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Characterization of dextrans by size-exclusion chromatography on unmodified silica gel columns, with light-scattering detection, and capillary electrophoresis with laser-induced fluorescence detection

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Abstract

Unmodified silica gel size-exclusion columns were used in an on-line combination with light-scattering detection for a size characterization of dextrans. The influence of electrostatic interactions on analyte retention was briefly investigated. Size-exclusion chromatography was also used for evaluation of the fluorescence labeling procedure for dextrans with 8-aminonaphthalene-1,3,6-trisulfonic acid. The derivatives obtained through this procedure were used for electrophoretic measurements with laser-induced fluorescence detection. A comparison between the size-exclusion data and capillary electrophoresis indicates that the effectiveness of fluorescent labeling decreases with molecular mass of the dextran analytes.
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1. Introduction

Characterization of water-soluble polymers in complex mixtures has become of importance in a number of scientific and technological disciplines. Traditionally, size-exclusion chromatography (SEC) has been applied to separate various mixtures of proteins, nucleic acids and polysaccharides. Although retention volumes correlate to some degree with the molecular sizes of biopolymers undergoing separation by SEC, there is a need to determine accurately the molecular masses, or the degree of polymerization, for such separated fractions. The recent advances in the on-line combination of SEC with light-scattering (LS) detection have made this task possible in a number of situations [1], and additional techniques such as capillary electrophoresis (CE) and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) are also potentially beneficial in assessing certain structural features of oligomeric mixtures.

The high-performance versions of SEC have improved the scope of polymer analysis in terms of speed and resolution. Both the semi-rigid organic gels and modified silica gel particles are applicable to the high-performance mode. The major criteria for their utility are: (a) sufficient stability under the operating temperatures and pressures; (b) availability

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of particle size in the range of 5–10 μm; (c) a complete surface wettability by the mobile phase, with minimum swelling effects and (d) availability in the adequate pore-size range.

Although some polymer gel packings are viewed as preferable for the separations of polysaccharides [2], some interesting results have also been achieved with silica-based SEC packings [3–8]. Water, aqueous buffers and mixed solvents (e.g., water–dimethyl sulfoxide) appear to be popular mobile phases in the SEC analysis of polysaccharides.

Natural or chemically modified polysaccharides can occur as electrostatically neutral (e.g., dextrans and dextrins), negatively charged (e.g., heparins, hyaluronic acid or polygalacturonic acid), or positively charged (e.g., aminodextrans or chitosans) molecules. The silica packings tend to be negatively charged due to the presence of residual, weakly acidic silanol groups on their surface [9–12]. Negatively charged solutes can be repelled from the surface of a packing and, short of additional matrix effects, they are eluted earlier than expected on the basis of sieving alone. The positively charged polymers are attracted by the packing surface and eluted later than expected. The electrostatic interactions can be suppressed through an increased ionic strength of the mobile phase [13,14], although higher ionic strengths tend to promote hydrophobic interactions on the organically modified silica materials. The hydrophobic adsorption of analytes can be reduced by adding organic solvents [15]. Dextrans (uncharged polysaccharides) have often been used in characterization of the SEC processes [16–22].

Capillary electrophoresis (CE) has now been increasingly explored in the separation of complex polysaccharide mixtures because of its extraordinarily high resolving power [23–26]. The requirement for CE with neutral polysaccharides has been the use of Tris–borate buffers [25,26] to impart a charge on the sugar molecules. In addition, attaching a chromophore, or preferably a fluorophore (for reviews, see Refs. [23,24]), to the oligosaccharides' end-group is essential to detection in CE.

Since SEC can potentially be employed as an effective fractionation tool (prior to CE) in polysaccharide analysis, it has been important to assess the value of both separation techniques in the accurate representation of polymeric mixtures. As a model system, we have chosen here to analyze different dextran mixtures, using both the native polysaccharides and those labeled with 8-aminonaphthalene-1,3,6-trisulfonate (ANTS) [26,27].

2. Experimental

The chromatographic system used in our SEC–LS detection experiments was a laboratory-built setup (Fig. 1) consisting of the following main components: a Shimadzu LC-5A pump (used in the isocratic mode) with Shodex degas system (Model KT-27, Showa Denko KKI, Japan) and a Shodex pulse dampener; a Rheodyne (Cotati, CA, USA) injection valve with a 20-μl loop; a Bio-Rad SEC column with an appropriate (see below) working range; and three different detectors connected in series: a Model LC 240 fluorescence detector (Perkin-Elmer, Norwalk, CT, USA), Mini Dawn light-scattering detector (Wyatt Technology, Santa Barbara, CA, USA) and a Model 410 differential refractometer (Waters, Milford, MA, USA). While the response values of the differential refractometer and the LS detector were utilized in assessing solutes' molecular masses, the fluorescence detector was needed to monitor the ANTS-labeled dextran fractions.

Three unmodified silica columns (all produced by the Bio-Rad Labs., Hercules, CA, USA) were tested for the retention of polysaccharides: Bio-Sil SEC-125, Bio-Sil-250 and Bio-Sil-400. All columns were 300×7.8 mm I.D., packed with 5-μm particles. The chromatographic experiments were carried out at room temperature, using three different mobile phases: distilled water; 0.0125 *M* phosphate buffer (pH 6.8) and 0.05 *M* phosphate buffer (pH 6.8). The flow-rate of the degassed mobile phase was maintained at 1.0 ml/min. The saccharide samples were

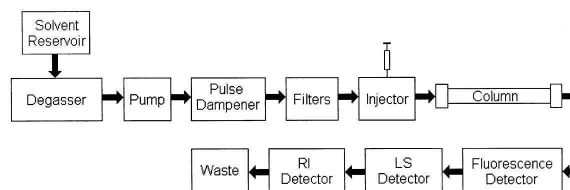

Fig. 1. Schematic representation of the experimental setup.

introduced onto the column at 0.5–10 mg/ml concentrations.

The laboratory-built CE instrument with laser-induced fluorescence (LIF) detection was described elsewhere [28]. A high-voltage power supply (Spellman High Voltage Electronics, Plainview, NY, USA) was operated at 20 kV. The on-column fluorescence excitation (325 nm) was provided by a Model 56X helium-cadmium laser (Omnichrome, Chino, CA, USA), while fluorescence emission was measured at 514 nm. The separation capillary [50 cm (35 cm effective length)×50 μm I.D.] originated from Polymicro Technologies (Phoenix, AZ, USA). The inner surface of the capillary was modified by the attachment of linear polyacrylamide [29]. The fluorescent labeling procedure for carbohydrates was described earlier [25,26]. 0.1 *M* Tris–borate buffer (pH 8.5) was used as the electrolyte.

All common saccharides and the dextran samples used in this study (weight-averaged molecular masses of 6000; 8800; 18 300; 39 000; 71 000; 135 000; 267 000; 526 000 and 2 000 000) were purchased from Sigma (St. Louis, MO, USA). 8-Aminonaphthalene-1,3,6-trisulfonic acid, disodium salt (ANTS) was received from Molecular Probes, (Eugene, OR, USA). All common chemicals for the preparation of buffer solutions were from Sigma.

3. Results and discussion

The solute retention values in SEC can be expressed as K_{SEC}, further described as

$$K_{SEC} = \frac{V_e - V_0}{V_t - V_0} \quad (1)$$

where V_e is the solute elution volume, and V_0 is the interstitial (void) volume, characterizing elution of the solutes with molecular diameters larger than the most accessible packing pores. V_t is the total volume describing elution of the low-molecular-mass solutes. The data describing the three columns used in this work are summarized in Table 1.

In the absence of additional (i.e., non-sieving) retention mechanisms, the calibration curves appear with the well-known S-shaped characteristics containing a linear part. The slope of the linear part is an indication of the homogeneity of the pore structure. Three calibration curves (Fig. 2) were measured (without LS detection) with 0.05 *M* phosphate buffer as the mobile phase and the manufacturer's data [30] were used for their construction. The elution volumes were taken at the bisectrix of the peak area [7], with the exception of the largest dextrans that were considered as partially excluded. For the larger polysaccharides, the elution volumes were taken at the peak maximum. This is likely to be the reason for observed deviations of the M_w 526 000 dextran data on the Bio-Sil SEC-400 column. It follows from the slopes of the measured calibration curves that Bio-Sil SEC-125 and SEC-250 columns have a narrower pore-size distribution than Bio-Sil 400 and, correspondingly, a greater value in resolving (in their respective size-exclusion range) the solutes with similar molecular volumes. Conversely, the Bio-Sil SEC-400 column can separate a broader range of

Table 1
Comparison of interstitial (V_0) and total (V_T) volumes for all columns used

Column	V_0 (ml)	V_T (ml)
SEC-120	4.85	9.75
SEC-250	5.10	10.75
SEC-400	4.80	11.20

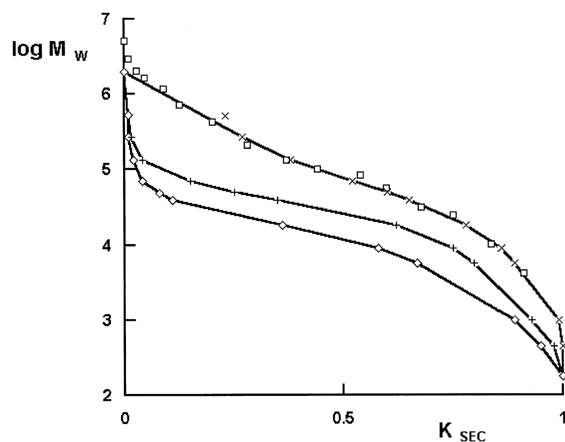

Fig. 2. Calibration curves for the three columns used. Eluent, 0.05 *M* phosphate buffer; calibrants: polydisperse dextrans. (◇) Bio-Sil SEC-125, (+) Bio-Sil SEC-250, (×) Bio-Sil SEC-400 (the three calibration curves are based on the manufacturer's data [30]); (□) Bio-Sil SEC-400 (based on the LS data). For details, see Section 2.

high-molecular-mass dextrans. It appears that Bio-Sil SEC-125 can be used for the range of dextrans from 500 to 70 000, Bio-Sil SEC-250 for 1000 to 100 000, and Bio-Sil SEC-400 from 3000 to 1 000 000 (Fig. 2).

Because our interests were in separation of the higher-molecular-mass polysaccharides, we concentrated on the characterization of the Bio-Sil SEC-400 column using LS detection. We found a good agreement between the manufacturer's data and our LS detection experiments within the linear part of the calibration curve (Fig. 2). Slight deviations were observed at the lower-molecular-mass end, and somewhat larger differences at the higher-molecular-mass end of the calibration curve. The latter deviations are likely to be a consequence of the secondary elution effects caused by hydrodynamic interactions of the excluded macromolecules with the flowing mobile phase that are confined to the relatively narrow channels, formed within the monodisperse and small packing particles [7,31–34].

Using the LS detector, it is possible to use the Bio-Sil SEC-400 column for dextran characterization up to molecular mass 5 000 000, although selectivity in the non-linear part of the curve will naturally be lowered. Molecular masses of the dextrans used and their distributions measured on the Bio-Sil 400 column, using 0.05 *M* phosphate mobile phase, are summarized in Table 2. The results agree with the manufacturer's data [30].

As stated above, unmodified silica materials feature weakly acidic silanol groups that give the packings a negative surface charge. This charge influences the retention of charged solutes. However, the effect can be minimized through an increase in the mobile phase ionic strength. Such electrostatic properties of our columns were demonstrated with the use of small, charged saccharides. At a low ionic strength (0.0125 *M* phosphate buffer), the negatively charged solutes (glucuronic or galacturonic acid) were eluted before the neutral solutes (glucose and galactose), while the cationic sugars (glucosamine or galactosamine) were retained longest. At a higher ionic strength (0.05 *M* phosphate buffer), all solutes were eluted at the same time.

In agreement with the previous studies [4–6,8], we found a slight influence of ionic strength on retention of dextrans (Fig. 3). Certain small peaks were observed at low retention with water as the mobile phase, but they were eliminated in 0.05 *M* phosphate (Fig. 4). It appears that these minor peaks were caused by the repulsive electrostatic interactions between the negatively charged packing and the dextran derivatives containing a few negative charges [13]. We have used here the dextran with average molecular mass of 18 300 as an example,

Table 2
Characterization of molecular mass distribution of dextrans by SEC–LS

Dextran	M_n	M_w	M_z	Polydispersity $\sigma_1 = \frac{M_w}{M_n}$	Polydispersity $\sigma_2 = \frac{M_z}{M_n}$	$M_w^{PeakMax}$
D-6K Sigma 6000	4510	6236	10 600	1.38	2.35	5303
D-8K Sigma 8800	6567	8600	15 290	1.31	2.32	6245
D-18K Sigma 18300	15 210	17 540	34 390	1.15	2.26	14 660
D-39K Sigma 39000	22 740	39 490	64 940	1.74	2.86	26 390
D-71K Sigma 71000	62 860	75 890	96 420	1.21	1.53	50 030
D-Q1M Sigma 135000	69 340	152 500	321 800	2.20	4.64	86 150
D-Q2M Sigma 267000	123 600	260 900	459 500	2.11	3.72	239 800
D-Q5M Sigma 526000	369 200	602 500	1 024 000	1.63	2.77	373 500
D-2M Sigma 2000000	669 800	2 181 000	4 584 000	3.12	6.55	589 000

Number-average molecular mass: $M_n = \sum_i n_i M_i / \sum_i n_i$, where n_i represents a number of polymer chains with mass M_i.

Weight-average molecular mass: $M_w = \sum_i n_i M_i^2 / \sum_i n_i M_i$.

Z-average molecular mass: $M_z = \sum_i n_i M_i^3 / \sum_i n_i M_i^2$.

$M_w^{PeakMax}$ is the weight-average of the molecular masses measured at the maxima of chromatographic peaks.

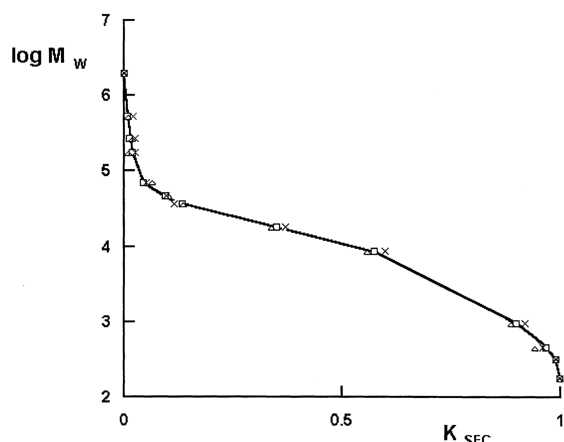

Fig. 3. Influence of the mobile phase ionic strength on retention of dextrans. Bio-Sil SEC-125 data: (△) water; (□) 0.125 *M* phosphate buffer; (×) 0.05 *M* phosphate buffer.

but a similar phenomenon was observed with all dextran samples.

Highly efficient separations of various dextran oligomers by CE were previously reported [23–25]. Defined dextran fractions can now be used as electromigration standards in CE of glycoconjugates. For the sake of detection and quantification, the oligosaccharides are often labeled at their reducing end by a suitable fluorophore. One of the more popular fluorescent tagging reagents, ANTS, also introduces into the derivatized saccharide molecules three negative charges, which has a visible effect [25] on the migration of dextrans, or other uncharged oligosaccharides.

ANTS labeling was also expected to have a measurable effect in SEC of dextrans on silica materials. As the labeled and unlabeled molecules exhibit different retention, their resolution can be utilized in investigations of the quantitative aspects of the ANTS labeling procedure. The dextran preparation of M_w 18 300 (same as in Fig. 4) was used for this investigation. The elution profiles of the reaction mixture in water and the phosphate mobile phases are compared in Fig. 5.

Three peaks were recorded (Fig. 5) using the differential refractometer and one peak using the fluorescence detector when water was used as the mobile phase. The first fraction contains all fluorescent compounds which are excluded because of their negative charge. The second small peak corresponds to the negatively charged portion of the original dextran fraction, and the third peak accounts for the remainder of unlabeled dextrans. The presence and distribution of the labeled (negatively charged) dextrans was confirmed through electrophoresis (Fig. 6).

With 0.05 *M* phosphate buffer as the mobile phase, one positive peak (corresponding to both labeled and unlabeled dextrans due to the suppression of electrostatic interactions) and one negative peak (corresponding to the refractive index differ-

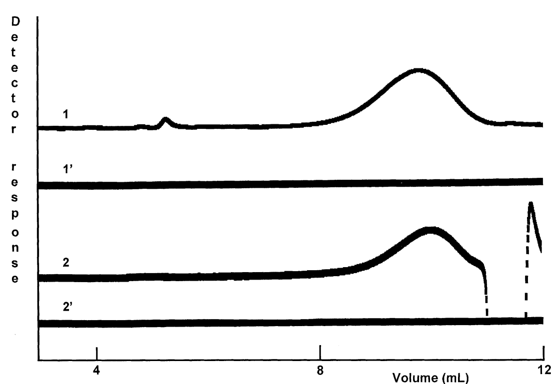

Fig. 4. Comparison of the chromatograms from M_w 18 300 dextran obtained with water and 0.05 *M* phosphate buffer on the Bio-Sil SEC-400 column with differential refractometer (DR) and fluorescence detection (FD) (λ_{ex} = 360 nm, λ_{em} = 514 nm). Curves: (1) water, DR; (1′) water, FD; (2) 0.05 *M* phosphate buffer, DR; (2′) 0.05 *M* phosphate buffer, FD.

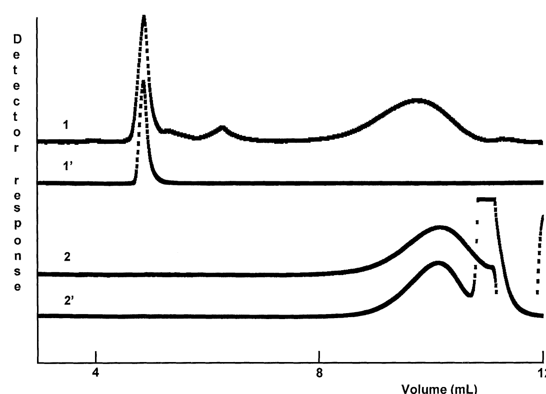

Fig. 5. Comparison of chromatograms from the ANTS-labeled M_w 18 300 dextran obtained with water and 0.05 *M* phosphate buffer on the Bio-Sil SEC-400 column with differential refractometer and fluorescence detection. Experimental conditions and designation of the curves are the same as in Fig. 4.

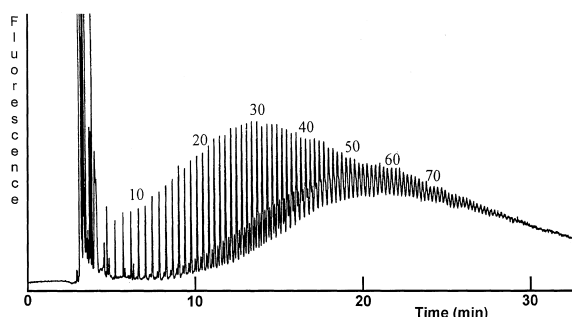

Fig. 6. Electrophoretic separation of the ANTS-labeled M_w 18 300 dextran.

ence between 0.05 *M* phosphate buffer and the low-molecular-mass components of the labeling mixture) were recorded with the differential refractometer. The fluorescence detection also produced two peaks: the first fraction, corresponding to the fluorescently labeled dextrans (still, mixed with the unlabeled components), and the second peak due to the low-molecular-mass by-products of the labeling reaction mixture.

Unfortunately, our results cannot be utilized directly to calculate accurately the yield of the ANTS labeling procedure. However, a reasonable estimate can be made on the basis of masses calculated from the LS data, with two assumptions: (a) labeled and unlabeled dextrans have a similar change of refractive index with concentration; and (b) contribution of the low-molecular-mass, fluorescent labeling by-products is small. From the data analysis of three independent labeling experiments, we estimate that only 30–50% of the original dextran sample has been labeled with ANTS.

Comparison of the calculated weight-averaged molecular masses (M_w) of the original sample and the remainder, unlabeled dextran fraction (after reaction with ANTS) shows that there may be a relation between the sizes of dextran molecules and the extent of labeling. We found that the weight-averaged molecular mass of the original sample measured by LS detection was lower ($M_w = 17\,540$) than the one corresponding to the remainder of unlabeled dextrans after the reaction with ANTS ($M_w = 23\,650$). It thus appears that the smaller dextrans have been labeled more effectively than the large ones. Whether a lesser accessibility of the molecules' reducing end to ANTS (due to a higher-order structure) or some kinetic reasons are responsible for this remains unclear.

As demonstrated in Fig. 6, the M_w 18 300 dextran preparation can be resolved into roughly 90 oligomers, which is comparable to the previously reported results [25], while the small satellite peaks are presumably due to the branched isomers. However, the molecular mass of the highest-resolved oligomer is M_w 14 598, which is lower than the M_w given by the manufacturer and the data obtained in our SEC–LS measurements. In order to obtain additional information on the dextran profile analysis through CE–LIF detection, we calculated the molecular masses for the samples of M_w 6000 and 8800 (Fig. 7). These dextran preparations were chosen because all observable oligomers could be fully resolved on CE. (The larger dextrans were usually resolved only up to 90-mer).

During the analysis of larger dextran ($M_w >$

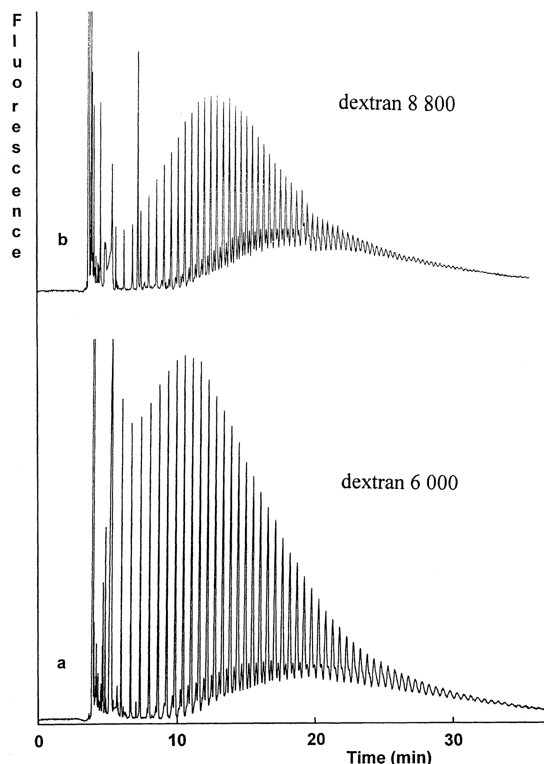

Fig. 7. Electrophoretic separations of the ANTS-labeled dextrans (M_w 8800 and 6000).

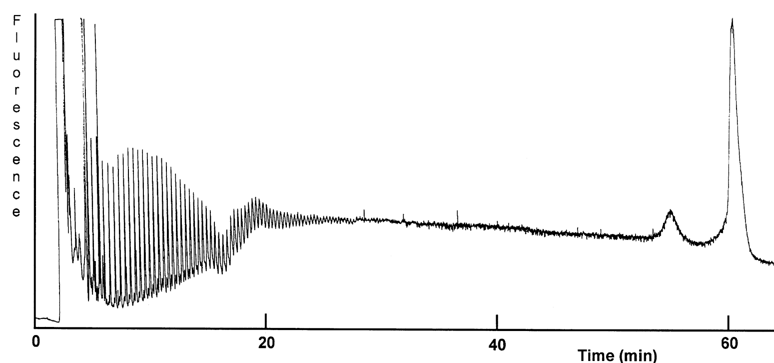

Fig. 8. Electrophoretic separation of the ANTS-labeled M_w 135 000 dextran.

50 000) fractions by CE, we have regularly observed late-migrating large peaks (or ''humps'') – for illustration, see Fig. 8. While the origin of these fluorescent signals is currently unclear, some form of molecular entanglement of the previously labeled component appears likely. Large polysaccharides, and particularly the random-coil entities such as dextrans, are known [35] to form aggregates quite readily.

The molecular-mass calculations from the CE–LIF detection data have been based on the assumption that the signals originating from all oligomer molecules are the same because there is only one reducing end in an oligomer molecule. While the peak heights have been used in such calculations, the molecular mass, M_{CE}, determined by this procedure corresponds to the number-averaged molecular mass (M_n). It means that M_{CE} should be compared to M_n (from SEC–LS measurements) rather than the manufacturer's data corresponding to M_w. Nevertheless, calculated values of M_{CE} for two dextran fractions were significantly lower than corresponding M_n (for 6000 dextran: $M_{CE}=2500$, $M_n=4510$; for 8800 dextran: $M_{CE}=3719$, $M_n=6567$). This supports the notion that the yield of labeling reaction is not the same for the shorter and larger oligomers.

The difference between the SEC–LS and CE–LIF detection data can be also increased by some other effects, e.g., light-scattering of the emitted fluorescent light due to larger dextran oligomers, or a partial quenching of the fluorescent signals inside the dextran molecules [36]. Similarly, a dramatically lower apparent concentration of the large polygalacturonic acid oligomers determined by CE–LIF detection, in comparison with HPLC, has recently been reported by Mort and Chen [37].

Although the CE–LIF detection combination provides a powerful tool in the analysis of glycans due to its unprecedented resolving power and detection sensitivity, the analytical results obtained from samples with a broad molecular-mass distribution should be interpreted with caution. A search for more quantitative labeling procedures and a calibration technique appears in order.

4. Conclusions

The unmodified SEC silica columns can be successfully utilized in the size determinations of dextrans (and, potentially, other polysaccharides) provided that the electrostatic interactions are suppressed through an increased ionic strength of the mobile phase. The rigid silica packings have the distinct advantage of working at relatively high flowrates (~1 ml/min), with the elution times shorter than 10 min, as often needed in the industrial analysis. When electrostatic interactions are enhanced, they can be utilized during the separations and characterization of charged and uncharged biological polysaccharides. The LS detection becomes a powerful and accurate adjunct to SEC.

The oligosaccharide analysis through CE–LIF detection brings an additional advantage of resolving some isomeric species [26], however, as indicated in this study, its utilization with samples of a broad molecular-mass distribution needs further evaluation.

Acknowledgements

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