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Observation of Ficoll charge using size-exclusion chromatography

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Abstract

The retention volumes of Ficoll samples of varying molecular weight were determined on porous glass and Superose columns, and compared with those of Pullulan. The retention of Ficoll is pH- and ionic strength-dependent, indicating that it bears a weak negative charge at moderate pH. Comparisons were made with similar charge repulsion effects for proteins on Superose to provide an estimate of the extent of charge on Ficoll, with the conclusion that only a few charges exist per molecule at neutral pH. © 1998 Elsevier Science B.V.

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1. Introduction

When size-exclusion chromatography (SEC) is used to characterize macromolecules, it is generally assumed that their elution volumes are controlled by steric repulsion. Such ideal SEC conditions are approached when interactions between the solute and the stationary phase are negligible. However, non-ideal SEC behaviour is common in aqueous systems due to hydrophobic [1] or electrostatic interactions [2] between the solute and the SEC packing material.

Although non-ideal SEC behaviour may introduce difficulties in macromolecular characterization, it can also provide some useful molecular information. For example, Dubin et al. used SEC to evaluate theories for the permeation of charged particles into cavities of similar charge [3]. Other investigations demonstrate that it is possible to separate steric interactions from either electrostatic [4,5] or hydrophobic [6] contributions in SEC. Therefore, a similar approach

may be employed to study the behaviour of a biotechnologically important polysaccharide, Ficoll.

Ficoll, a water-soluble highly cross-linked (densely branched) copolymer of sucrose and epichlorohydrin, is used to form density gradients for ultracentrifugation [7,8], to enhance the electrofusion of cells of different densities [9], and to separate proteins via polymer two-phase partitioning [10]. Furthermore, the permeability of biological membranes has been studied using Ficoll as a model solute [11,12]. In view of these numerous applications, it is important to understand how the behaviour of Ficoll solutions arise from the structural features of Ficoll molecules. Based on hydrodynamic properties, it appears that the behaviour of Ficoll approximates that of a compact sphere [13]. It is furthermore commonly assumed that Ficoll is electrically neutral. However, the present SEC results indicate that Ficoll is weakly charged.

The observation of weak anionic charge on an ostensibly neutral polysaccharide is not new. Porsch et al. [14] observed two elution peaks from SEC of

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Table 1
Molecular weight and intrinsic viscosity of Pullulan standards

$M_w \times 10^{-3}$	$[\eta]$ (dL/g) ^a
100.0	0.459
48.0	0.286
23.7	0.181
12.2	0.119
5.8	0.079

^a In water, at 25°C, from manufacturer.

dextran in pure water. They explained this result in terms of a minute negative charge on dextran, presumably 1 or 2, which interacts with the negative charges on the surface of the column packing to produce a fast-eluting peak (5–10% compared to the normal elution peak). They further speculated that the presence of charged groups is a general feature of polysaccharides. In this paper, the elution behaviour of Ficoll and Pullulan are studied on both Superose-12 and CPG columns. We detect an electrostatic contribution to the SEC elution of the former polysaccharides.

2. Materials and methods

Pullulan standards, Shodex P-82, were obtained from Showa Denko (Tokyo, Japan) with molecular weight and intrinsic viscosity listed in Table 1. Ficoll fractions were obtained from Dr. Rune Andersson of Pharmacia, with molecular weight and intrinsic viscosity shown in Table 2. Intrinsic viscosities $[\eta]$ (dL/g), shown in Table 2 are calculated via [16]

$$[\eta] = 2.94 \cdot 10^{-4} M^{0.30} \quad (1)$$

Stainless-steel columns (15×0.3 cm I.D.) were dry packed with 30–60 μm grain size Controlled

Table 2
Molecular weight and intrinsic viscosity of Ficoll samples

$M_w \times 10^{-3}$	$[\eta]$ (dL/g) ^a
175.5	0.133
105.2	0.114
60.7	0.096
37.4	0.083
21.3	0.070

^a Interpolated from Ref. [15], 25°C in water, via Eq. (1).

Pore Glass (Schott–Geräte, Mainz, Germany), either CPG-30 (145 Å pore diameter, 136 m² g⁻¹ surface area) or CPG-50 (199 Å pore diameter, 86 m² g⁻¹ surface area). A Superose 12 column (30×1 cm I.D.) was obtained from Pharmacia (Uppsala, Sweden). The chromatography system also included a Milton Roy mini-pump (St. Petersburg, FL, USA), a 20 μl injection loop, and a differential refractometer R401 (Waters, Milford, MA, USA). Flow-rates were maintained around 0.4 ml min⁻¹ (±0.3%) for both Superose and CPG columns. NaH₂PO₄–Na₂HPO₄ and Tris–HCl buffer of the desired pH and ionic strength were used as mobile phases. ²H₂O and Pullulan 1600 ($M_w = 1600 \cdot 10^3$) were used to measure total elution volume (V_t) and void column volume (V_0), respectively. The numbers of theoretical plates obtained from the elution peak of 10% ²H₂O were 8.5·10² and 2.3·10⁴ for CPG and Superose columns, respectively.

The SEC partition coefficient was obtained as

$$K_{\text{SEC}} = \frac{V_e - V_0}{V_t - V_0} \quad (2)$$

where V_e is the sample elution volume. The viscosity radius of the solute was obtained as [17]

$$R_\eta = \left[\frac{3[\eta]M}{10\pi N_A} \right]^{1/3} \quad (3)$$

where M is the molecular weight of solute, and N_A is Avogadro's number. Because pH and ionic strength effects on R_η are negligible in the range of experimental conditions [16], $[\eta]$ values measured in pure water at 25°C were used to calculate R_η for Pullulan and Ficoll.

3. Results and discussion

In order to detect non-steric effects in SEC, it is useful to fit the data to some model that should apply to ideal SEC. According to the cylindrical pore model [18]

$$K_{\text{SEC}} = \left(1 - \frac{R}{r_p} \right)^2 \quad (4)$$

where R is the solute radius, and r_p is the pore radius. While neither Superose nor CPG is expected

to have pores of particularly well-defined geometry, there is strong empirical evidence in support of Eq. (4), namely that plots of $K_{SEC}^{1/2}$ versus R for various solutes yield straight lines with slope of $1/r_p$ for both CPG [19] and Superose [20].

Data for Pullulan plotted in Fig. 1 as $K_{SEC}^{1/2}$ versus R_η (solid circles) show an intercept of $K_{SEC}=1$ at $R_\eta \rightarrow 0$, and a slope corresponding to $r_p=114 \pm 3 \text{ \AA}$. The error limits shown here and elsewhere are obtained from the standard deviation of the slope. As seen in Fig. 1, Ficoll samples (solid squares) elute more rapidly than Pullulan of equal size. The absence of an effect of pH on the elution of Pullulan from Superose columns [21] indicates that Pullulan can be treated as a neutral molecule at the lowest ionic strength used. Therefore, the fast elution of Ficoll relative to Pullulan suggests repulsion between Ficoll and the CPG column.

The apparent value of r_p for Ficoll, $82 \pm 2 \text{ \AA}$, can be analyzed in terms of a hypothetical portion of the pore volume that is penetrable to nonionic solutes but impenetrable to polyion. The thickness of this hypothetical electrostatic barrier to permeation is

$$X_e = r_p^{geom} - r_p^{app} \quad (5)$$

where r_p^{geom} is the geometric pore radius, and r_p^{app} is the apparent pore radius measured for a charged polymer. Therefore, X_e is obtained as $(114 \pm 3) - (82 \pm 2) = 32 \pm 4 \text{ \AA}$ for Ficoll on CPG at pH 9.0 and

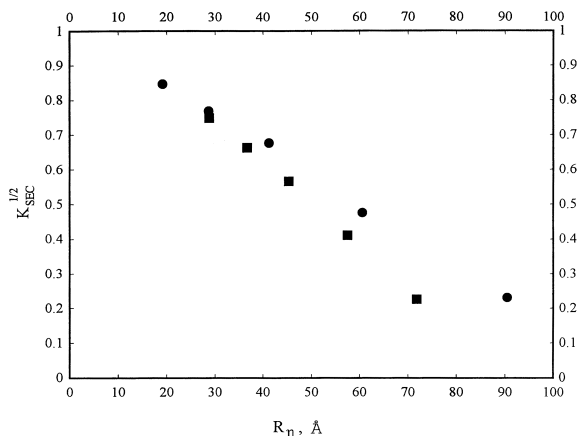


Fig. 1. $K_{SEC}^{1/2}$ versus R_h for Pullulan (●) and Ficoll (■) on CPG-30 column. Mobile phase: pH 9.0, $I=0.005$, Tris buffer.

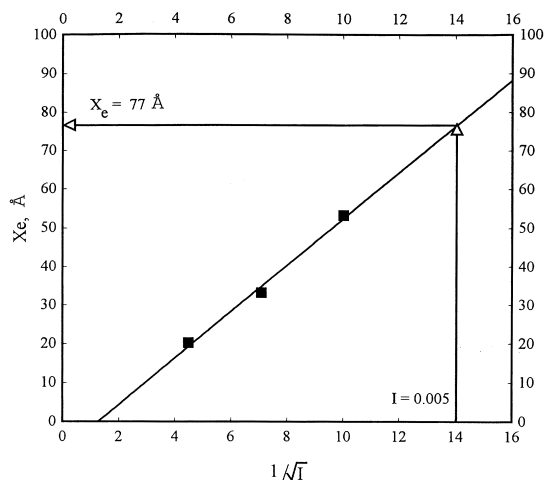


Fig. 2. X_e versus $I^{-1/2}$ for CPG column using NaPSS as probe. Mobile phase: pH 5.0 phosphate buffer.

$I=0.005$. The repulsive force between charged particles in solution is a function of ionic strength, or more exactly of the Debye–Hückel parameter, $\kappa \sim I^{1/2}$. X_e should therefore depend on the Debye length κ^{-1} . The value of X_e for Ficoll on CPG may be compared with the value for polystyrenesulphonate (a strong polyelectrolyte) on CPG [4]. Extrapolation to $I=0.005$, as shown in Fig. 2, gives $X_e = 77 \pm 11 \text{ \AA}$, more than twice the value for Ficoll.

From the results obtained in the same mobile phase on a Superose 12 column shown in Fig. 3, we find $r_p^{geom} = 170 \pm 10 \text{ \AA}$, $r_p^{app} = 146 \pm 6 \text{ \AA}$, and conse-

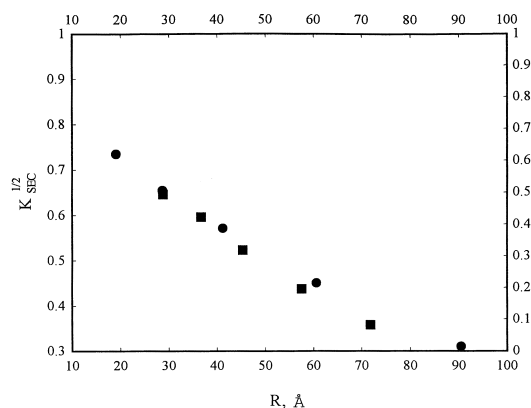


Fig. 3. Universal calibration for Pullulan (●) and Ficoll (■) on Superose-12 column. Mobile phase: pH 9.0, $I=0.005$, Tris buffer.

quently $X_e = 24 \pm 12 \text{ \AA}$. Although a smaller value of X_e is expected for Superose 12 due to its low charge density (only about 1/10 that of the CPG, see Fig. 4), X_e for Superose, $24 \pm 12 \text{ \AA}$, is not significantly smaller than the value for CPG, $32 \pm 4 \text{ \AA}$. The unexpectedly small effect of the packing surface charge density on X_e may be explained as follows. If the preferred orientation of Ficoll is one in which the charge is far from the surface, the effective potential at this location (ψ_x) may be small compared to the surface potential ($\psi_{x=0}$). Similarly, $\psi_x(\text{CPG}) - \psi_x(\text{Superose})$ may be small even when $\psi_{x=0}(\text{CPG}) - \psi_{x=0}(\text{Superose})$ is large. Therefore, the difference between $X_e(\text{CPG})$ and $X_e(\text{Superose})$ could be insignificant.

Under charge repulsion conditions, the observed SEC partition coefficient (K_{obs}) is a function of solute surface charge density ($\sigma = Z/4\pi R^2$). An empirical relationship between ΔK ($K_{\text{obs}} - K_{\text{ideal}}$) and σ (at constant ionic strength) may be constructed with either polyelectrolytes or proteins. Because Ficoll is densely branched, its charge groups, unlike those of a polyelectrolyte, may be considered to be fixed in space. At the ionic strength corresponding to our experimental conditions, the interior charge groups should not contribute to the repulsion between Ficoll and SEC packing materials. Proteins, with charges confined to their surfaces, thus provide a better model, and an empirical relationship of ΔK versus σ of protein may be applied to Ficoll mole-

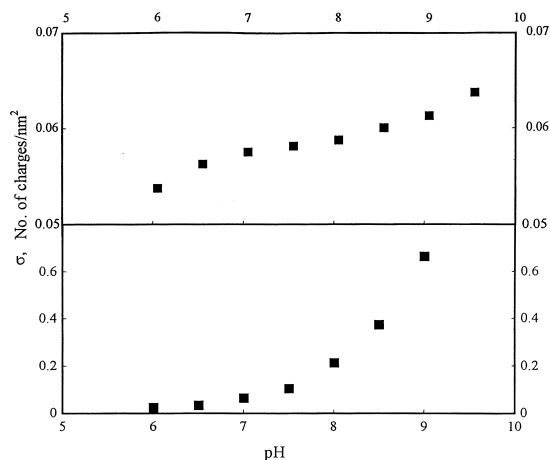


Fig. 4. Surface charge density as function of pH. Upper: Superose 12 (Ref. [22]), $I=0.005$. Lower: CPG (Ref. [15]), $I=0.01$.

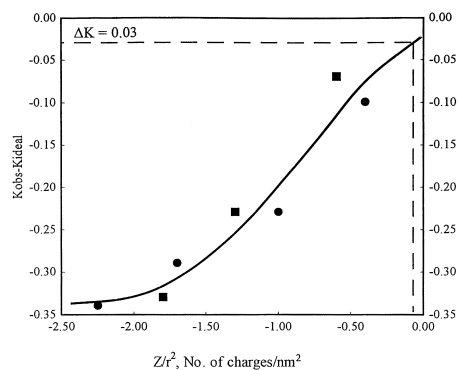


Fig. 5. ΔK versus protein surface charge density Z/r^2 (Ref. [23]). Mobile phase: $I=0.01$, phosphate buffer at pH 9.5, 10, 10.5, and 10.9. Myoglobin (●) and RNAse (■)

cules. Cai et al. [23] obtained such empirical relationship on a Superose 12 column for different proteins, as shown in Fig. 5, in which K_{ideal} is the value for a non-interacting neutral molecule of equal size. $\Delta K = 0.03 \pm 0.02$ for the highest molecular-weight sample of Ficoll gives by extrapolation Z/r^2 ca. -0.1 . With a radius of 7 nm for this sample, we then obtain an effective surface charge of about -4 for Ficoll.

The interaction between Ficoll and either CPG or Superose may be fully suppressed by an increase in ionic strength. Fig. 6 shows that Pullulan and Ficoll

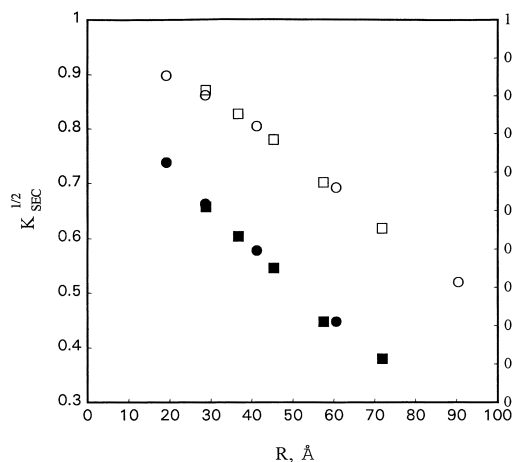


Fig. 6. Universal calibration for Pullulan (●) and Ficoll (■) on Superose-12 column, pH 6.2, $I=0.10$, phosphate buffer; for Pullulan (○) and Ficoll (□) on CPG-50 column, pH 9.0, $I=0.10$, Tris buffer.

plots converge either on CPG-50 at pH 9.0, $I=0.1$, or on Superose 12 at pH 6.2, $I=0.1$. These results confirm that Ficoll is only weakly charged at pH 9.0.

The source of the negative charge on Ficoll is not known. Porsch [14] suggested that carboxyl groups are formed by oxidation of the aldehyde end groups of dextran. Since Ficoll is highly cross-linked, it has numerous end groups and the probability of carboxylic acid end groups is consequently much higher than for Pullulan. Attempts to separate Pullulan and Ficoll by capillary electrophoresis at pH 9.0 and $I=0.005$ were unsuccessful. This suggests that SEC is very sensitive to weak electrostatic interactions between the solute and the stationary phase. Porsch et al. [14] reported that SEC can detect non-ideal behaviour of polysaccharides that bear only one or two carboxyl groups, which is consistent with our results.

In conclusion, we observed electrostatic interaction between weakly charged Ficoll and negatively charged chromatographic packing materials at high pH and low ionic strength. It is shown that SEC is a sensitive method to detect such weak electrostatic interactions. Such interaction should be considered when Ficoll is studied in charged systems.

Acknowledgements

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