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Journal of Chromatography A, 946 (2002) 69–81

JOURNAL OF CHROMATOGRAPHY A

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Molecular mass distribution analysis of ethyl(hydroxyethyl)cellulose by size-exclusion chromatography with dual light-scattering and refractometric detection

Bedrich Porsch[a,*], Mats Andersson[b], Bengt Wittgren[c], Karl-Gustav Wahlund[b]

[a]*Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, 162 06 Prague, Czech Republic*
[b]*Division of Technical Analytical Chemistry, Center for Chemistry and Chemical Engineering, Lund University, P.O. Box 124, S-221 00 Lund, Sweden*
[c]*Substance Analysis, Analytical Development, PAR&D, AstraZeneca R&D Mölndal, SE-431 83 Mölndal, Sweden*

Received 31 August 2001; received in revised form 26 November 2001; accepted 28 November 2001

Abstract

Dual low-angle light scattering and refractometric detection coupled to size-exclusion chromatography provided proof for the presence of a low amount of stable aggregates/particles in ethyl(hydroxyethyl)cellulose. Unlike the correct size-exclusion chromatographic behavior of the parent polysaccharide itself, the aggregates exhibit variable size-dependent weak retention as a function of flow-rate and of ionic strength of the aqueous mobile phase. Therefore, determination of the molecular mass of non-aggregated polymer is possible in aqueous mobile phase containing 0.1 *M* NaCl under conditions at which aggregates are completely adsorbed on the column packing irrespective of the flow-rate used. Flow-rate and ionic strength-dependent variations of aggregate behavior as well as model size-exclusion experiments with latex particles indicate that they partly carry a minute charge and have a compact structure. Their weak retention under the separation conditions used suggests a difference in their surface chemistry when compared with the dissolved polymer coils which exhibit a correct size-exclusion behavior.

Keywords: Detection, LC; Ethyl(hydroxyethyl)cellulose

1. Introduction

Ethyl(hydroxyethyl)cellulose (EHEC) belongs to the class of water-soluble cellulose derivatives with reduced solubility at elevated temperatures [1]. It is produced in large quantities, in general has a broad molecular mass distribution (MMD), and finds many industrial applications; among others, being an associative polymer, as a thickener. A delicate balance between interactions of hydrophobic (ethyl) and hydrophilic (hydroxyethyl) groups as well as hydration of its backbone determines its solution behavior. Its application behavior is frequently related not only to its average molecular mass (*M*) but also to the shape and width of its molecular mass distribution. In particular, differences in low- and high-*M* tails of MMD of different batches may be detrimental in specialized applications. The precise knowledge of MMD is thus highly desirable. The methods of choice to determine MMD of EHEC are size-exclu-

*Corresponding author. Tel.: +420-2-2040-3111; fax: +420-2-3535-7981.
E-mail address: porsch@imc.cas.cz (B. Porsch).

0021-9673/02/$ – see front matter
PII: S0021-9673(01)01572-2

sion chromatography (SEC) or field-flow fractionation (FFF). In SEC, three basic approaches are used, (i) calibration with narrow standards, (ii) universal calibration procedure (using calibration with narrow standards different from the polymer investigated and congruence of plots log [ηM] against elution volume for different polymers) and (iii) dual light scattering (LS)/concentration (RI) detection. The first approach was not further pursued, as no narrow calibration standards were available for the polymer under investigation. Universal calibration procedure fails with water-soluble polyelectrolytes [2] and is highly questionable also with many uncharged water-soluble polymers [3]. Hence, only the direct (calibration-less) method of *light scattering/concentration detection* remains and will be used in this paper.

Already in 1956 Manley [4] provided evidence for the presence of particles and/or aggregates in ethyl(hydroxyethyl)cellulose including a warning that this seems to be a general feature of all water-soluble cellulose derivatives [4]. Using dynamic light scattering (DLS), it was shown recently [5] that ethyl(hydroxyethyl)cellulose contains a low amount of two filterable (i.e. stable) particle/aggregate populations differing in hydrophobicity. A more or less general presence of stable particles/aggregates in water-soluble cellulose derivatives was conclusively proved by Burchard and co-workers [6–8]. The structure suggested by these authors, termed fringed micelles, consists of a microcrystalline core and a 'brush-like' corona of hydrophilic chains, which have been appropriately derivatized to ensure solubility in water. The general presence of such structures in water-soluble cellulose polymers is not surprising, because the first step in derivatization, i.e. solubilization in sodium hydroxide solution [1], as the generally applied technique, still preserves structural integrity of a very small amount of highly microcrystalline regions during subsequent derivatization [7]. Hence, MMD characterized by a long high-molecular mass tail, which however, is only relatively small with respect to its weight fraction should be frequently expected in MMD analysis of water-soluble cellulose derivatives. The compositional heterogeneity of EHEC as an inevitable result of modification chemistry [1] is especially relevant here. The zero content of ethyl or hydroxyethyl groups is the theoretical limit of heterogeneity, i.e. hydroxyethyl cellulose and ethyl cellulose, the latter being almost insoluble in water. Also, unreacted cellulose particles might be present and must be accepted as a sample component.

Light-scattering (LS) detection is considered as extremely useful to detect a minor population of aggregates in polymer samples. This is certainly an advantage for aggregating systems. On the other hand, already low amounts of impurities (especially dense, i.e. strongly scattering) of comparable and larger particle size than the dissolved polymer coil itself may often easily be misinterpreted as aggregates. The presence of some impurities in industrial water-soluble polymer samples must be taken into consideration, because it is well-known [9] that contaminants and other components in the atmosphere, e.g. hydrocarbons, compounds from tobacco smoke, etc., are enriched at the surface of the aqueous layer. This is valid for both polymer preparation (when large volumes of water are used as a solvent) and preparation of solutions prior to MMD measurement. The particle size of these micro droplets and particles may be as small as 50 nm and fairly extends above the μm size. Hence, filtration of samples through a 0.22 μm filter 'to remove dust' in most light-scattering studies may be insufficient in aqueous solutions for water-soluble polymers. This kind of impurity (micro droplets) was recently shown to be present even in high-quality poly(ethylene oxide) standard samples [10]. Therefore, a key issue is the correct differentiation between true aggregates (stable or dynamic) as a component of a dissolved polymer sample and additional impurity particles of an entirely different chemical composition possibly collected during preparation of the polymers which occur as apparent aggregates. Fortunately, the cellulose derivatization techniques [1] used to prepare EHEC and other water-soluble cellulose derivatives use neither large volumes of water as a dispersant nor any solution/precipitation steps. Hence, impurities like those found in poly(ethylene oxide) are not very likely here and enrichment of such impurities during preparation of EHEC solutions for SEC analysis can be easily reduced.

The problem of the presence of a minor amount of aggregates/impurities in basic research studies of polymer MMD focuses on their complete removal. The users of water-soluble polymers especially in the

medical field are in a more difficult situation. Impurities (pyrogens!) and aggregates of the polymer in question may sometimes considerably affect successful application. The same holds true for tablet-coating procedures. Correct analysis including the presence of a minor amount of particles/aggregates is of primary importance.

Various water-soluble derivatives were recently characterized by SEC with low-angle laser light-scattering (LALLS)/concentration detection using silica-based diol-modified SEC packings [11]. EHEC was eluted with a methanol–water (50:50) mobile phase and, no general evidence of the presence of stable aggregates/particles was found. If the above mentioned LS and DLS studies are to be considered as a conclusive proof for the presence of a minor particle/aggregate component in EHEC polymer, the active role of the used SEC column materials in complete adsorption would conveniently explain this apparent contradiction.

This paper reports on MMD of EHEC in aqueous mobile phases and addresses the following issues related to the presence of aggregates and/or impurities in this polymer: (i) explanation of a contradiction between molecular masses obtained using static light scattering and SEC observed earlier, (ii) possibility of determination of MMD of non-aggregated polymer, (iii) elucidation of possible SEC column activity affecting aggregate behavior when the experimental conditions are varied and (iv) evidence that particles/aggregates present in a minor mass amount are the sample component.

2. Experimental

2.1. Materials

EHEC was a sample EHM0 (Ethyl Hydroxy Modified 0, i.e. not hydrophobically modified). According to the manufacturer (Akzo-Nobel Surface Chemistry, Stenungsund, Sweden) the molecular mass is about 100–200 000 based on viscosimetric measurements. Model polymer lattices were dispersions of an ethyl acrylate copolymer with a low content of methacrylic acid crosslinked with *N*,*N*′-methylenebisacrylamide [12]. Two samples with hydrodynamic diameters $d_H = 52$ and 114 nm (determined by dynamic light scattering) were used.

2.2. Chromatography

The modular chromatograph consisted of a Degasser X-act (Your Research, Onsala, Sweden), a Constametric 3200 MS pump (Thermo Separation Products, Riviera Beach, FL, USA), a Pharmacia injection valve V-7 with 200-μl loop (Pharmacia & Upjohn, Uppsala, Sweden), a Chromatix KMX-6 LALLS detector (LDC/Milton Roy, Sunnyvale, CA, USA) and an R-401 differential refractometer (Waters, Milford, MA, USA) connected through a Black Star 2308 A/D converter (Black Star, Huntingdon, UK) to an IBM-compatible computer. The on-line refractive index (RI)-LALLS arrangement allows simultaneous determination of M and c at any elution volume ('slice'). The following relation is valid for Rayleigh scattering from a polydisperse polymer/solvent system at low angle (6–7°):

$$(K^*c)/R_\Theta = 1/M_w + 2A_2c \quad (1)$$

where c is the concentration of scattering species, R_Θ is the excess Rayleigh scattering factor, M_w is the weight-average molecular mass of scattering species and A_2 is the second virial coefficient. $K^* = (2\pi n^2/N_A\lambda^4)\nu^2$ where n is the refractive index of the solvent, λ is the wavelength in vacuo (633 nm), N_A is the Avogadro number and ν is the refractive index increment of the scattering species in the solvent used. In the case of using small slices, the polymer seen at a slice is assumed to be monodisperse. The angular dependence of the scattered light is neglected at the low angle used. Due to the fact that the sample is considerably diluted by polydispersity and/or column band broadening, the term A_2c may be neglected if the concentration of the injected solution is low enough and therefore, calibration is directly obtained by plotting log M vs. elution volume (V_e). Laboratory-written software (M. Netopilík, Institute of Macromolecular Chemistry, Prague, Czech Republic) allows on-line data accumulation and all calculations of molecular mass distributions and their averages.

Two stainless-steel columns (250×6 mm I.D., supplied by Tessek, Prague, Czech Republic) packed

with diol-modified LiChrospher 1000 and 4000 (Merck, Darmstadt, Germany) packings prepared according to a recently described procedure [13] connected in series were used for SEC of model latexes.

Three stainless-steel columns (250×8 mm) in series packed with hydrophilized GMB 200, 1000, 5000+ poly(glycidyl methacrylate) packings (Labio, Prague, Czech Republic) were used for SEC of EHEC.

A PTFE capillary (0.5 m×0.5 mm) was used to substitute SEC columns in flow-injection determinations of M_w of EHEC.

All solutions were prepared by mass. Water from a Millipore Milli-Q_{PLUS} ultrapure water purification unit (Millipore, Bedford, MA, USA) was used. Analytical reagent-grade NaCl and methanol were obtained from Merck and used without further purification. Millipore syringe filters Millex GV_{13} and HV_{13} (diameter 13 mm, 0.22 μm and 0.45 μm, respectively, hydrophilic Durapore membrane) and FH_{13} (diameter 13 mm, 0.5 μm, PTFE membrane), were used for sample filtration.

2.3. Asymmetrical flow FFF–MALLS

The asymmetrical flow field-flow fractionation system utilized a trapezoidal channel shape and was identical to that one described elsewhere [14]. The channel thickness w was 104 μm. The carrier solvent for FFF experiments was 10 m*M* NaCl. The EHEC sample had a concentration of 1 mg/ml and 100 μl, i.e. a total amount of 100 μg, were injected. The applied inlet flow-rate F_{in} was 0.8 ml/min which divides into the crossflow-rate F_c 0.21 ml/min and the outlet (detector) flow-rate F_{out} 0.59 ml/min. The hydrodynamic diameter d_H is obtained from the retention time t_r following the expression:

$$d_H = \frac{2kTV^0}{\pi\eta t^0 F_C w^2} \cdot t_r \quad (2)$$

where k is the Boltzmann constant, T the temperature in Kelvin, V^0 the channel volume, t^0 is the void time, and η the viscosity of the carrier liquid. For a more complete description of experimental procedures and details the reader is referred to other publications [14,15].

The multiangle light scattering (MALLS) photometer was a DAWN-DSP (Wyatt Technology, Santa Barbara, CA, USA) with a laser wavelength of 633 nm. The RI detector was an Optilab DSP (Wyatt Technology) also using a wavelength of 633 nm. Computer software, ASTRA for Windows 4.5 (Wyatt Technology), was used for data collection and calculations of the light scattering data. Molecular mass data were obtained from a linear Berry fit of the angular dependence of the scattered light.

3. Results and discussion

3.1. Separation with salt-containing eluent systems

A typical SEC separation of the EHM0 sample (0.1% solution) at a flow-rate 0.11 ml/min in 0.1 *M* aqueous NaCl is presented in Fig. 1. Another experiment at 0.45 ml/min was made to verify the absence of shear degradation of this polymer at higher flow-rate. The same behavior as in Fig. 1 was observed with no change in the noise level of LS signal at both flow-rates. The overlay of log $M = f(V_e)$ curves at 0.11 and 0.45 ml/min in Fig. 2 confirms the absence of degradation as both curves match quite well. Accordingly, as follows from Fig. 3, a fair agreement of MMDs determined at both flow-rates is found. An inspection of Fig. 1 reveals no indication of the presence of large particles/aggregates (as spikes and/or increased noise of the LS trace). However,

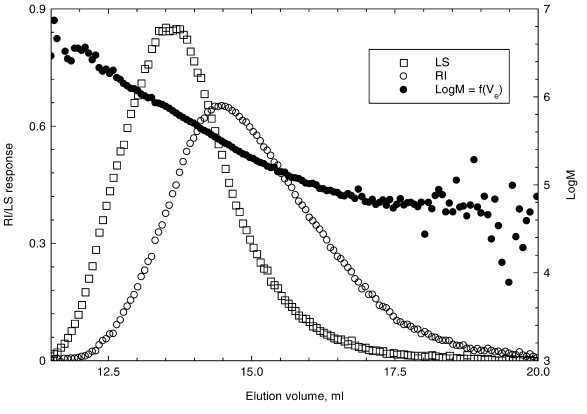

Fig. 1. MMD determination of EHM0 by SEC–RI–LALLS in 0.1 *M* NaCl at flow-rate of 0.11 ml/min.

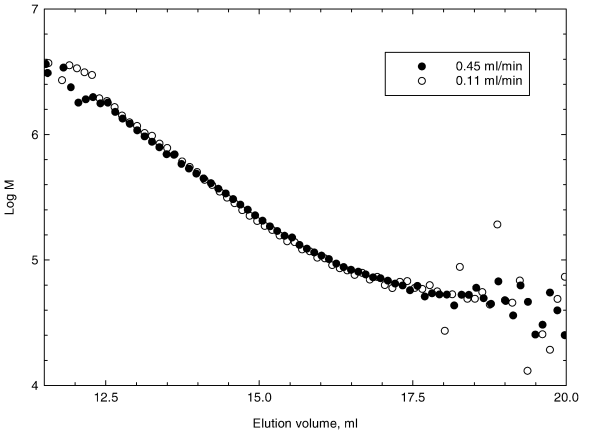

Fig. 2. Comparison of EHM0 calibrations log $M = f(V_e)$ obtained from SEC–RI–LALLS at 0.11 ml/min and 0.45 ml/min in 0.1 *M* NaCl.

such particles were clearly detectable in a DLS study [5] of other EHEC polymers at an angle of 90° and their LS signal, if present, should be more pronounced at an angle of 6–7° used in LALLS detection. To verify the possibility that the aggregates are fully retained by the column set used at a high salt content of the mobile phase, the analysis was repeated using 0.01 *M* NaCl at both flow-rates. No difference in the chromatographic pattern was observed at a flow-rate 0.45 ml/min and the experiment was fully comparable with the behavior in 0.1 *M* NaCl except for a lower polydispersity index

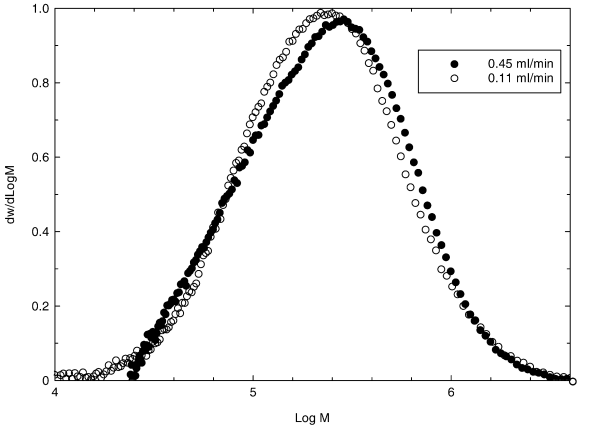

Fig. 3. Comparison of EHM0 molecular mass distributions obtained from SEC–RI–LALLS at 0.11 ml/min and 0.45 ml/min in 0.1 *M* NaCl.

Table 1
Summary of molecular mass data of EHM0 based on SEC measurements

Mobile phase	Flow-rate (ml/min)	M_w	M_n	M_w/M_n
0.1 *M* NaCl	0.45	352 400	156 800	2.3
0.1 *M* NaCl	0.11	356 900	141 700	2.5
0.01 *M* NaCl	0.45	353 400	175 900	2.0
0.01 *M* NaCl	0.11	301 200	108 200	2.8
Water	0.11	482 000	–	–

(Table 1), indicating a retention of aggregates also in 0.01 *M* NaCl at 0.45 ml/min.

A surprisingly different LS behavior was observed in 0.01 *M* NaCl at flow-rate 0.11 ml/min. After a set of previous SEC experiments at 0.45 ml/min (LS peak in Fig. 4A as an example, no baseline noise) and after change of flow-rate from 0.45 ml/min to 0.11 ml/min, an immediate 'dusting' was seen. The LS signal increased above zero (baseline was normally set to −350) with many spikes making an immediate injection impossible. The average LS signal still decreased after 110 min of rinsing when an injection of the same EHEC solution as that one used at 0.45 ml/min was tried (Fig. 4B). To obtain still noisy but a constant baseline like in Fig. 4D where another injection of the same solution was made, columns had to be rinsed longer than 24 h.

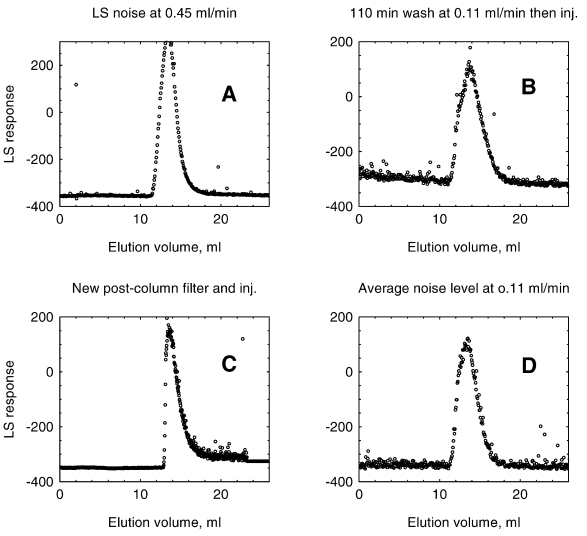

Fig. 4. LALLS noise behavior as a function of flow-rate, column rinsing time and choice of post-column filter.

These observations indicate that the baseline noise observed here reflects particles/aggregates in the sample retained within the columns when a high flow-rate is used. Replacement of the normally used post-column hydrophilic filter (0.45 μm HV_{13}) by a hydrophobic one (PTFE membrane) Millex FH_{13} immediately removed baseline noise (Fig. 4C baseline in front of the peak). This can be explained by adsorption of these particles on the filter membrane due to hydrophobic interaction. This observation agrees with the results of our recent DLS studies of EHEC [5] and poly(ethylene oxide) [10] where we have also seen removal of aggregates/particles dependent on the chemical properties of the filter material. The first injection of the same EHM0 solution using this new post column filter (Fig. 4C) shows adsorption of a small amount of sample (the front part of the peak is missing when compared with Fig. 4D) until the hydrophobic post column filter becomes saturated. Being saturated with adsorbed EHEC, the filter stops removing both particles/aggregates and EHEC coils. There seems to be a similarity in adsorption affinity to hydrophobic surface of both aggregates and EHEC. Then, a sudden appearance of particle noise appears—both EHEC and aggregates contained in the sample become visible. An increased, apparently constant noisy baseline (experiment stopped at 21.5 ml) again supports the assumption that aggregates are not eluted in the SEC elution mode. Fig. 4D then shows the next EHM0 injection under the apparent constant baseline condition. The baseline increase might contribute to a decrease in M in this case (see Table 1). To check the size range of particles responsible for the observed baseline noise, the normally used post-column hydrophilic HV_{13} filter (0.45 μm pore size) was substituted by a filter GV_{13} differing from the previous one only in the pore size (0.22 μm). A significant reduction of baseline noise was immediately perceptible confirming the presence of stable aggregates in the size range 0.22–0.45 μm. When the 0.22 μm filter was tested as a sample filter, its clogging after filtration of several ml of EHEC solution (0.1%) was observed, indicating that filterable aggregates, present in low amount not detectable by RI, belong to the sample. Therefore, all experiments were made using 0.45 μm pore size of hydrophilic filters to avoid a significant change of sample composition. It is worth noting that no increase of system pressure was observed using 0.45 μm HV_{13} post-column filter when multiple injections were made, indicating extremely low (if any) content of aggregates/particles larger than this filter size. Accordingly, sample recovery was very close to 100% in all experiments irrespective of the salt content in the mobile phase used.

Table 1 summarizes the results in terms of molecular mass averages and polydispersity indices M_w/M_n. A reasonable agreement is found with the exception of the experiment at 0.11 ml/min in 0.01 M NaCl. The values of M_w and M_n decreased in this case by 15 and 30%, respectively, which certainly exceeds the expected experimental error. The origin of this discrepancy is unclear at present; a possible explanation might consist in uncertainty of the selection of true LS baseline due to increased baseline noise (see above). A relevant conclusion here is that low salt content and flow-rate conditions should be avoided when analysis of non-aggregated EHEC is of interest.

A comparison of calculated log $M = f(V_e)$ calibrations obtained in 0.01 M NaCl at both flow-rates is presented in Fig. 5. A significant difference is observed here. It follows from Fig. 5 that an apparent increase in molecular mass at higher elution volumes is accompanied by a decrease in M at lower elution volumes at high flow-rate. In hitherto unpublished investigations of large coils (hyaluronic acid and

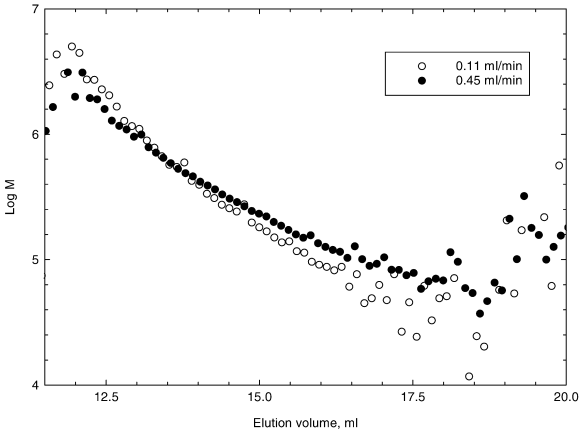

Fig. 5. Comparison of EHM0 calibrations log $M = f(V_e)$ obtained from SEC–RI–LALLS at 0.11 ml/min and 0.45 ml/min in 0.01 M NaCl.

poly(ethylene oxide)) having molecular mass of several millions we have observed the same effect using markedly different flow-rates. The condition of the equilibrium distribution of a solute between stationary solvent inside pores and interstitial mobile phase is substantially affected in the case of very high molecular mass coils having very low diffusion coefficients, in particular at higher flow-rates. The corresponding diffusion term in the Van Deemter equation increases with molecular mass, becomes more and more significant and the resulting non-equilibrium at higher flow-rate introduces an increased tailing, which is higher, the higher is the molecular mass. For this reason, such coils are eluted at higher elution volumes. For example, in the case of a polydisperse polymer with M in the interval 10^5–10^7, the coil size and thus its diffusion coefficient should roughly change by a factor of 10. This means that the effect of non-equilibrium diffusion may also increase about 10 times. This could explain data in Fig. 5 if M_w exceeds 10^6; however, it is difficult to get accustomed with the values in Table 1. Moreover, $\log M = f(V_e)$ curves match at high salt content (Fig. 2) and there is no reason to argue that a change in the salt content might decrease the van Deemter diffusion term.

An increase of LS noise at low salt concentrations and flow-rates can be regarded as indicative for the presence of strongly scattering particles. This observed spiking is only visible in the chromatogram when their number per scattering volume is low. Under the conditions used, the LALLS system was shown to be able to observe under optimum conditions compact dense individual particles as spikes if their diameter exceeds 50 nm [9]. It is worth noting that our LALLS unit at the setting used starts to detect compact particles (increased baseline noise as the onset of spiking) somewhere between 100 and 150 nm. Such spiking cannot be related to any size of dissolved EHEC coils having diameter below the pore size of the used sample and post-column filters. This follows from a simple comparison of the coil segment density (mass of the macromolecule/volume of the coil) with a particle of unit density having the same d_H. It is well known that typical coil densities of soluble cellulose derivatives are around 10^{-3} or less [6] and vary considerably among various polysaccharides [16]. Particle mass of a coil and of a dense sphere of the same diameter may thus differ by a factor of about 10^3. As scattering intensity is proportional to $c_i M_i$, the polymer coil is expected to scatter light to a lower degree when compared with a solid particle of the same hydrodynamic size. On the other hand, already an amount of only 0.1% of such dense particles/aggregates present in EHEC solution would increase intensity of scattered light by a factor of two. In fact, a purity of 99.9% is a very high value in the case of an industrial sample. Assuming a correct separation according to size and a very high M (i.e. low diffusion coefficient) of these aggregates, this would explain only a noisy LS peak and a slope change in Fig. 5. The apparent continuous noisy LS baseline, only observed under conditions of low salt concentrations and flow-rates, requires an assumption of the presence of such dense particles moving through the column by a different mechanism than separation with respect to size, i.e. separation is superimposed by a small contribution of adsorption. The observed baseline noise may be then related to previous sample injections.

3.2. Separation with pure water as the mobile phase

The experiments reported in Section 3.1 indicated that the retention of aggregates/particles presumably results from their weak adsorption dependent on salt concentration. Hence, it may be anticipated that such aggregates should be even more visible in the absence of salt in the mobile phase. At the same time, it follows from our previous work [5] that EHEC carries a minute negative charge and exhibits ion-exclusion in methanol–water (50:50) as a mobile phase. Assuming such a small charge to be present in the EHM0 sample, the charge repulsion in pure water should lead also to the ion-exclusion of particles/aggregates, provided they are charged. On the other hand, in a salt-containing eluent, formation of larger aggregates from primary particles present in the sample should take place due to hydrophobic interaction among them. This secondary aggregation should disappear in pure water in the case of charged particles. Fig. 6 illustrates that ion-exclusion behavior of the EHM0 sample in pure water takes place in a similar way to other polysaccharides. In agree-

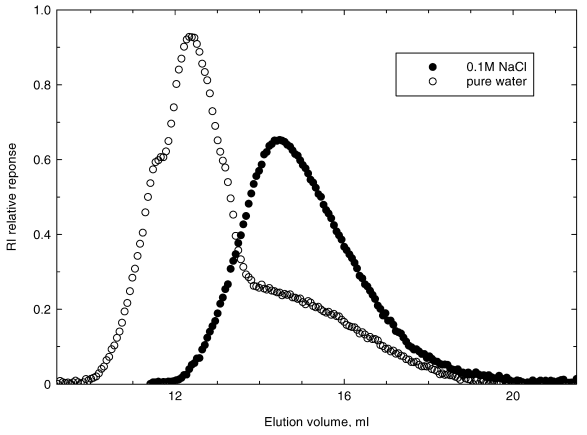

Fig. 6. Ion-exclusion of EHM0 by SEC in pure water and its suppression in 0.1 *M* NaCl at 0.11 ml/min.

ment with our previous observations [5], two partially resolved excluded peaks are observed, most probably related to EHEC macromolecules carrying one and two carboxyl groups (as a result of partial oxidation of EHEC end groups), similar to other mono and dicarboxy polysaccharide derivatives found earlier [17,18]. It follows from Fig. 6 that more than 50% (w/w) of the sample carries some charge.

Fig. 7 illustrates SEC–LALLS analysis of EHM0 sample in pure water. A frequent spiking of the LS trace is observed only near to the exclusion volume, indicating superposition of polymer signals and those attributable to the individual strongly scattering species. Such extensive spike phenomena have never been observed in salt-containing mobile phases at any elution volume. This observation may thus be indicative that this particle component is fully trapped in the columns when salt is added. Accordingly, $M_w = 482\,000$ was obtained using the data in Fig. 7 which is markedly higher than the value of 350 000 observed when a mobile phase containing 0.1 *M* NaCl was used. Contrary to experiments in 0.01 *M* NaCl no release of particles was observed from the columns after a change of flow-rate from 0.45 ml/min to 0.11 ml/min and LS traces at both flow-rates were identical. Log $M = f(V_e)$ calibrations obtained in pure water and in 0.1 *M* NaCl are compared in Fig. 8. A shift of the presumably charged macromolecules along the elution volume axis toward the exclusion limit without a marked increase in molecular mass is clearly visible. The increased scatter of points near to the column exclusion volume is attributable to an additional contribution of strongly scattering particles/aggregates presumably containing a negative charge, which are completely absent when 0.1 *M* aqueous NaCl was used as a mobile phase. We can thus conclude that at least negatively charged aggregates having size up to 0.45 μm (sample filter diameter) can be made visible in the absence of salt in the mobile phase. Nevertheless, positively or uncharged particles/aggregates similar in size and structure may be trapped in the columns also in pure water.

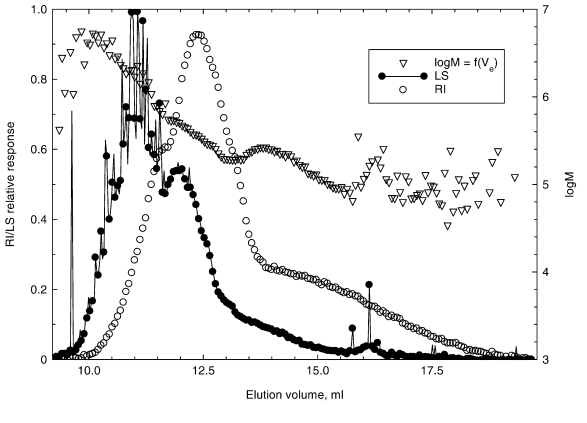

Fig. 7. SEC–LALLS analysis of EHM0 in pure water at 0.11 ml/min.

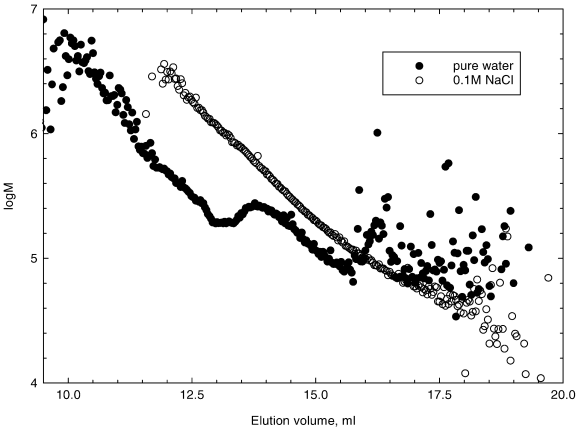

Fig. 8. Log $M = f(V_e)$ calibrations of EHM0 obtained by SEC–RI–LALLS in pure water and 0.1 *M* NaCl at 0.11 ml/min.

3.3. Flow-injection experiments

Replacement of the column set by a connecting capillary, the SEC system with dual LALLS–RI detection can be transformed to a flow-injection set-up [19] that allows to investigate the total sample content (polymer coils + aggregates) in terms of average molecular mass. M_w is defined as $\Sigma c_i M_i / \Sigma c_i$. The LS peak area is proportional to $\Sigma c_i M_i$ at low angles and concentrations used and the RI peak area is proportional to Σc_i. The M_w thus should be obtained by division of integrated LS and RI signals if proper instrumental constants are introduced. When a 0.45 μm sample filter is used, M_w values thus obtained should reflect the contribution of all particles smaller than the filter pore size, provided that negligible adsorption of the sample components takes place in the capillary and other system components. A short column (microporous and assumed to be inert to the polymer in question) is normally used in the flow-injection system [19] to resolve low-molecular-mass impurities and/or sample solvent from a polymer injected and to improve the peak shape of the polymer investigated. However, it is evident that such a column cannot be used in our case. Rather complex peak shapes are to be expected with polymers having high molecular masses as diffusion-convection equilibria cannot be reached [20] in a connecting capillary of reasonable length. Therefore, a certain loss of data precision in comparison to the SEC experiment must be accepted.

The results of flow-injection measurements of EHM0 sample are summarized in Table 2 as a function of the salt content in the mobile phase. The flow-rate used (0.1 ml/min) closely matches the lower flow-rate used in our SEC experiments to provide similar LS response of aggregates/particles in both experiments.

Table 2
Flow-injection determination of M_w of EHM0 at 0.1 ml/min as a function of salt content in mobile phase

NaCl concentration (M)	M_w		
	Total	Despiked	Decrease (%)
0.00	429 000	368 000	9
0.01	802 000	717 000	11
0.10	613 000	552 000	10

An approximate agreement of total M_w in Table 2 with M_w obtained from an SEC experiment in pure water is found. A 12% difference may be simply ascribed to an increased error of these experiments. After de-spiking of the LS signal (the closest unbiased points in front of and behind a 'spike' were connected by a straight line manually), M_w almost approaches the value obtained from SEC experiment in 0.1 M NaCl. The 'de-spiked' values roughly reflect removal of aggregates having sizes above ~150 nm. A significant increase in M_w was found in 0.01 M NaCl. Formation of larger aggregates from smaller primary dense ones is thus indicated. A decrease in M_w in 0.1 M NaCl is somewhat difficult to explain. Assuming hydrophobic attraction as the origin of aggregation in 0.01 M NaCl, an increased salt content may increase this tendency and larger aggregates than 0.45 μm may be formed and filtered off by the sample filter used. Nevertheless, the observed increase in M_w values in Table 2 in comparison with SEC experiments agrees with our previous investigation of various water-soluble cellulose derivatives [11], where M_w values from classical static light scattering were found to be few tens of percent higher than those obtained from SEC measurement for all polymers investigated. It is now not surprising that M_w values obtained by flow-injection analysis are higher because they reflect a total sample content (polymer + aggregates) contrary to the column SEC experiments in the previous section where the aggregates were retained depending on the experimental conditions used. Also, the more or less general appearance of aggregates/particles in water-soluble cellulose derivatives is confirmed again.

3.4. Latex model experiments

Studies of attachment/detachment processes of colloids in packed beds are of utmost importance in waste water treatment and water purification [21]. Non-porous sand or silica-based beds are usually used and latex particles serve as model particles. A process called slow deposition seems to be relevant to our observations of a weak retention of EHEC aggregates [22]. Using a pulse-injection chromatographic technique to investigate deposition and release of colloidal particles, it was found [23] that latex particles begin to elute at the void volume of

the packed bed and their elution continues over the next 5–6 void volumes under certain conditions. A delicate balance of several interactions and hydrodynamic forces is needed in this case and a repulsive barrier to particle attachment is required to observe this slow deposition. A minor or no dependence of the attachment efficiency on particle size was observed when non-porous glass beads were used as a packed bed [24]. It is worth noting that this kind of retention is not possible with polydisperse polymer coils under isocratic conditions [25,26] due to the extreme sensitivity of their adsorption behavior to the number of monomer units.

Our SEC packings are macroporous, i.e. colloid particles have a possibility to enter the pores where the hydrodynamic shear force cannot act on them. Thus, a fundamental difference between force field in the inter particle space and inside pores exists. Therefore, we decided to check the behavior of model solid particles under slow deposition conditions in the case of wide-pore packing. To approach as much as possible the deposition experiments mentioned above, silica-based columns were used for these preliminary experiments.

Model experiments with polymer lattices (i.e. dense spherical particles) were made to check the behavior of particles of two different diameters under conditions similar to those used in the SEC of EHM0 sample. Firstly, pure water was used as mobile phase. Both particles of different size were eluted by an ion-exclusion mechanism around the column's exclusion volume and no significant change of elution patterns was found at both flow-rates (0.4 and 0.1 ml/min) used. As expected, separation according to molecular size in the hydrodynamic chromatography mode [20] was observed because both column packing and lattices are negatively charged and the long-range electrostatic repulsion force effectively prevents any attractive interaction. The LS behavior of latex particles having average size of 52 nm in an SEC experiment using 0.25 m*M* NaCl as the mobile phase is shown in Fig. 9. Retardation of these particles at 0.4 ml/min in comparison with 0.1 ml/min is clearly visible. In addition, the LS signal does not decrease to the initial baseline level at the end of the experiment, i.e. these particles continue to elute after the SEC separation interval. A close similarity of this behavior to that observed in Ref. [23]

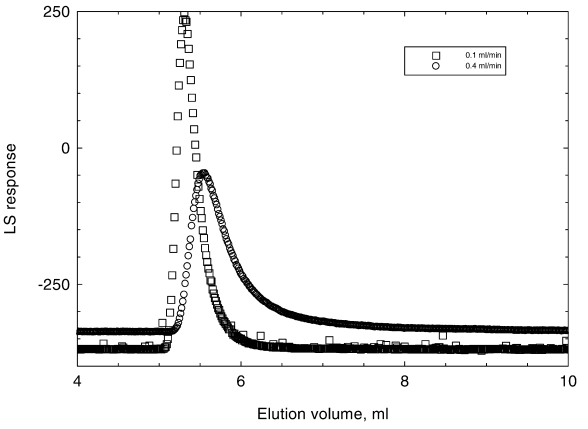

Fig. 9. SEC flow-rate dependence of elution of 52-nm latex particles in 0.00025 *M* NaCl.

indicates the slow deposition regime mentioned above. The calibrations log $M = f(V_e)$ corresponding to both flow-rates are depicted in Fig. 10. A more pronounced retardation of latex particles at higher flow-rate similar to that found for EHM0 in Fig. 5 is observed. This result supports the conclusion that the retardation of an aggregate component of the EHM0 sample may result from a slow deposition regime. An alternative explanation by a possible effect of slow diffusion in the mobile phase within the pores may be rejected as the hydrodynamic diameter of these particles is fairly small.

The behavior of latex particles with $d_H = 114$ nm under the same conditions is presented in Fig. 11. It

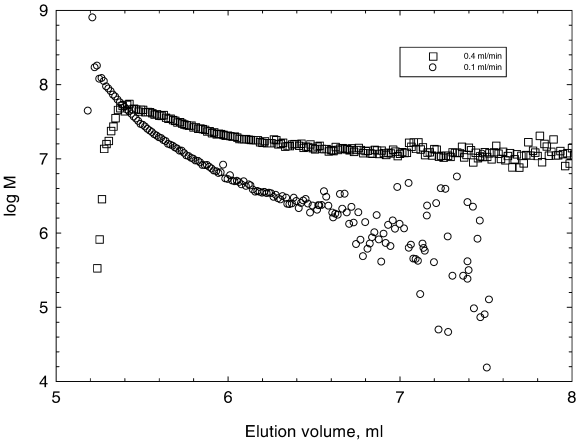

Fig. 10. Log $M = f(V_e)$ calibrations of 52-nm latex obtained from SEC–RI–LALLS as a function of flow-rate in 0.00025 *M* NaCl.

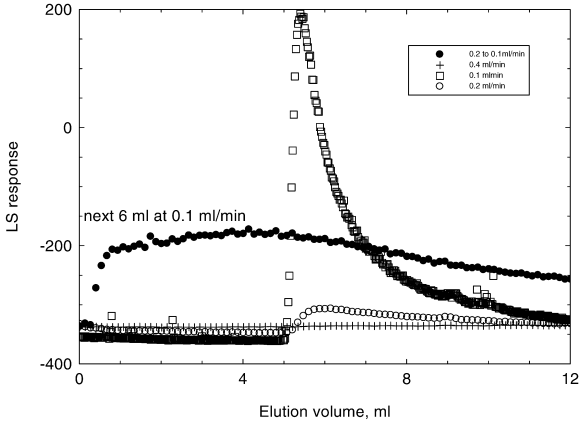

Fig. 11. SEC flow-rate dependence of elution of 114-nm latex particles in 0.00025 *M* NaCl.

is observed that particles of this diameter were not eluted at all at a flow-rate of 0.4 ml/min. At a flow-rate of 0.2 ml/min only a small part of the sample was eluted during the SEC elution volume interval as follows from the low peak area and still higher baseline. When the flow-rate was changed from 0.2 ml/min to 0.1 ml/min at the end of the SEC separation interval, the retained part of the sample was slowly released from the column. Even at flow-rate of 0.1 ml/min, only partial elution was observed in the elution range of SEC. A significant part of the sample injected still remained to be eluted. It can be concluded from these experiments that larger particles having diameter of about 200 nm and more, would not elute at all at any flow-rate under these conditions. When the salt concentration in the mobile phase was increased to 0.0005 *M*, the repulsive barrier determining the slow deposition behavior was suppressed and both lattices were not eluted at all at any flow-rate due to their total adsorption in the fast deposition regime [23].

The occurrence of noise in EHM0 experiments when the flow-rate is lowered from 0.45 ml/min to 0.11 ml/min in 0.01 *M* NaCl is thus conveniently explained provided that the sample also contains such (and larger) particles, which are eluted in the slow deposition regime. When SEC columns were rinsed with pure water after a set of analyses of EHM0 in a salt-containing mobile phase, a huge LS peak appeared when the salt has been completely displaced by pure water, as is also seen with RI detection, which means that pure water acts as a real 'displacing solvent'. This observation may be regarded as a confirmation that aggregates having sizes up to the filter diameter are present in the EHEC sample. The largest fraction of them is quantitatively retained in the column in salt-containing eluents but in contrast, they are eluted with pure water, which in turn implies that they presumably carry a negative charge. Accordingly, large spikes were observed only in the SEC experiment in pure water (Fig. 7). Assuming that the slow deposition regime determines the behavior of EHEC aggregates in the EHM0 SEC experiment in 0.01 *M* NaCl, a change in salt concentration to 0.1 *M* NaCl should also change the slow deposition into a fast deposition regime [23].

3.5. Asymmetrical flow FFF

An asymmetrical flow FFF system was assumed to be less prone to create weak retention conditions due to a much lower total surface of the system as compared with SEC columns and, therefore, will be better suited for characterization of large components. Also, the absence of a stagnant mobile phase as being the case within the pores of the SEC packing should reduce the flow-rate dependence of aggregate elution if present. A preliminary FFF analysis of EHM0 (Fig. 12) has shown a second peak clearly visible in the MALLS trace. This peak relates to these large aggregates which may be indicative for the presence of dense strongly scattering particles in minor amounts. It follows from Fig. 12 that the particle size of the aggregates extends up to 400 nm, which approximately corresponds to the maximum pore size of the usually available SEC packings. Hence, it would be difficult to analyze such large particles by SEC even in the absence of weak retention. At the same time, molecular mass of these aggregates seems to increase up to more than 10^{10}, which confirms the SEC result that these components should consist of more or less compact dense particles. Moreover, the weight-average molecular mass, M_w, obtained for sample constituent eluting in the range between 0.65 and 5.0 min, where aggregates do not appear, is in fairly good agreement with SEC measurements.

The great potential of the FFF technique to

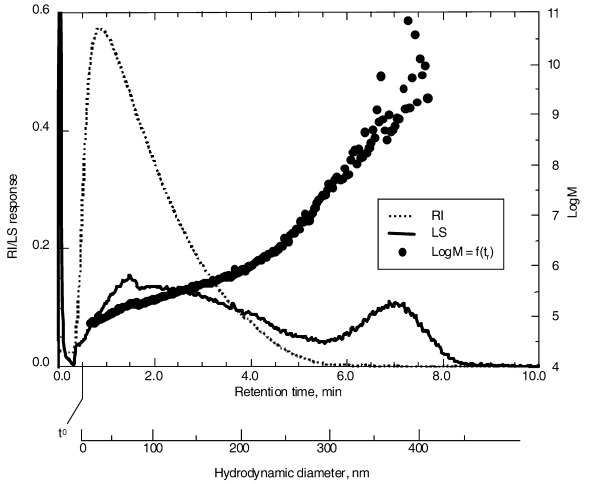

Fig. 12. RI and MALLS 90° signals and obtained molecular masses as a function of elution time after separation by flow FFF. $F_c = 0.21$, $F_{in} = 0.8$, $F_{out} = 0.59$ ml/min, $t^0 = 0.50$ min.

analyze both components simultaneously even in the cases when SEC retains the larger component, i.e. the aggregate, is thus confirmed. At the same time, a combination of SEC and FFF techniques allows to quantify both aggregates of extreme *M* as well as the dissolved polymer coils. The details of the related FFF study will be published elsewhere [14]. In addition, it is important to note that the FFF experiment gives size information about the system investigated.

The last issue addresses a possible structure of aggregates contained in the EHM0 sample, noting that their more detailed investigation, although in principle feasible, is beyond the scope of this paper. The above experiments indicate that most probably the aggregates are compact and dense particles. The model of fringed micelle proposed by Burchard and co-workers [6–8], i.e. comprising a compact core with a brush-like hydrophilic and thus water-soluble corona of EHEC polymer chains, implies the same interaction behavior of the aggregate as the soluble EHEC polymer itself. The concept of weak adsorption, observed for such aggregates and being in contrast to the correct SEC behavior of water-soluble EHEC coils, is difficult to understand, unless the corona is formed from markedly hydrophobic and insoluble chains rich in ethyl cellulose content. Such chains may be then expected to collapse around the crystalline core. This particle could then behave in solution like a solid one. Most probably, these aggregates are cellulose crystallites that remained either intact during synthesis of the EHEC polymer, or more or less undergo surface modification with ethyl and hydroxyethyl groups. These primary aggregates might then form larger secondary particles due to hydrophobic interaction among them when the salt content in the solvent increases. Actually, fringed micelles might be also present in the sample but they should be expected to be eluted in the correct SEC mode. Hence, these structures, if present, would be difficult to separate from the EHEC macromolecules if they were evenly distributed within the same size range as EHEC coils.

Acknowledgements

The authors wish to thank The Centre for Amphiphilic Polymers, Lund University (projects 31 and 39) for financial support.

References

[1] R. Dönges, Polym. J. 23 (1990) 315.
[2] W.F. Reed, Macromol. Chem. Phys. 196 (1995) 1539.
[3] M. Potschka, Macromolecules 24 (1991) 5023.
[4] R.St.J. Manley, Arkiv Kemi 9 (1956) 519.
[5] B. Porsch, S. Nilsson, L.-O. Sundelöf, Macromolecules 30 (1997) 4626.
[6] W. Burchard, R. Schulz, Papier 43 (1989) 665.
[7] R. Schulz, W. Burchard, Papier 47 (1993) 1.
[8] W. Burchard, H.J. Vogel, Comput. Theor. Polym. Sci. 10 (2000) 133.
[9] W. Kaye, J. Colloid Interface Sci. 46 (1974) 3443.
[10] B. Porsch, L.-O. Sundelöf, Macromolecules 28 (1995) 7165.
[11] S. Nilsson, B. Porsch, L.-O. Sundelöf, Carbohydr. Polym. 28 (1995) 265.
[12] O. Quadrat, L. Mrkvičková, Z. Walterová, L. Titkova, P. Bradna, J. Šňupárek, Colloid Polym. Sci. 276 (1998) 879.
[13] B. Porsch, J. Chromatogr. A 653 (1993) 1.
[14] M. Andersson, B. Wittgren, K.-G. Wahlund, Anal. Chem., submitted.
[15] B. Wittgren, K.-G. Wahlund, H. Derand, B. Wesslén, Macromolecules 29 (1996) 268.
[16] A. Huber, Makromol. Chem. Macromol. Symp. 61 (1992) 248.
[17] B. Porsch, L.-O. Sundelöf, J. Chromatogr. A 669 (1994) 21.
[18] Y. Zhu, M. Potschka, P.L. Dubin, Ch. Cai, Macromol. Chem. Phys. 202 (2001) 61.

[19] J.W. Lyons, D. Poche, F.C.-Y. Wang, B. Smith, Adv. Mater. 12 (2000) 1847.
[20] E.P.C. Mes, W.Th. Kok, H. Poppe, R. Tijssen, J. Polym. Sci. B 37 (1999) 593.
[21] Ch.R. O'Melia, Environ. Sci. Technol. 14 (1980) 1052.
[22] N.P. Ryde, E. Matijevič, Colloids Surf. A 163 (2000) 59.
[23] Y.D. Yan, Langmuir 12 (1996) 3383.
[24] M. Elimelech, Ch.R. O'Melia, Langmuir 6 (1990) 1153.
[25] D.W. Armstrong, K.H. Bul, Anal. Chem. 54 (1982) 706.
[26] P. Jandera, Chromatographia 26 (1988) 417.