by the resolution of RNase and insulin.

Fig. 6 is the separation electropherograms of these two proteins by the five procedures; and the resolutions calculated according to Ref. [32] are compared in Fig. 7. These results also indicated that the procedure C had the best resolution among the five procedures. The semi-aqueous system without PEO coating (Figs. 6D and 7D) gave the second best result. For the aqueous systems with PEO coating (Figs. 6B and 7B and 6E and 7E), both gave lower resolution than the semi-aqueous systems (Figs. 6C and 7C and 6D and 7D). In a sentence, the situation is similar to that of PEGylated proteins.

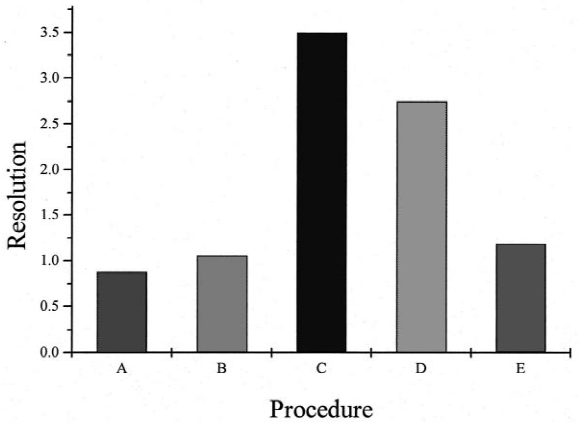

Fig. 7. Resolution of RNase and insulin by the five procedures. Procedures A–E are as presented in Table 1. All other conditions are described in Fig. 2.

4. Conclusion

A semi-aqueous buffer prepared by acetonitrile–water (1:1, v/v) was found suitable for characterization of PEGylated proteins with the aid of PEO 8×10^6. Both acetonitrile and PEO could enhance the resolution by decreasing the protein adsorption to the inner wall of the silica capillary. While PEO achieved the effect through its coating, acetonitrile might reduce the adsorption by decreasing zeta potential of the inner wall of the silica. It was also found that acetonitrile had better effect than PEO to enhance the resolution. Low current, low EOF mobility of the semi-aqueous system might also contribute to the better resolution. And this system could be used to monitor the PEGylation process, conveniently.

For natural proteins, this semi-aqueous procedure also showed good resolution. Considering that acetonitrile's volatility is welcome in CE–MS analysis, we predict that this system would have advantages in CE–MS analysis of some proteins, such as PEGylated proteins. As the lipophilicity of the PEG chain is similar to that of lipids, these results might also give some new ideas with regard to the CE separation of lipoproteins.

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References

[1] R.L. Lundblad, R.A. Bradshaw, Biotechnol. Appl. Biochem. 26 (1997) 143.
[2] C. Monfardini, F.M. Veronese, Bioconjugate Chem. 9 (1998) 418.

[3] New Drug Approvals in 1990, Pharmaceutical Manufactures Association, Washington, DC, 1991.
[4] E. Teske, G.R. Rutteman, P. van Heerde, W. Misdorp, Eur. J. Cancer 26 (1990) 891.
[5] C.-J.C. Jackson, J.L. Charlton, K. Kuzminski, G.M. Lang, A.H. Sehon, Anal. Biochem. 165 (1987) 114.
[6] A.F.S.A. Habeeb, Anal. Biochem. 14 (1966) 328.
[7] S.J. Stocks, A.J.M. Jones, C.W. Ramey, D.E. Brooks, Anal. Biochem. 154 (1986) 232.
[8] M.J. Roberts, J.M. Harris, J. Pharm. Sci. 87 (1998) 1440.
[9] D.L. Ladd, R.A. Snow, Anal. Biochem. 210 (1993) 258.
[10] F.M. Veronese, P. Caliceti, O. Schiavon, L. Sartore, in: J.M. Harris (Ed.), Poly(Ethylene Glycol) Chemistry: Biotechnical and Biomedical Applications, Plenum Press, New York, 1992, p. 129.
[11] M.M. Kurfurst, Anal. Biochem. 200 (1992) 244.
[12] P. McGoff, A.C. Baziotis, R. Maskiewicz, Chem. Pharm. Bull. 36 (1988) 3079.
[13] L. Banci, I. Bertini, P. Caliceti, L. Monsu' Scolaro, O. Schiavion, F.M. Veronese, J. Inorg. Biochem. 39 (1990) 149.
[14] E. Watson, B. Shah, R. DePrince, R.W. Hendren, R. Nelson, Biotechniques 16 (1994) 278.
[15] J.W. Jorgenson, K.D. Lukacs, Anal. Chem. 53 (1981) 1298.
[16] R. Kuhn, S. Hoffstetter-Kuhn, in: Capillary Electrophoresis: Principles and Practice, Springer, Berlin, 1993.
[17] R.L. Cunico, V. Gruhn, L. Kresin, D.E. Nitecki, J. Chromatogr. 559 (1991) 467.
[18] J. Bullock, S. Chowdhury, D. Johnston, Anal. Chem. 68 (1996) 3258.
[19] M. Lázníček, O. Schiavon, P. Caliceti, F.M. Veronese, Pharmacol. Res. 28 (1993) 153.
[20] O. Schiavon, P. Caliceti, L. Sartore, F.M. Veronese, Farmaco 46 (1991) 967.
[21] S. Pacor, E. Giacomello, A. Bergamo, K. Clerici, M. Zacchigna, E. Boccu, G. Sava, Anticancer Res. 16 (1996) 2559.
[22] T. So, H.O. Ito, T. Koga, T. Ueda, T. Imoto, Immunol. Lett. 49 (1996) 91.
[23] A. Abuchowski, G.M. Kazo, C.R. Verhoest Jr., T. Van Es, D. Kafkewitz, M.L. Nucci, A.T. Viau, F.F. Davis, Cancer Biochem. Biophys. 7 (1984) 175.
[24] Y. Iwashita, K. Ajisaka, K. Iwasaki, US Pat. 4 412 989 (1983).
[25] N. Iki, E.S. Yeung, J. Chromatogr. A 731 (1996) 273.
[26] N.J. Adamson, E.C. Reynolds, J. Chromatogr. B 699 (1997) 133.
[27] V.L. Ward, M.G. Khaledi, J. Chromatogr. A 859 (1999) 203.
[28] H.J. Bode, FEBS Lett. 65 (1976) 56.
[29] S. Zalipsky, C. Lee, in: J.M. Harris (Ed.), Poly(Ethylene Glycol) Chemistry: Biotechnical and Biomedical Applications, Plenum, New York, 1992, p. 351.
[30] C. Schwer, E. Kenndler, Anal. Chem. 63 (1991) 1801.
[31] H. Salimi-Moosavi, R.M. Cassidy, Anal. Chem. 67 (1995) 1067.
[32] Beckman P/ACE Station Software Reference Manual. Beckman Instruments, Fullerton, 1996, Appendix B-17.