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AQUEOUS SIZE-EXCLUSION CHROMATOGRAPHY OF ANIONIC AND NON-IONIC WATER-SOLUBLE POLYMERS ON SILICA GEL WITH BONDED HYDROPHILIC GROUPS

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SUMMARY

The elution behaviour of sodium polystyrene sulphonates (NaPSS) and linear polysaccharides (pullulan) on a column packed with porous silica gel with bonded glyceropropyl groups was studied. Phosphate buffer solution of various concentrations was used as the mobile phase. Early elution of NaPSS relative to non-ionic polymers was observed at low concentrations. Calibration graphs of $\log [\eta]M$ vs. K_{SEC} of NaPSS ($[\eta]$ = intrinsic viscosity of the polymer in the mobile phase; M = molecular weight; K_{SEC} = distribution coefficient) converged to the pullulan calibration graph with increasing concentration, crossed it, and then diverged on the other side. Late elution regarding the calibration graph for pullulan was caused by the hydrophobic interactions. The addition of a simple electrolyte suppressed the ion-exclusion effect, but the addition of an excess did not suppress the hydrophobic interactions. It is not possible to conclude that "ideal SEC" is obtained when the calibration graph for NaPSS approaches that for pullulan. The retention volume of NaPSS results from a combination of three effects: size-exclusion, ion-exclusion and hydrophobic interactions.

INTRODUCTION

Aqueous size-exclusion chromatography (ASEC) has been used for the determination of molecular weights (MW) of proteins and water-soluble synthetic polymers. One of the packing materials used for ASEC is porous silica gel chemically treated with γ-glycidyloxypropyltrimethoxysilane to form glyceropropyl groups on the surface[1]. There are several commercially available supports of this type and most of them exhibit weak cation-exchange properties[2]. These properties are due to electrostatic interactions with anionic polyelectrolytes, resulting in early elution relative to non-ionic water-soluble polymers[3]. The suppression of this electrostatic effect was observed at high ionic strenght[4], but "ideal SEC" (unperturbed by polymer–substrate interactions) cannot be achieved in this manner[5].

Barth[6] has summarized several problems associated with ASEC. Non-size-exclusion effects were divided into two types, ionic and adsorption effects. Ion-exchange, ion-exclusion, ion-inclusion and intramolecular electrostatic effects are ac-

counted for by ionic interaction between polyelectrolytes and substrate. Adsorption effects may arise from hydrogen bonding, hydrophobic and ionic interactions. Several studies have indicated that a mobile phase ionic strength of 0.2 is sufficient to eliminate these ionic interactions. However, the adsorption effects (hydrophobic interactions) of a number of protiens were observed by increasing the ionic strength of the mobile phase from 0.36 to 3.5 M[7]. These hydrophobic interactions can be eliminated by the addition of ethylene glycol[7] or an organic modifier (alcohol or glycol)[8].

Several commercially available supports for ASEC have already been examined extensively[2,4,6], and were found to exhibit ion-exclusion, cation-exchange and hydrogen partitioning effects under certain conditions. Recently a silica gel with bonded glyceropropyl groups became commercially available, *i.e.* Shodex Protein WS (Showa Denko, Tokyo, Japan). This paper is concerned with the elution behaviour of non-ionic polymers such as polyethylene oxides (PEO) and polysaccharides (pullulan) and anionic polymers such as sodium polystyrene sulphonates (NaPSS) on Shodex Protein WS-803. Mainly the influence of the ionic strenght of the mobile phase on retention volume and peak broadening was examined.

EXPERIMENTAL

ASEC measurements were performed on a Jasco TRIROTAR-V high-performance liquid chromatograph (Japan Spectroscopic, Tokyo, Japan) with a Model VL-611 loop injector. A UVIDEC-100V ultraviolet (UV) absorption detector was used at 254 nm for NaPSS samples and a Model SE-11 refractive index (RI) detector (Showa Denko) was used for other samples.

Shodex PROTEIN WS-803 was packed in a 50 cm × 8 mm I.D. stainless-steel column. The number of theoretical plates (N) was determined to be 28 000 plates per 50 cm by injecting 0.025 ml of a 0.5% ethylene glycol (EG) solution at a flow-rate of 1 ml/min of deionized water.

Samples were PEO standards (Tosoh, Tokyo, Japan) (MW $2.5 \cdot 10^4$, $4.0 \cdot 10^4$, $7.3 \cdot 10^4$, $1.5 \cdot 10^5$, $2.8 \cdot 10^5$, $6.6 \cdot 10^5$ and $1.2 \cdot 10^6$), polyethylene glycols (PEG) (MW 300, 600, 1000, 3400 and 5000), pullulan standards (Showa Denko) (MW 5800, $1.22 \cdot 10^4$, $2.37 \cdot 10^4$, $4.80 \cdot 10^4$, $1.00 \cdot 10^5$, $1.86 \cdot 10^5$, $3.80 \cdot 10^5$ and $8.53 \cdot 10^5$) and NaPSS (Pressure Chemical, Pittsburgh, PA, U.S.A.) (MW 4000, 6500, $1.60 \cdot 10^4$, $3.10 \cdot 10^4$, $8.80 \cdot 10^4$, $1.77 \cdot 10^5$, $6.90 \cdot 10^5$ and $1.00 \cdot 10^6$) (henceforth the numbers given after the sample name represent the MW). These samples were dissolved in the solution used as the mobile phase at a concentration of 0.1% for NaPSS, 0.2% for PEO and pullulan and 0.5% for PEG. Sodium *p*-toluenesulphonate was also used as a model monomer sample of NaPSS. The sample injection volume was 0.025 ml in all experiments.

The mobile phase for non-ionic polymers was deionized water and that for NaPSS was made up from Na_2HPO_4 and NaH_2PO_4 to the desired concentration at pH 8.0. The flow-rate of the mobile phase was 1 ml/min.

RESULTS AND DISCUSSION

The retention volume of EG was 20.0 ml, which was assumed to be equivalent to the total permeation volume. Size-exclusion chromatograms of non-ionic, water-soluble polymers obtained with water as the mobile phase are shown in Fig. 1. All
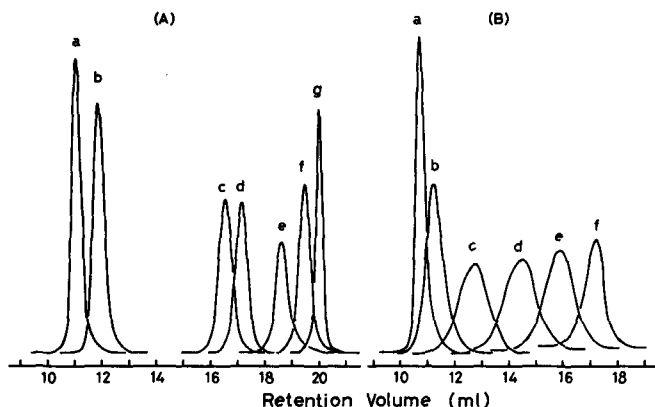
Fig. 1. Size-exlusion chromatograms of (A) PEO and PEG and (B) pullulan. Mobile phase, deionized water; detector, RI. Attenuation: (A)(a), (b) and (B), × 4, (A)(c) − (g), × 16. MW: (A)(a) 7.3 · 10^4, (b) 4.0 · 10^4, (c) 5000, (d) 3400, (e) 1000, (f) 600, (g) EG; (B)(a) 1.86 · 10^5, (b) 1.00 · 10^5, (c) 4.8 · 10^4, (d) 2.37 · 10^4, (e) 1.22 · 10^4, (f) 5800.

chromatograms are symmetrical and eluted before V_R = 20 ml, indicating that these polymers eluted mainly by size exclusion and no adsorption effects occurred.

The elution behaviour of anionic polymers, NaPSS, with water as the mobile phase was different from that of non-ionic polymers. Examples are shown in Fig. 2. Two peaks were observed for each sample, one at V_R = 10.45 ml and the other at V_R = 20.3–20.5 ml. The first peak corresponds to polystyrenesulphonic acid (PSSA) and the second to sodium hydroxide. These compounds originated from the NaPSS sample which separated into two components during elution by ion-exchange between ionic groups on the stationary phase and NaPSS. The packing material used in this experiment exhibits weak cation-exchange properties owing to the residual silanol groups. The Na$^+$ ions in NaPSS might undergo ion-exchange interactions with the
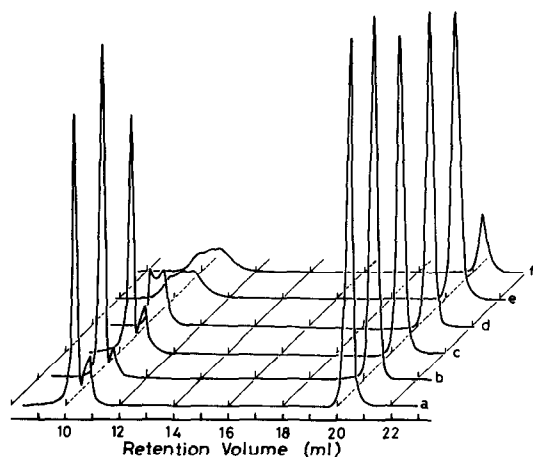

Fig. 2. Size-exlusion chromatograms of NaPSS in a mobile phase of deionized water. Detector, RI; attenuation, × 4. MW: (a) 4000; (b) 6500; (c) 3.1 × 10^4; (d) 1.77 · 10^5; (e) 6.9 · 10^5; (f) 1.0 · 10^6.

H^+ ions of the silanol groups, and PSSA elutes at the retention volume corresponding to the exclusion volume owing to ion-exclusion interactions between $PSSA^-$ and SiO^- ions. The reasons why the first peak split into a large and a small peak and why the peaks of high-MW NaPSS such as $1.0 \cdot 10^6$ and $6.9 \cdot 10^5$ were broad are unclear; probably one represents PSSA and the other the residual NaPSS. However, when $1.15 \cdot 10^{-3}$ M phosphate buffer solution was used as the mobile phase, no splitting occurred and only one sharp peak was observed at the exclusion volume ($V_R = 10.45$ ml). The second peak at $V_R = 20.3$–20.5 ml also disappeared.

The retention volume of NaPSS increased with increasing phosphate buffer concentration in the mobile phase. Fig. 3 shows an example using NaPSS 6500, which eluted at the exclusion volume when the phosphate buffer concentration in the mobile phase was $1.15 \cdot 10^{-3}$ M and eluted after the exclusion volume at higher concentrations. The retention volume of NaPSS 6500 continued to increase with increasing phosphate buffer concentration in the mobile phase and finally the NaPSS peak almost disappeared above the total permeation volume. The negative peak near the total permeation volume may be due to the difference in concentration of phosphate ions between the mobile phase and the sample solution. Although this negative peak interrupted the NaPSS 6500 peak at high phosphate buffer concentrations in the mobile phase, it was obvious that NaPSS 6500 eluted after the total permeation volume and the peak became very broad, indicating the existence of secondary effects other than ion-exclusion when the phosphate buffer concentration in the mobile phase was 0.3 M. The peak width of NaPSS 6500 also increased with increasing concentration of phosphate buffer in the mobile phase, mainly owing to the size-exclusion effect as it eluted between the size-exclusion volume and the total permeation volume.

Sodium p-toluenesulphonate can be assumed to be a model monomer compound of NaPSS. Its retention volume increased with increasing phosphate buffer concentration in the mobile phase, similarly to NaPSS. However, the peak of this compound was broad at low phosphate buffer concentrations in the mobile phase (below $1.0 \cdot 10^{-2}$ M) and sharp at concentrations above $2.76 \cdot 10^{-2}$ M. The results are
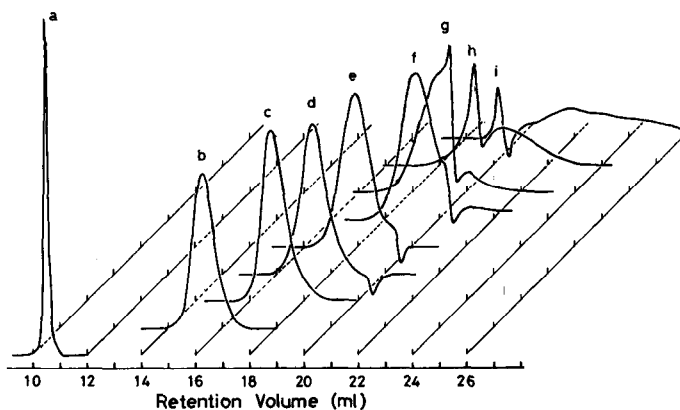

Fig. 3. Size-exclusion chromatograms of NaPSS 6500 in mobile phases with various phosphate buffer concentrations (M): (a) $1.15 \cdot 10^{-3}$; (b) $2.76 \cdot 10^{-2}$; (c) $6.0 \cdot 10^{-2}$; (d) $8.0 \cdot 10^{-2}$; (e) 0.10; (f) 0.12; (g) 0.15; (h) 0.20; (i) 0.30, UV attenuation: (a), (c), (d), $\times$ 0.16; (b), (e)–(i), $\times$ 0.08.
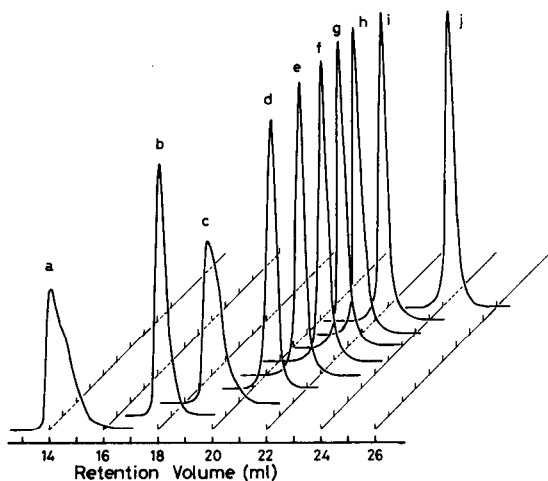
Fig. 4. Chromatograms of sodium p-toluenesulphonate. Concentration of phosphate buffer in the mobile phase (M): (a) $1.15 \cdot 10^{-3}$; (b) $5.0 \cdot 10^{-3}$; (c) $1.0 \cdot 10^{-2}$; (d) $2.76 \cdot 10^{-2}$; (e) $5.0 \cdot 10^{-2}$; (f) $6.0 \cdot 10^{-2}$; (g) 0.10; (h) 0.12; (i) 0.15; (j) 0.40 UV. attenuation: $\times$ 0.16.

shown in Fig. 4. Peaks e–j eluted above the total permeation volume and were sharp as peak d eluted at the total permeation volume (V_R = 20 ml). The widths of peaks d–j were identical. The elution behaviours of the polymer and the monomer may differ.

An example of the size-exclusion chromatograms of several NaPSS in a mobile phase with a phosphate buffer concentration of 0.05 M is shown in Fig. 5. The peaks that eluted at the exclusion volume were sharp and those eluting between the exclusion volume and the total permeation volume were broad. This peak broadening may be due mainly to the size-exclusion effect and implies that the NaPSS samples have molecular weight distributions, although they were supposed to have narrow distri-
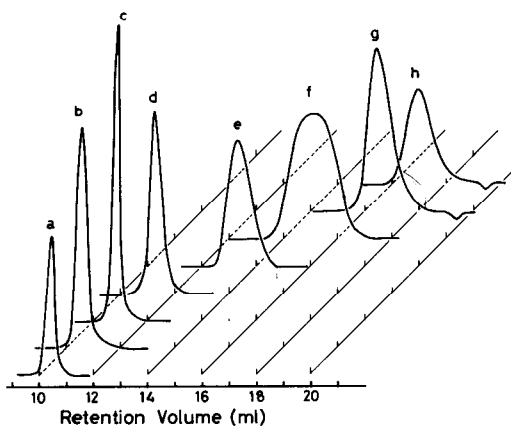

Fig. 5. Size-exclusion chromatograms of NaPSS in a mobile phase of 0.05 M phosphate buffer solution of pH 8.0. MW: (a) $1.0 \cdot 10^{6}$; (b) $6.9 \cdot 10^{5}$; (c) $1.77 \cdot 10^{5}$; (d) $8.8 \cdot 10^{4}$; (e) $3.1 \cdot 10^{4}$; (f) $1.6 \cdot 10^{4}$; (g) 6500; (h) 4000. UV attenuation: (a)–(d), $\times$ 0.16; (e)–(h), $\times$ 0.08.

butions. The pH of the mobile phase used here was 8.0; the same results were obtained at pH 4.7.

The retention volume, V_R, in SEC can be expressed by the equation

$$V_R = V_o + K_{SEC} V_i$$

where V_o is the interstitial volume (the exclusion volume) of the column, V_i is the inner volume of the packing material in the column and K_{SEC} is the distribution coefficient. V_o was taken as the retention volume of NaPSS in a mobile phase consisting of deionized water or phosphate buffer of concentration $1.15 \cdot 10^{-3}$ M. The validity of this assumption was supported by observations that all the NaPSS tested in these two mobile phases appeared at $V_R = 10.45$ ml. V_i was measured as the difference between the retention volume of EG and V_o, and was 9.55 ml.

Fig. 6 shows plots of log M vs. K_{SEC}. Pullulan and PEO (and PEG) eluted according to their molecular weights and were not affected by the concentration of the mobile phase; their K_{SEC} values were between 0 and 1. K_{SEC} for NaPSS in deionized water was zero and increased with increasing phosphate buffer concentration in the mobile phase. The NaPSS peaks also broadened with increasing retention volume. NaPSS of MW $6.9 \cdot 10^5$ and $1.0 \cdot 10^6$ appeared at V_o and were not affected by the phosphate buffer concentration in the mobile phase. NaPSS 6500 and 4000 appeared near $V_o + V_i$ with a phosphate buffer concentration in the mobile phase of 0.15 M and were retained in the column with concentrations over 0.20. NaPSS above MW $1.6 \cdot 10^4$ appeared with phosphate buffer concentrations in the mobile phase of up to 0.30 M. At buffer concentrations above 0.40 M all NaPSS were retained in the
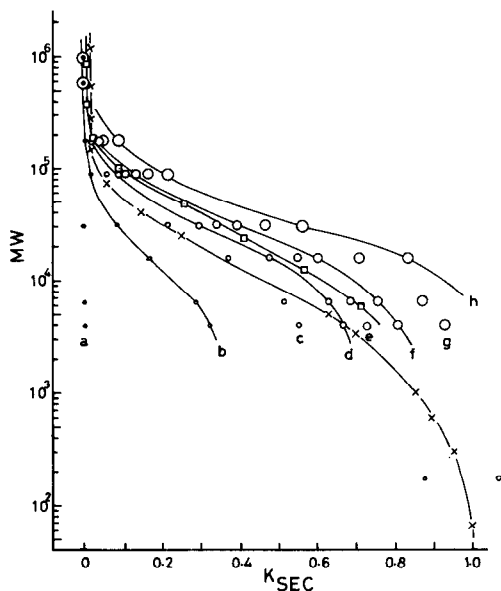

Fig. 6. Calibration graphs of log MW vs. distribution coefficient for ($\square$) pullulan, ($\times$) PEO and PEG and ($\bigcirc$) NaPSS. Concentration of phosphate buffer in the mobile phase (M): (a) 0; (b) $1.0 \cdot 10^{-2}$; (c) $2.76 \cdot 10^{-2}$; (d) $5.0 \cdot 10^{-2}$; (e) $7.0 \cdot 10^{-2}$; (f) 0.10; (g) 0.15; (h) 0.20. The mobile phase for pullulan, PEO and PEG was deionized water.
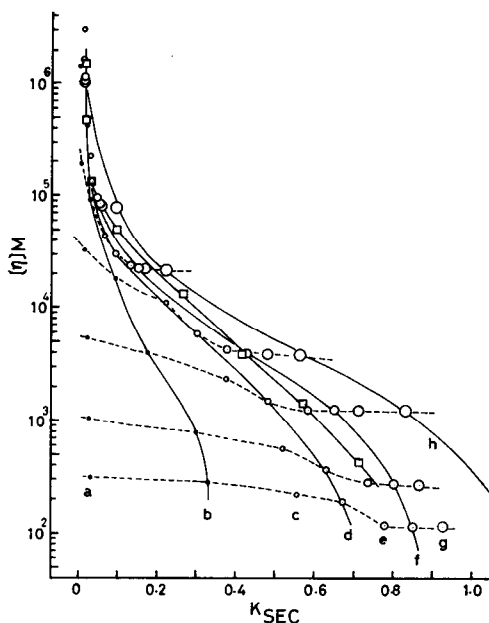
Fig. 7. Calibration graphs of log $[\eta]M$ vs. distribution coefficient for ($\square$) pullulan and ($\bigcirc$) NaPSS. Concentration of phosphate buffer in the mobile phase (M): (a) $1.15 \cdot 10^{-3}$; (b) $1.0 \cdot 10^{-2}$; (c) $2.76 \cdot 10^{-2}$; (d) $5.0 \cdot 10^{-2}$; (e) $9.0 \cdot 10^{-2}$; (f) 0.12; (g) 0.15; (h) 0.20. The mobile phase for pullulan was deionized water.

column and only sodium p-toluenesulphonate eluted; NaPSS are more hydrophobic than the latter.

NaPSS of MW $6.9 \cdot 10^5$ and $1.0 \cdot 10^6$ could not enter the pores of the packing material and their peak widths were unchanged until they were retained in the column. Other NaPSS showed broadened peaks when they could enter the pores. Peak broadening may be due mainly to the size-exclusion effect in mobile phases with low concentrations of phosphate buffer. Intramolecular chain expansion of polyelectrolytes leads to early elution relative to non-ionic polymers and this expansion can be suppressed by the addition of simple electrolytes[3]. To discuss the elution behaviour of NaPSS from the viewpoints of secondary effects, the variable of intramolecular chain expansion should be removed. The solution is to use the hydrodynamic volume parameter $[\eta]M$, where $[\eta]$ is the intrinsic viscosity of the polymer in the mobile phase and M is the molecular weight. Fig. 7 shows plots of log $[\eta]M$ vs. K_{SEC} for pullulan standards and NaPSS. The hydrodynamic volume of NaPSS decreased rapidly with increasing concentration of phosphate buffer in the mobile phase up to $9.0 \cdot 10^{-2}$ M. It is evident that retention volume of NaPSS of the same molecular size increased with increasing phosphate buffer concentration in the mobile phase.

It is possible to compare the retention volumes of NaPSS and pullulan of the same hydrodynamic volume by using Fig. 7. The retention volume of NaPSS converged to that of pullulan of the same molecular size with increasing concentration of phosphate buffer in the mobile phase and then diverged to the other side. The phosphate buffer concentration in the mobile phase at the crossing point of the pullulan calibration graph for low-MW NaPSS was $9.0 \cdot 10^{-2}$ M and that for high-MW

NaPSS was $1.2 \cdot 10^{-1}$ M. The retention volume of NaPSS continued to increase and finally NaPSS was retained in the column.

In conclusion, it is evident that the early elution of NaPSS relative to non-ionic polymers was caused by the ion-exclusion effect and late elution after the pullulan calibration graph by hydrophobic interactions. A size-exclusion effect would occur after V_o. The addition of a simple electrolyte suppressed the ion-exclusion effect. However, the addition of an excess of simple electrolyte (high concentration in the mobile phase) did not suppress the hydrophobic interactions, and it therefore cannot be said that "ideal SEC" will be obtained when the calibration graph for NaPSS approaches that for pullulan or when it overlaps that for pullulan. Elution between V_o and $V_o + V_i$ will be a combination of three effects: size-exclusion, ion-exclusion and hydrophobic interactions.

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