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Macroporous, monodisperse particles and their application in aqueous size exclusion chromatography of high molecular weight polysaccharides

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Three different types of 15 μm macroporous polymer particles (MP) based on poly(styrene-*co*-divinylbenzene), all with an extreme degree of monodispersity, were prepared by the method of 'activated swelling'. The type and relative content of low molecular weight compounds used as pore-forming agents (porogens) ensured that the total pore volume was confined to large pores with radii larger than 300 Å, and with an increasingly larger fraction of the pore volume shifted towards very large pores (> 2000 Å). A hydrophilic layer was covalently linked to the particles to eliminate their hydrophobic character, and they were subsequently tested in aqueous size exclusion chromatography (SEC). Favourable chromatographic properties generally associated with monosized particles were obtained, and column packing as well as the separations were performed with very low back pressures. The particles were found to be suitable for SEC of a series of high molecular weight polysaccharides, irrespective of their charge (anionic, cationic and neutral). The shift in pore size distribution towards very large pores resulted in a corresponding shift in the range of molecular weights (strictly, hydrodynamic volumes) where separation took place. For particles with 70% of the pore volume confined to pores with radii larger than 2000 Å, it was estimated that the molecular weight range of polysaccharides of the pullulan type accessible to SEC was extended up to ca. 10^8. The corresponding value for an extended, rod-like, triple-helical polysaccharide (scleroglucan) was 10^7. Copyright © 1996 Elsevier Science Limited.

INTRODUCTION

Size exclusion chromatography (SEC) is commonly used for determining molecular weights and molecular weight distributions (MWD) of polymers in solution. The polymers are separated according to their molecular sizes (hydrodynamic volume), and the pore size distribution of the column material should therefore correspond to the hydrodynamic size distribution of the polymer. The hydrodynamic volume of a polymer in solution is proportional to the product $M[\eta]$, where M is the molecular weight and $[\eta]$ is the intrinsic viscosity. Most separation media for SEC have a region of elution volumes (V) where there is a linear relationship between $\log(M[\eta])$ and V, almost independent of the shape of the molecules. This is commonly referred to as the universal calibration (Benoit *et al.*, 1966), although different polymers do give different relationships between $\log(M[\eta])$ and V. In particular, rod-like chains such as schizophyllan deviate from the general trend (Dubin, 1994).

Many biopolymers, including a large number of polysaccharides, have very large hydrodynamic sizes which may prevent the use of SEC for molecular size analysis. For example, xanthan polysaccharides with molecular weights larger than 10^6 are not separated on SEC columns containing TSK-GEL G6000PWXL (Christensen *et al.*, 1993), which is one of the column materials currently available for aqueous SEC that contain the largest pores.

In this work we report on the preparation and application of macroporous, highly monodisperse polymer

particles (diameter 15 μm) with pore radii concentrated in the range of 300–10 000 Å. The particle matrixes are poly(styrene-*co*-divinylbenzene) with a hydrophilic layer covalently linked to the surface of the pores. A high content of divinylbenzene corresponding to a high degree of crosslinking ensures excellent mechanical and hydrodynamic stability, and in addition provides a high content of unreacted vinyl groups which facilitate the coupling of the hydrophilic layer (Ellingsen *et al.*, 1990, 1993). As this process only involves formation of stable ether bonds the particles are chemically robust, and permit elution with organic solvents, strong acids or strongly alkaline solutions without swelling or cleavage of labile bonds.

Special advantages associated with monodispersity include efficient packing of columns, uniform flow, low back pressure and an optimal combination of resolution and speed (Ellingsen *et al.*, 1990).

The basis for preparation of the highly monodisperse particles described in the present paper is the 'activated swelling' principle invented by Ugelstad (1982, 1984). The theoretical basis and practical implementation of the method have been described in detail in a series of papers (Ugelstad *et al.*, 1980, 1983, 1992; Ellingsen *et al.*, 1993), and is only briefly outlined here. In the first step of the particle preparation a 'swelling agent' consisting of a highly water insoluble compound of relatively low molecular weight, is introduced into small monodisperse polymer particles in a volume ratio (swelling agent to particles) of 1:1 to 5:1. An aqueous dispersion of such 'activated' particles is then swollen with slightly water soluble compounds such as appropriate vinyl and divinyl monomers and, optionally for macroporous particles, inert pore-forming agents (porogens). The 'activated' particles may absorb from 100 to more than 1000 times their own volume of the compounds added in the second step. The corresponding value for particles consisting of pure polymer is 1 to 10. In many cases the swelling agent itself may be a highly water insoluble, oil soluble initiator, which then may act both as a swelling agent and as an initiator for polymerization. A most important feature is that all ingredients are introduced into the particles before polymerization. This ensures a very high degree of monodispersity and an exceptional batch to batch reproducibility.

Previous experiments with polydisperse particles prepared by suspension polymerization have revealed that the main factor in determining the pore size and pore size distribution of macroporous particles is the type of porogen applied. Porogens with solubility parameters considerably lower or higher than that of the polymer formed tend to give large pores while porogens with solubility parameters close to the polymer lead to small pores (Seidl *et al.*, 1967). In the present investigation carboxylic acids are used as porogens to achieve large pores. With the 'activated swelling' method the seed polymer may account for less than 1% of the porogen. This allows a very flexible and controlled pore size distribution and most importantly, an easy removal of porogens including the polymer seed even in highly crosslinked systems.

The easy and reproducible preparation and the favourable chromatographic properties of monodisperse particles prepared by the 'activated swelling' method have been demonstrated both for size exclusion chromatography in organic solvents (Kulin *et al.*, 1990, Ellingsen *et al.*, 1990) and for ion-exchange chromatography (Ellingsen *et al.*, 1990, 1992; Barnes, 1993). More recently, the method of 'activated swelling' has been adopted by a number of scientists for preparation and studies of highly monodisperse macroporous particles (Wang *et al.*, 1992; Smigol & Svec, 1992, 1993; Fréchet, 1993; Smigol *et al.*, 1993; Hosoya & Fréchet, 1993; Galia *et al.*, 1994). It is so far the only method available which allows preparation of extremely monodisperse, highly crosslinked particles, combined with a practically unlimited choice of monomers and porogens and thereby of chemical composition and morphology of the final particles.

EXPERIMENTAL

Preparation of particles

Three particle types, I, II, and III, all with diameters of 15 μm, were prepared essentially as described earlier (Ellingsen *et al.*, 1993). The seed particles applied were in all cases monodisperse polystyrene particles with diameter 3 μm. Aqueous dispersions of these particles were swollen with an oil soluble initiator which was added in the form of finely dispersed droplets (Ellingsen *et al.*, 1993). Vinyl compounds and porogens were then added and became absorbed by the 'activated' seed particles. The composition of the polymerization mixture is given in Table 1. Following polymerization the surface of the pores was covered with a covalently linked hydrophilic layer. This involves the formation of a crosslinked polyhydroxy compound covalently linked to the pore surface as described earlier (Ellingsen *et al.*, 1993; Barnes, 1993; Pharmacia, 1994).

Table 1. Particle characteristics

Particle type	Porogen type	Relative volume ratios[a]	
		Monomer[b]	Porogen
I	Branched alkanoic acid	13	30
II	Branched alkanoic acid	11	32
III	Linear alkanoic acid	13	30

[a]Relative to seed volume.
[b]Monomer composition: mono- and divinyl compounds (20:80).

Packing into chromatographic columns

An aqueous suspension of particles was washed several times with aqueous 0.1 M NaCl. A vertically mounted column (Pharmacia HR 10/30, $ID = 10$mm, $l = 300$mm) was connected to an extension tube ($ID = 10$mm, $l = 1000$mm) and filled with solvent (50–60ml). The slurry was then poured into the extension tube before connecting the tube to a pump operating at a flow rate of 1.0ml/min. During packing the connector between the column and the extension tube was vibrated at 50Hz by mounting an aquarium pump in physical contact with the connector. When the packing was finished the extension tube and the connector were removed and the height of the packed material was adjusted to 30cm before placing the top adaptor.

Size exclusion chromatography

SEC was performed at room temperature or at 40°C using a HPLC pump operating at a flow rate of 0.5ml/min. Injected samples (100–200μl) contained 0.02–2mg/ml of dissolved polymer. High temperature (40°C) and low polymer concentrations (0.02–0.2mg/ml) were used for samples with extremely high molecular weights (scleroglucans) to minimize effects caused by the viscosity of the injected samples. The elution was monitored by a refractive index detector (Shodex RI SE-61) and a light scattering photometer (Chromatix KMX-6) equipped with the standard HPLC cell. Data acquisition and molecular weight calculations were performed by the PCLALLS software. The value of the refractive index increment, dn/dc, was 0.150 in all cases.

The samples were dissolved directly in the solvent or in pure water followed by adjustment of the ionic strength, and then filtered using filters with pore sizes depending on the polymer (typically 0.22–0.8μm). A second in-line filter containing the same filter type was used during sample injection. The mobile phase was 0.2M ammonium acetate (pH4.5) for chromatography of chitosans. In the other cases the mobile phase was 0.1 M NaCl.

Polymer samples

Pullulan standards covering molecular weights (number average) in the range from $1.62 \cdot 10^4$ to $2.61 \cdot 10^6$ were obtained from Hayashibara Biochemical Laboratories, Japan. Scleroglucan was obtained as a fermentation broth from Norferm (Stavanger, Norway). The sample was purified and depolymerized with H_2O_2/Fe^{2+} as described previously (Hjerde *et al.*, 1994), yielding samples with weight average molecular weights (M_w) ranging from $0.46 \cdot 10^6$ to $11 \cdot 10^6$. Algal alginate isolated from *Macrocystis pyrifera* was obtained from Kelco Division of Merck, USA. A bacterial alginate rich in β-D-mannuronic acid was prepared in the laboratory. Chitosans with varying degrees of *N*-acetylation (Anthonsen *et al.*, 1994) were produced in the laboratory.

Calculations

The selectivity coefficient, K_{av}, was calculated as $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e is the peak elution volume, V_0 is the void volume and V_t is the total volume. The use of K_{av} instead of V_e facilitates a direct comparison of the selectivities for columns with different sizes.

The number of theoretical plates (N) was calculated from the relationship $N = 5.54(t_r/t_{w,1/2})$, where t_r is the retention time and $t_{w,1/2}$ is the peak width at 50% of the peak maximum. The peak for glucose was used as a basis for the calculations.

Since monodisperse water soluble polymer standards are not available in the very high molecular weight range the relationships between log M and K_{av} were obtained from polydisperse samples by means of the combined light scattering and refractive index detector (Stuting & Krull, 1990). For each volume increment (ΔV) of the elution, the corresponding concentration (c) and calculated Raleigh factor (R_θ) gives the weight average molecular weight (M_w). For the low scattering angle (θ) used in the Chromatix KMX-6, we obtain $M_w = R_\theta/Kc$, where K is an optical constant. If axial dispersion can be neglected, the material eluting within each volume element is considered monodisperse, and the log $M - K_{av}$ relationships are then directly obtained. In addition, averages of the molecular weights (e.g. M_n, M_w, and M_z) for the whole sample may be directly computed.

The use of a light scattering detector also facilitated the determination of the void volume (V_0), which was taken as the volume where an abrupt increase in the scattering level was observed when using scleroglucan as test material.

RESULTS AND DISCUSSION

Particle analysis

Figure 1 shows scanning electron micrographs of particles I, II and III. The uniform size distribution and the perfect spherical shape are clearly seen. The particle diameter is 15μm for all types. It can be visually observed that II contain larger pores than I, and that III contain even larger pores. Figure 1 also gives the pore size distributions (measured by mercury porosimetry). The change of porogen from a branched alkanoic acid (I and II) to the linear type (III) has a marked effect on the pore size distribution. Even if the amount of porogen in the latter case is 70%, as in particle I, particle III

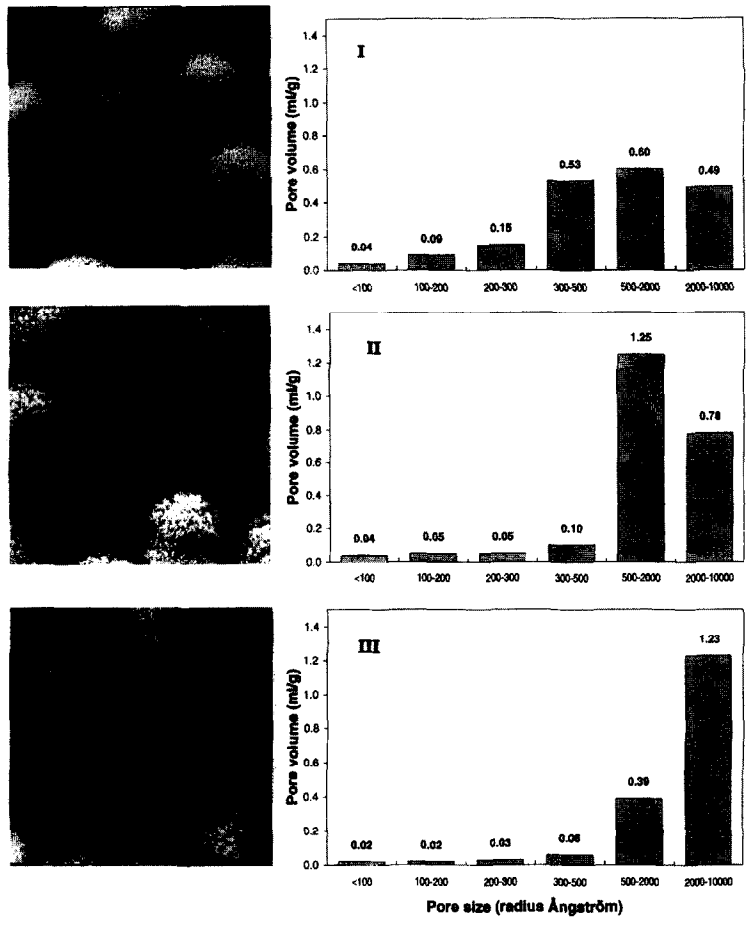

Fig. 1. Scanning electron micrographs and pore size distributions (determined by mercury porosimetry) of particles I, II and III. The diameter is 15 μm for all types.

contains more pores in the higher regions than particle II. For particle II, 89% of the pore volume consists of pores larger than 500Å. For particle III the value is 96%. Corresponding values for pores in the range 2000 to 10000Å are 35% and 70% for particles II and III, respectively. A comparison with nonhydrophilic particles (not shown) indicates that the hydrophilic layer does not affect the size of the larger pores. The pore sizes obtained for particles II and III suggest that SEC with very large molecules can be expected. The pore size distribution of TSK-GEL 6000 PWXL (not shown) was found to be intermediate between particles I and II.

Characteristics of packed columns

SEC columns (30cm) with particles I, II and III were packed. The number of theoretical plates (N) was 9900, 6600 and 6500, respectively, which is close to the value of 9000 obtained for the smaller particles (average particle diameter of 13 μm) of a commercial TSK-GEL G6000PWXL column (30cm). This shows that good packing of MPP could be obtained by simple packing methods. The pressure drop was approx. 3 bar at a flow-rate of 1.0ml/min.

Pullulans

In Fig. 2 are shown SEC chromatograms using columns I, II and III with pullulan standards with number average molecular weights (M_n) ranging from $0.137–2.61 \cdot 10^6$ (values obtained from supplier). The corresponding calibration curves (M versus elution volumes at the peak maxima) are given in Fig. 3, where in addition a comparison with TSK-GEL G6000PWXL is made. It should be noted that the two largest pullulan standards are polydisperse, giving rise to broader peaks, and the elution volume corresponding to the given average molecular weight cannot be assigned without considering the molecular weight distribution (Lecacheux *et al.*, 1982). However, the elution volumes corresponding to the peak maxima allow a consistent comparison between the different particle types provided that the standards do not elute too close to V_0 or V_t.

The plots in Fig. 3 are essentially parallel, but are shifted towards higher molecular weights when going from I to II and further to III. This shift is in qualitative agreement with the shift in pore size distribution towards larger pores (Fig. 1). Compared to TSK-GEL

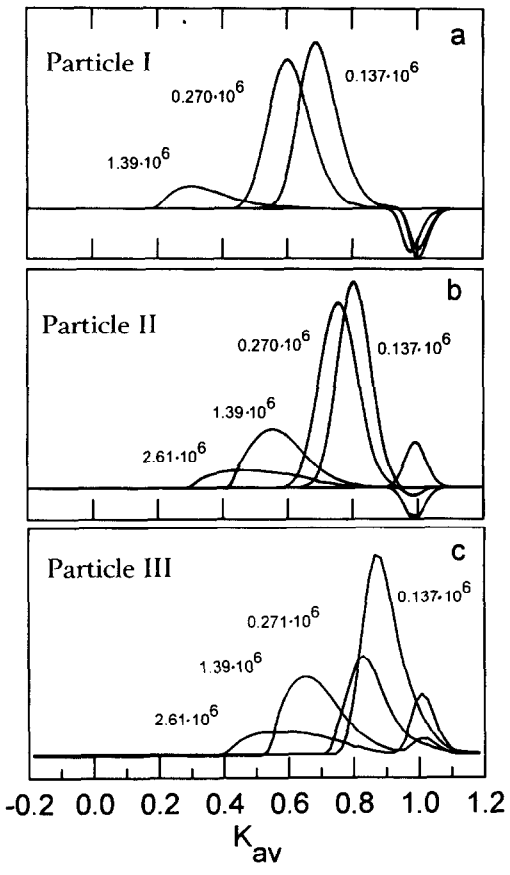

Fig. 2. Chromatograms obtained with pullulan standards using columns packed with particles I (a), II (b) and III (c). Molecular weights given in the figure are values obtained from the supplier.

G6000PWXL the effective separation range is shifted by a factor of 0.8, 2.9 and 6.0 in molecular weight (measured at $K_{av}=0.6$) for particles I, II and III, respectively. Due to the lack of available pullulan standards with molecular weights larger than $2.6\cdot10^6$, the full separation potential cannot be directly assessed in the high molecular weight region, which is of primary interest here. However, extrapolation of the data obtained for III to $K_{av}=0.2$ suggests that effective separation of pullulans, and hence other polysaccharides of similar chain flexibility and shape, can be expected for molecular weights up to ca. 10^8.

Chitosans

Chitosans are linear copolymers of *N*-acetyl-D-glucosamine and D-glucosamine linked by $\beta(1\rightarrow4)$ linkages. The fraction of *N*-acetylated units (F_A) may vary, depending on the degree of de-*N*-acetylation of the native chitin. Most chitosans are only soluble in acidic solutions when the free amino groups are positively charged. As polycations, chitosans are susceptible to adsorption onto media containing negatively charged groups such as carboxylate or sulphonate. Due to the

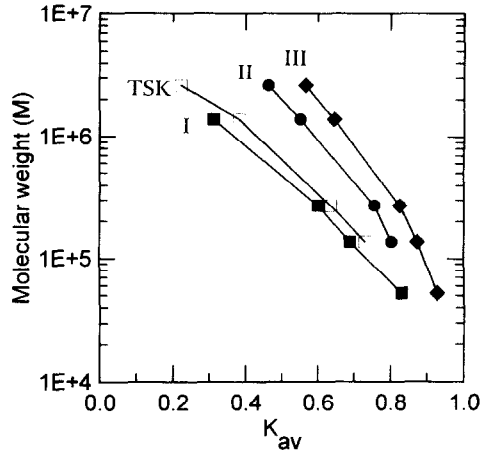

Fig. 3. Calibration curves of pullulan standards for columns packed with particles I (■), II (•), III (◆) and TSK-GEL G6000PWXL (□).

high acetyl content, hydrophobic interactions may also take place, as suggested by total adsorption to Sepharose columns (K.M. Vårum, personal communication). In addition chitosans may associate into larger aggregates, depending largely on the polymer concentration (Anthonsen *et al.*, 1994). Figure 4(a) shows the elution curves of two high molecular weight chitosans with F_A values of 0.20 and 0.52, respectively, using particle II. The elution was performed in a 0.2M ammonium acetate buffer at pH4.5. Both samples are well separated according to the molecular weight, even in the high molecular weight range. Linear plots of log *M* versus elution volume (Fig. 4(b)) were obtained for molecular weights ranging from $0.16\text{–}1.6\cdot10^6$. The linear range

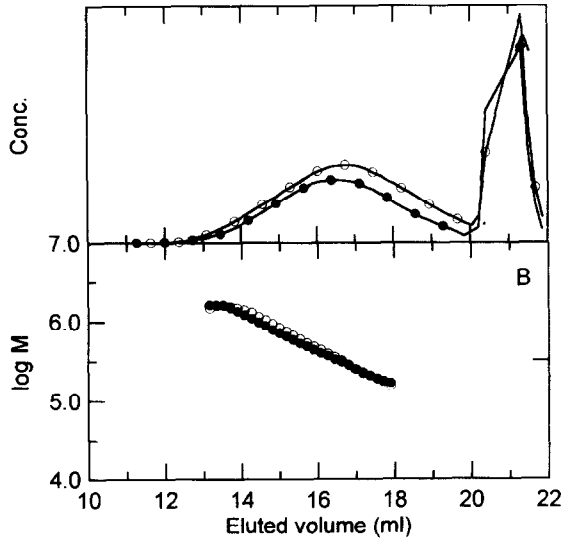

Fig. 4. Chromatograms (a) and calibration curves (b) obtained for a column with particle II for chitosans with varying degree of *N*-acetylation. $F_A=0.52$ (•) and $F_A=0.20$ (○). Symbols in (a) are inserted at every 12th data point. The *M* values were obtained by combining data from the light scattering and refractive index detectors (see Methods).

may well extend towards both higher and in particular lower molecular weights. This could not be assessed accurately in these experiments due to the low signal-to-noise ratio in the light scattering signal, particularly in the low molecular weight region. The sample recoveries were in both cases calculated to be approx. 90%.

Alginates

Alginates are linear copolymers of β-D-mannuronic acid (M) and α-L-guluronic acid (G), both linked in a (1→4) manner. Different alginates vary considerably in the relative content and distribution of the two monomers (Skjåk-Bræk *et al.*, 1986a, b), and the chain stiffness is in the range of chitosan and cellulose derivatives (Smidsrød & Christensen, 1991). As polyanions with pK_a of approx. 3.5, alginates are negatively charged under normal aqueous conditions. Some bacterial alginates have very high molecular weights and elute in the void volume of most commercial SEC media. Figure 5 shows the elution profiles and the corresponding plots of log M versus elution volume for particle II of two alginates, a typical commercial alginate (isolated from the kelp *Macrocystis pyrifera*) and a bacterial alginate. The figure clearly shows that the latter has a molecular weight distribution which is shifted towards higher values of M than the former, which is consistent with the differences in intrinsic viscosities (unpublished data). The log M–V curves in Fig. 6(b) appear to have quite different slopes. This is in part ascribed to differences in the chain flexibilities, which is largely determined by the content of alternating MG sequences (Stokke *et al.*, 1993a).

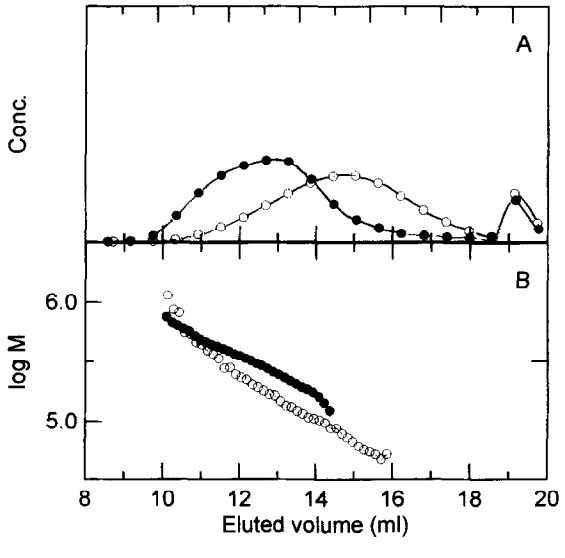

Fig. 5. Chromatograms (a) and calibration curves (b) obtained for a column with particle II for a bacterial alginate rich in β-D-mannuronic acid (●) and an algal alginate (○). The M values were obtained by combining data from the light scattering and refractive index detectors (see Methods).

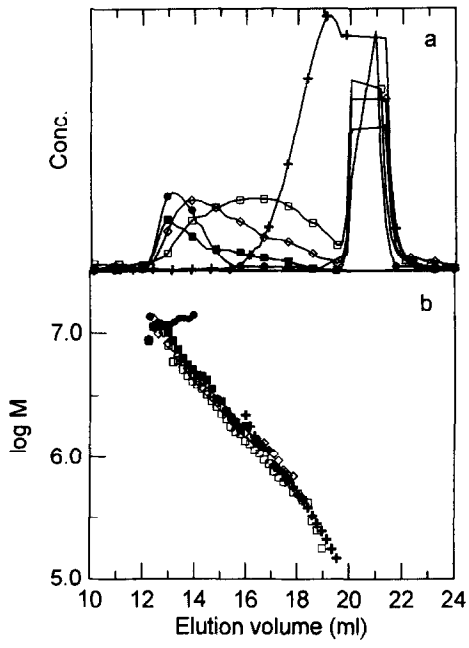

Fig. 6. Chromatograms (a) and the corresponding calibration curves (b) obtained with particle III for undegraded scleroglucan (●, $M_w = 11 \cdot 10^6$) and partially depolymerized scleroglucan with $M_w = 5.9 \cdot 10^6$ (■), $3.4 \cdot 10^6$ (◇), $2.0 \cdot 10^6$ (□) and $0.46 \cdot 10^6$ (+). The M values were obtained by combining data from the light scattering and refractive index detectors (see Methods). Symbols in (a) are inserted at every 15th data point.

Scleroglucan

Scleroglucan is a neutral β(1→3) linked D-glucan with a single D-glucose unit attached in position 6 of approximately every third residue in the main chain. Scleroglucans have M_w-values in the range $1–10 \cdot 10^6$. It has further an extended, triple-helical conformation giving rise to very large intrinsic viscosities ([η] = ca. 12000 ml/g for $M_w = 5.7 \cdot 10^6$) (Norisuye *et al.*, 1980) and the corresponding hydrodynamic volume is about 50 times larger than that of the largest pullulan standard. In electron micrographs, scleroglucans appear as worm-like chains which become more rod-like at lower molecular weights (Stokke *et al.*, 1993b).

In addition to the native scleroglucan, partially depolymerized samples (Hjerde *et al.*, 1994) were analysed on a column with particle III. The M_w-values of the 5 samples investigated were $11 \cdot 10^6$, $5.9 \cdot 10^6$, $3.4 \cdot 10^6$, $2.0 \cdot 10^6$ and $0.46 \cdot 10^6$, respectively. The corresponding elution curves and plots of log M versus elution volume are shown in Fig. 6. Undegraded scleroglucan ($M_w = 11 \cdot 10^6$) eluted mainly in the void volume, and the on-line calculated values of M were independent of the elution volume. However, the sample with $M_w = 5.9 \cdot 10^6$ was well separated, as demonstrated by the linear decrease in log M with increasing elution volume for the entire sample. This is further verified by the lower M_w samples, which all gave calibration plots which overlapped and extended that of the $5.9 \cdot 10^6$

sample. In total, the column with particle III demonstrates good separation according to M in the range from about 10^5 to 10^7. This is a significant improvement compared to what may be obtained with e.g. TSK-GEL G6000PWXL.

CONCLUSIONS

The results presented here with particle types I, II and III demonstrate that the range of molecular sizes accessable for aqueous SEC has been extended towards larger values compared to existing SEC media. The high molecular weight limit obtained with particle III was estimated to ca. 10^8 for pullulans, a family of coil-like polysaccharides, whereas the limit for the much more rigid and extended scleroglucans was ca. 10^7. In addition, essential chromatographic properties such as uniform packing combined with low pressure drop are obtained. A major advantage is the use of a very stable and inert particle material, i.e. highly cross-linked poly(styrene-*co*-divinylbenzene). It is likely that even more porous MPP of this type can be produced with the current method, and work along these lines will continue in our laboratories. Likewise, the corresponding non-hydrophilic particles should give good separation in the high molecular weight range for synthetic polymers in organic solvents. The simple handling and packing of these particles provides an excellent basis for preparative SEC using larger columns.

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