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Ethanol–sodium chloride–phosphate mobile phase for size-exclusion chromatography of poly(ethylene glycol) modified proteins

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Abstract

The use of an aqueous sodium chloride–phosphate mobile phase for size-exclusion chromatography of poly(ethylene glycol) modified (PEGylated) proteins resulted in premature degradation of silica-based gel filtration columns. This column degradation was manifested as peak tailing and loss of resolution. An aqueous ethanol–sodium chloride–phosphate mobile phase was identified which extended column lifetimes without loss of resolution or change in peak shape. We describe the effects of sodium chloride, phosphate, and ethanol concentrations on column performance. Peak shape and column lifetimes were better when an aqueous ethanol–sodium chloride–phosphate mobile phase was used than with an aqueous salt mobile phase. Also, circular dichroism was utilized to investigate possible solvent effects on the conformation of each protein.

Keywords: Mobile phase composition; Proteins; Poly(ethylene glycol)-modified; Ethanol

1. Introduction

Poly(ethylene glycol) modified (PEGylated) proteins were investigated by size-exclusion chromatography (SEC) using a silica-based column with an aqueous salt mobile phase containing NaCl and phosphate (PO_4^{3-}) at pH 6.9. Premature loss of resolution resulted after ~20 injections of either PEG–r-HuMGDF totalling ~220 μg or PEG–r-metHuBDNF totalling ~200 μg. Degraded resolution was observed as a steady decrease or abrupt loss of multimer peaks and/or tailing of the main peak.

In general, salts such as NaCl have proven effective in reducing undesirable ionic interactions between the silica packing surface and protein [1–5]. After increasing the NaCl concentration in aqueous PO_4^{3-} mobile phases for SEC of PEG–r-HuMGDF or PEG–r-metHuBDNF, columns still degraded as described above; therefore, another additive was required to eliminate silica packing interactions.

With silica-based column packings, certain polar, organic compounds can reduce protein–packing interactions [6]. Ethanol was identified as one such additive.

For SEC sequences of each protein in mobile phases with or without ethanol, performance characteristics of beginning and ending injections were compared as an indication of column degradation. In addition, the concentration of each mobile phase component was investigated, and performance characteristics were monitored for optimal resolution. For a set of SEC mobile phases with increasing ethanol concentration, changes in protein secondary

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structure were monitored by circular dichroism (CD).

2. Experimental

2.1. Chemicals, mobile phases, and protein samples

Inorganic chemicals were ACS reagent grade. Ethanol, 200 proof, anhydrous, was purchased from Quantum Chemical Co. (Anaheim, CA, USA). Water was purified with a Milli-Q UV water purification system. Mobile phases were prepared with NaH_2PO_4, NaCl, and ethanol; pH adjusted with aqueous NaOH; and filtered through a 0.2 μm filter unit before use. Aqueous Mobile Phase 1 was composed of 0.1 *M* NaH_2PO_4–0.5 *M* NaCl at pH 6.9. Aqueous Mobile Phase 2 was composed of 0.1 *M* NaH_2PO_4–0.2 *M* NaCl at pH 6.9. PEG–r-HuMGDF is a medium sized, monomeric protein which has been chemically modified by the attachment of PEG. r-HuMGDF is an mpl ligand. PEG–r-metHuBDNF is a medium sized, dimeric protein which has been chemically modified by the attachment of PEG.

Prior to use, PEG–r-HuMGDF and PEG–r-metHuBDNF were stored at 4°C. Samples of PEG–r-HuMGDF were heated at 37°C for 4 weeks (37°C-4W), and samples of PEG–r-metHuBDNF were heated at 52°C for 1 week (52°C-1W) and 4 weeks (52°C-4W).

2.2. Instrumentation

SEC was performed on a Hewlett Packard 1050 high-performance liquid chromatography system (Palo Alto, CA, USA) using a TosoHaas TSK-GEL $G3000SW_{xl}$ column (5 μm particle size, 250 Å pore size, 7.8 mm I.D.×300 mm length) (Montgomeryville, PA, USA). Columns were equilibrated in the appropriate mobile phase. Protein samples, 20 μl (11 μg) of PEG–r-HuMGDF or 20 μl (10μg) of PEG–r-metHuBDNF, were analyzed over 30 min at a flow-rate of 0.7 ml/min. For experiments with PEG–r-HuMGDF, as shown in Figs. 6 and 8 (below), the injection volume was 68 μl. The detector wavelength was 215 nm. Hewlett Packard ChemStation software was utilized to calculate performance characteristics of retention time (t_R), peak height, peak width at half height, and peak symmetry at 5% of peak height.

CD measurements were performed at 25°C on a JASCO J-710 Spectropolarimeter (Easton, MD, USA) from 190–250 nm. The cell pathlength was 0.01 cm, scan rate was 100 nm/min, data was collected at 0.2 nm intervals, and 150 scans were time averaged. For CD, PEG–r-HuMGDF and PEG–r-metHuBDNF were dialyzed into SEC mobile phases. When the refractive index of the dialyzed mobile phase matched the starting mobile phase, dialysis was terminated. CD spectra of the dialysis mobile phases were subtracted from the protein spectra. CD spectra were corrected using A_{280} values.

3. Results and discussion

3.1. SEC mobile phases with and without ethanol

Figs. 1–4 give a visual comparison of SEC runs of PEG–r-HuMGDF or PEG–r-metHuBDNF run in Mobile Phase 1 with or without 10% ethanol (v/v). For runs with an ethanol containing mobile phase (Figs. 2 and 4), notice the constancy of t_R, and an

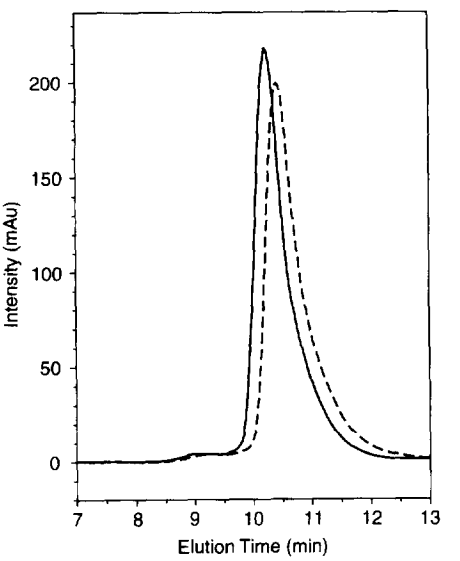

Fig. 1. PEG–r-HuMGDF, initial (—) and final (- - - -) injections from a SEC sequence of 150 injections in Mobile Phase 1.

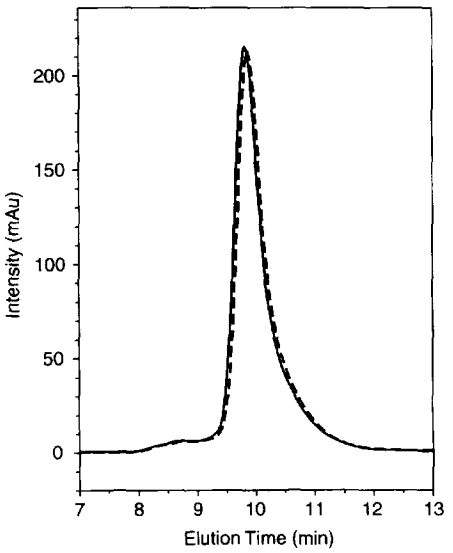

Fig. 2. PEG–r-HuMGDF, initial (—) and final (- - - -) injections from a SEC sequence of 150 injections in Mobile Phase 1 containing 10% ethanol (v/v).

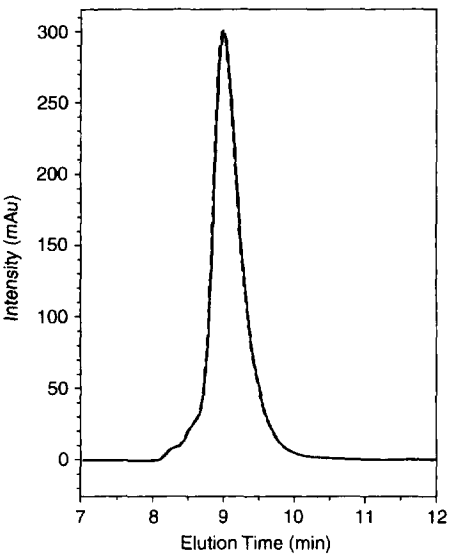

Fig. 4. PEG–r-metHuBDNF, initial (—) and final (- - - -) injections from a SEC sequence of 150 injections in Mobile Phase 1 containing 10% ethanol (v/v).

almost identical overlap of the initial and final injections from a sequence of 150 injections. These differences are shown graphically in Fig. 5. For both proteins run in Mobile Phase 1 (without ethanol),

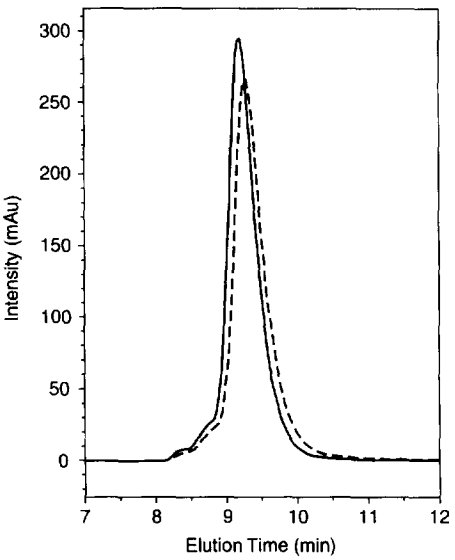

Fig. 3. PEG–r-metHuBDNF, initial (—) and final (- - - -) injections from a SEC sequence of 150 injections in Mobile Phase 1.

changes in peak height and peak width (column performance) are in the 6–11% range. Addition of 10% ethanol (v/v) to Mobile Phase 1 resulted in a dramatic improvement in column performance. The changes in column performance for PEG–r-HuMGDF and PEG–r-metHuBDNF were less than 1.6%, except for PEG–r-HuMGDF-37°C-4W which had height and width changes of 2.0% and 3.3%, respectively.

However, using Mobile Phase 2 with 10% ethanol (v/v), instead of Mobile Phase 1 with 10% ethanol (v/v), PEG–r-HuMGDF-37°C-4W was rerun, and the changes in t_R, peak height, and peak width were less than 0.6%, indicating that use of 0.2 *M* NaCl improves column performance.

3.2. Optimizing mobile phase compositions

Having established that addition of ethanol to SEC mobile phases significantly improved column lifetimes and resulted in minimal change in resolution, we investigated the sequential addition of PO_4^{3-}, NaCl, and ethanol to the mobile phases used for each protein and their effect on resolution of the main peak.

Throughout this study, pH was held at 6.9, as

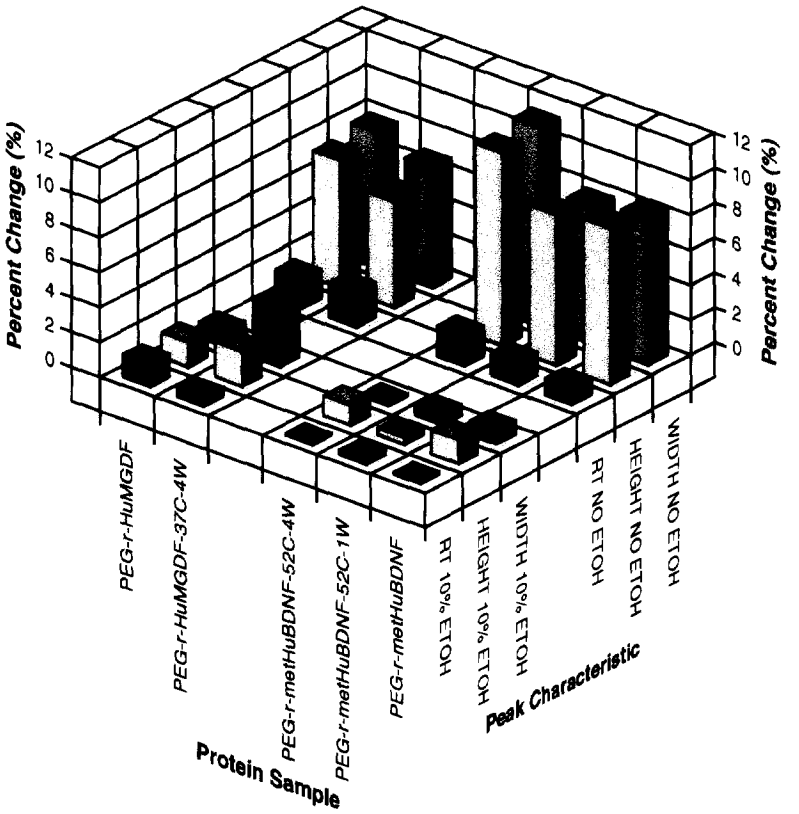

Fig. 5. Percent change for column performance characteristics between averaged initial and final injections for SEC sequences of 150 protein injections. Percent change of each performance characteristic was calculated from an average of the last five injections minus the first five injections. Results are reported as absolute values. PEG–r-HuMGDF and PEG–r-metHuBDNF were run in Mobile Phase 1 with and without 10% ethanol (v/v).

changes in pH from 6.3 to 7.3 had little effect on the chromatography of PEG–r-HuMGDF or PEG–r-metHuBDNF.

For both proteins, at PO_4^{3-} concentrations less than ~0.05 *M*, t_R values and peak widths increased and peak heights decreased which suggested that these proteins require a higher ionic strength [7]. From ~0.05 to 0.15 *M* PO_4^{3-}, the performance characteristics plateaued except for the peak height of PEG–r-metHuBDNF which showed a slight increase. A PO_4^{3-} concentration of 0.10 *M* was the median value in the range (0.05 to 0.15 *M* PO_4^{3-}) of uniform performance values; therefore, 0.10 *M* PO_4^{3-} was chosen for the next step, examination of the effect of changing NaCl concentration.

Holding PO_4^{3-} concentration constant at 0.1 *M* and varying NaCl concentration as the second added component (Fig. 6), an immediate improvement in resolution was observed for both proteins. For PEG–r-HuMGDF, all three performance parameters are fairly uniform from ~0.1 *M* to 0.5 *M* NaCl. NaCl at 0.2 *M*, is a reasonable distance from ~0.075 *M* NaCl where performance values are changing and at a concentration which showed improved column performance (Section 3.1); therefore, 0.2 *M* NaCl was chosen for further study.

For PEG–r-metHuBDNF, NaCl, in the range 0.3–0.5 *M*, gave peak heights and peak widths which are maximized and fairly uniform. Peak symmetry is maximized at 0.5 *M* NaCl as shown in Table 1, therefore, 0.5 *M* NaCl was chosen as the mobile phase for PEG–r-metHuBDNF.

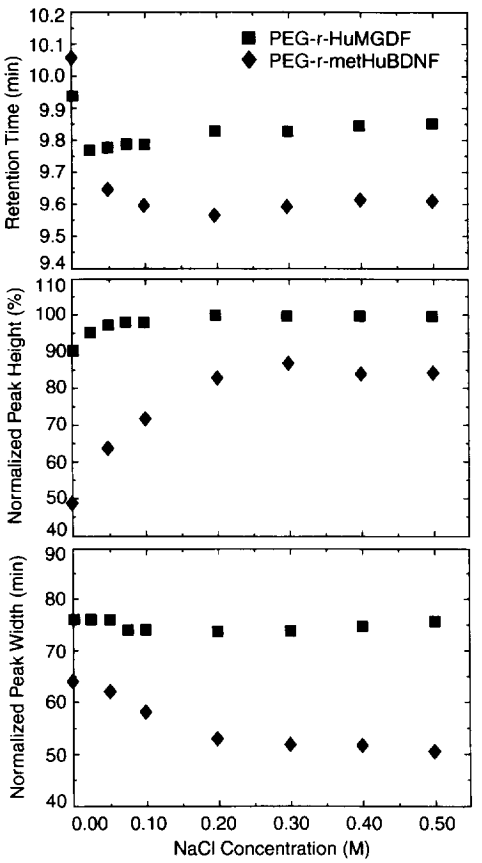

Fig. 6. Change in column performance characteristics for duplicate injections of PEG–r-HuMGDF or PEG–r-metHuBDNF in a 0.1 *M* PO_4^{3-} SEC mobile phase at pH 6.9 with varying NaCl concentrations.

3.3. Effect of ethanol

As ethanol is known to denature some proteins [8–10], there was concern that both proteins were denaturing and increasing in size. During SEC, this would cause a decrease in t_R, as observed. Another possible explanation is that ethanol may prevent PEG–silica gel interaction which would decrease residence time in the pores of the silica gel resulting in a decrease in t_R.

To decide between these two possibilities, PEG–r-HuMGDF and PEG–r-metHuBDNF were dialyzed into SEC Mobile Phases 2 and 1 respectively, with increasing ethanol concentration. The dialyzed samples were examined by CD, in order to identify secondary structural changes which can also be indicative of a volume change. As shown in Fig. 7, for mobile phases with up to 15% ethanol (v/v), the CD spectra are very similar, thus suggesting that the secondary structures of PEG–r-HuMGDF and PEG–r-metHuBDNF remain essentially unchanged. From these data, we can infer that the volume of each protein is basically uniform. Therefore, we conclude that these PEGylated proteins are most likely retarded by interaction with the silica surface, and ethanol diminishes this process. Thus, the CD data support an ethanol concentration in the range from 5–15% (v/v).

In Fig. 8, PEG–r-HuMGDF shows decreased peak height and increased peak width (loss of resolution) with added ethanol. But, a minimal volume of

Table 1
Peak symmetry values for PEG–r-metHuBDNF in 0.1 *M* PO_4^{3-} at pH 6.9 with varying NaCl concentrations, PEG–r-metHuBDNF in 0.1 *M* PO_4^{3-}–0.5 *M* NaCl at pH 6.9 with varying ethanol concentrations, and PEG–r-HuMGDF in 0.1 *M* PO_4^{3-}–0.2 *M* NaCl at pH 6.9 with varying ethanol concentrations.

PEG–r-metHuBDNF		PEG–r-metHuBDNF		PEG–r-HuMGDF	
NaCl concentration (*M*)	Peak symmetry	Ethanol concentration [% (v/v)]	Peak symmetry	Ethanol concentration [% (v/v)]	Peak symmetry
0.00	0.35	0	0.46	0	0.85
0.05	0.36	5	0.50	5	0.86
0.20	0.43	10	0.53	10	0.87
0.30	0.44	15	0.52	15	0.85
0.40	0.45			20	0.84
0.50	0.46				

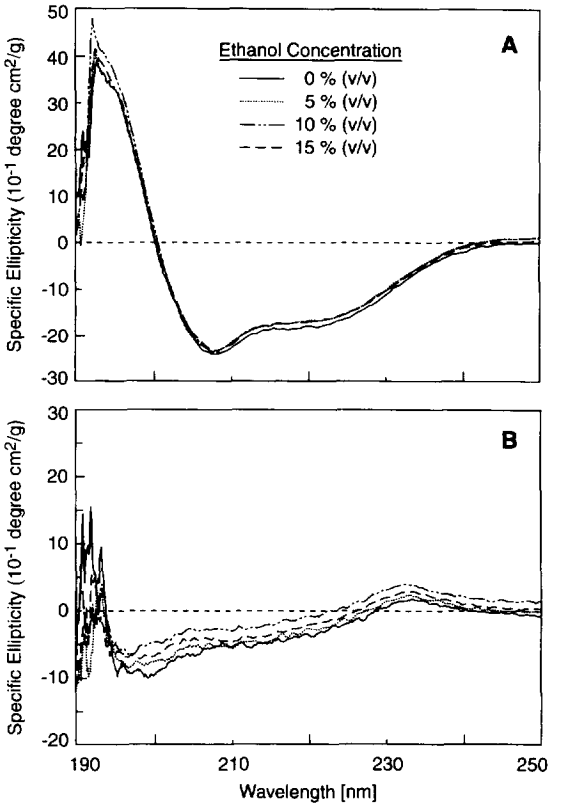

Fig. 7. (A) CD spectra of PEG–r-HuMGDF dialyzed into Mobile Phase 2 containing varying ethanol concentrations. (B) CD spectra of PEG–r-metHuBDNF dialyzed into Mobile Phase 1 containing varying ethanol concentrations.

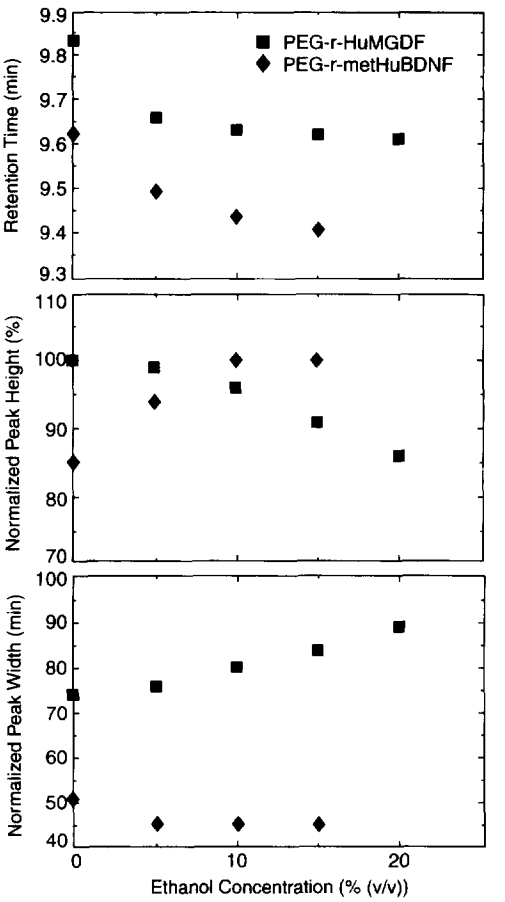

Fig. 8. Change in column performance characteristics for duplicate injections of PEG–r-HuMGDF in 0.1 *M* PO_4^{3-}–0.2 *M* NaCl at pH 6.9 or PEG–r-metHuBDNF in 0.1 *M* PO_4^{3-}–0.5 *M* NaCl at pH 6.9 both with varying ethanol concentrations.

ethanol is required to maintain increased column lifetime; therefore, 10% (v/v) was used for PEG–r-HuMGDF's mobile phase, as peak height decreased by only 4%, and peak width increased by only 6%. Moreover, for the ethanol concentration range examined as shown in Table 1, a peak symmetry value of 0.87 (the highest value) was obtained with 10% ethanol (v/v).

In Fig. 8, for PEG–r-metHuBDNF, ethanol addition to the mobile phase greatly improved peak height and peak width. At 10% ethanol (v/v), peak height increased 15%, and peak width decreased 6%, as compared to zero ethanol concentration. Peak symmetry was 0.53 (the highest value) as shown in Table 1; therefore, 10% (v/v) is an optimal concentration for the SEC analysis of PEG–r-metHuBDNF.

In summary, for PEG–r-HuMGDF, a mobile phase composition of 0.1 *M* PO_4^{3-}–0.2 *M* NaCl–10% ethanol (v/v) at pH 6.9 resulted in a 4% decrease in peak height and a 6% increase in peak width, and a 2% improvement in peak symmetry as compared to the same mobile phase without ethanol. For PEG–r-metHuBDNF, resolution improved substantially with addition of ethanol to the salt mobile phase. A mobile phase composition of 0.1 *M* PO_4^{3-}–0.5 *M* NaCl–10% ethanol (v/v) at pH 6.9 resulted in a 15% increase in peak height, a 6% decrease in peak width, and a 13% improvement in peak symmetry as compared to the same mobile phase without ethanol.

3.4. Column packing interactions

Nonideal SEC interactions of mobile phase, protein, and PEG with the silica packing surface of an SEC column are a combination of hydrogen bonding, dipolar effects, and ionic effects [4,5,11–15]. A few experiments were conducted to identify specific interactions.

An experiment was completed to address the question of whether an aqueous salt mobile phase, such as Mobile Phase 2, was responsible for performance loss of the SEC column. After equilibrating a new column with Mobile Phase 2, a sequence of 80 SEC runs was completed without injections. At the front, middle, and end of the sequence, duplicate injections of PEG–r-HuMGDF-37°C-4W were inserted. Chromatograms overlaid almost identically, thus strongly suggesting that interaction of PEGylated proteins with the packing material is the principal cause of column degradation.

For a few degraded columns, each front endcap and frit were carefully removed, and the solid-phase packing was visually inspected for void volumes, which were absent. A void volume would indicate dissolved or crushed silica packing particles.

Using the same loading as for PEGylated proteins, sequences of 150 injections of nonPEGylated r-metHuBDNF or r-HuMGDF were analyzed by SEC using Mobile Phases 1 or 2, respectively. Over the course of the sequence, there was no evidence of binding of protein or loss of resolution.

Using the same loading as for PEG–r-HuMGDF and using Mobile Phase 1, a sequence of 150 injections of PEG only was analyzed by SEC. In this case, loss of resolution occurred after ~20 injections, thus indicating that PEG interaction with the packing surface is the principal cause of the loss of resolution for PEG–r-HuMGDF and PEG–r-metHuBDNF.

In water, the surface of silica is known to contain residual negative charges above pH 4.0 due to ionization of silanol residues [12,16,17]. PEG–r-HuMGDF and PEG–r-metHuBDNF are well below their respective p*I* values; therefore, they contain net positive charges, and this would result in an ionic attraction during SEC. This was not observed with our nonPEGylated proteins, as even low NaCl concentration mitigated this interaction. TosoHaas SW-type packings are silica gel with chemically bonded hydrophilic groups which are terminated with a primary alcohol functional group [18]. We speculate that PEG is attracted to these modified hydrophilic groups or silica gel surface by either hydrogen bonding with the terminal –OH groups or other dipolar interactions with the $-(-OCH_2CH_2-)-$ groups of PEG [19].

4. Conclusions

Addition of 10% ethanol (v/v) to aqueous salt mobile phases significantly improved the performance of TSK-GEL G3000SW$_{xl}$ silica-based columns for SEC of PEGylated proteins. With addition of ethanol to aqueous salt mobile phases used in sequences of 150 injections, performance characteristics for 4°C and heated PEG–r-HuMGDF and PEG–r-metHuBDNF varied by less than 1.6% for initial versus final injections.

CD spectra of PEG–r-HuMGDF and PEG–r-metHuBDNF, dialyzed into SEC mobile phases with up to 15% ethanol (v/v), were very similar, suggesting that the PEGylated proteins exhibit little change in conformation.

Comparison of SEC results from PEG only, nonPEGylated proteins, and PEGylated proteins indicates that silica packing interactions occur principally through the PEG moiety.

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