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Aggregation of amphiphilic pullulan derivatives evidenced by on-line flow field flow fractionation/multi-angle laser light scattering

C. Duval, D. Le Cerf, L. Picton, G. Muller*

PBM, UMR 6522, Université de Rouen-CNRS, F-76821 Mont Saint Aignan Cedex, France

Abstract

Size-exclusion chromatography (SEC) is a useful steric separation technique for the analysis of water-soluble polysaccharides in aqueous solution. However, in the case of amphiphilic derivatives, the usefulness is limited because of interactions between hydrophobic segments and the stationary phase. Alkyl-bearing pullulans differing from the extent and the length of alkyl groups were characterized using flow-field flow fractionation with on-line coupling multi-angle laser light scattering (F^4/MALLS). Comparison of SEC and F^4 is presented and the interest of F^4 in the field of amphiphilic derivatives is demonstrated.

Keywords: Derivatization, LC; Pullulan

1. Introduction

Water-soluble amphiphilic polymers have been extensively studied over the last decades. Those compounds are made of both hydrophilic and hydrophobic segments. They give rise to various applications, owing to their texturing properties, and in a lesser extent to their interfacial properties. Those properties are modulated by the ''hydrophilic–lipophilic balance'' (HLB) characteristics of the polymers (nature, length and distribution of the hydrophobic segments, charge ratio). In aqueous solutions, hydrophobic segments self-associate. Depending on the concentration range, associations can be intra- or intermolecular, leading to the formation of aggregates or physical networks.

Characterization in aqueous solution is of great importance for the understanding of such systems, in order to design their properties according to the desired application. Many studies have been published using techniques such as viscometry [1,2], fluorescence [3–5], NMR [6], static and dynamic light scattering [7,8]. However, information about mass and aggregates distribution and conformation is still missing.

For hydrophilic polymers this information is commonly obtained using size-exclusion chromatography with on-line coupling multi-angle laser light scattering (SEC/MALLS). However, this technique is less reliable in the case of amphiphilic derivatives: because of interactions between the stationary phase and the hydrophobic segments, low recovery of the sample, incorrect separation and also fouling of the column may occur.

Among amphiphilic polymers, hydrophobically modified biopolymers with a natural, non-toxic and biodegradable backbone are of particular interest. In our group, amphiphilic polysaccharides have been

*Corresponding author. Fax: +33-235-14-6704.

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studied over the last years, mainly for their associative properties [9–14]. Therefore, most of the samples had a small amount of long pendent hydrophobic moieties. More recently, pullulan (Fig. 1) has been chosen as the hydrophilic backbone. This exopolysaccharide is highly flexible [15] and its chemical modification can be well modulated [7,12,14,16,17]. Pullulan and carboxymethylpullulans are well characterized in aqueous solution using SEC/MALLS [12,14,18]. However, despite the low degrees of hydrophobic modification, SEC was effectively revealed to be an inappropriate method for amphiphilic derivatives of pullulan.

In an attempt to develop new systems with interesting surface properties, amphiphilic pullulans with higher degrees of modification and smaller hydrophobic chains have been synthesized. Because of the problems already encountered with SEC/MALLS, flow-field flow fractionation (F^4) was used to characterize those new samples. This sterical separation technique appeared in 1966 [20], but the coupling with MALLS is more recent. Separation is based on the diffusive physico-chemical properties of the sample. As there is no stationary phase, the opportunities of interactions between amphiphilic samples and the system are reduced.

This work presents the F^4/MALLS characterization of a carboxymethylpullulan and alkyl-bearing pullulans of low molecular mass. SEC and F^4 are compared and the relevance of F^4 in the case of amphiphilic derivatives is evidenced.

2. Material and methods

2.1. Material

Pullulan of average molecular mass 150 000 g mol^{-1} (determined from SEC/MALLS measurements) was purchased from Hayashibara Biochemical Laboratory. All reagents and solvents were commercially available and used without further purification. Water was from a Milli-Q water reagent system.

2.2. Synthesis

The sodium salt of carboxymethylpullulan (CMP) was synthesized according to a procedure previously described [12,14,21]. The degree of substitution in carboxymethyl groups (DS) was determined by conductimetric titration using a conductimetric radiometer type CD810 (Tacussel) according to the method of Eyler et al. [22]. It is defined as a number of carboxymethyl groups per glucose units. In this study the CMP used had a DS=1.

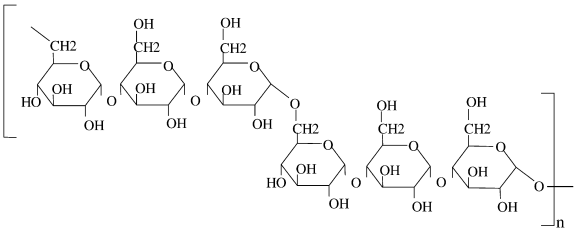

Fig. 1. Formula of pullulan.

Hydrophobic alkyl chains (octyl or decyl) were then grafted through ester linkages according to a synthetic pathway adapted from that used by Della Valle [23] for gellan and Fischer et al. [3] for pectin. The grafting extent x was determined by gas-phase chromatography measurement of the alcohols obtained after alkaline hydrolysis; x is expressed as the number of alkyl moieties per glucose unit.

To reduce molecular mass, ultrasonic degradation (Vibra Cell, Sonics and Materials) was then performed (2 g/200 ml, 20 W for 8 h). Contrary to all other samples, the octyl-bearing pullulan was degraded before hydrophobic modification, to allow the comparison with a degraded unmodified CMP.

In the following, samples are referred to CMP-xC$_n$, with n the number of carbon atoms in the grafted alkyl chain.

For all studied samples, the dn/dc has been determined and the value was 0.160 ml g^{-1} for CMP in both eluents used, and 0.158 ml g^{-1} for octyl- and decyl-bearing pullulan derivatives in Tris–hydroxymethylaminomethane (Tris–HCl).

2.3. Coupling size-exclusion chromatography–multi-angle laser light scattering measurements

Analysis of unmodified CMP was performed by coupling on-line SEC with a MALLS photometer (Dawn DSP-F laser photometer, Wyatt Technology) and a differential refractive index (RI) detector (ERC-7515A, ERMA CR). The SEC system con-

sisted of a OHpak SBG guard column (Showa Denko) and two Shodex OHpak SB-804 HQ and SB-806 HQ columns (Showa Denko) in 0.1 *M* $LiNO_3$. Data collection from MALLS photometer and RI detector were carried out and analyzed using ASTRA software (version 4.50 for Windows, Wyatt Technology). The sample ($C_p \approx 8$ g l^{-1} in 0.1 *M* $LiNO_3$) was filtered through a 0.45 μm Millex-HV type membrane (Millipore) and was eluted by 0.1 *M* $LiNO_3$ at a flow-rate of 0.6 ml min^{-1} (0.1 ml was injected) at room temperature.

2.4. Coupling flow-field flow fractionation–multi-angle laser light scattering measurements

Fig. 2 illustrates the separation principle. The sample is eluted through a thin channel from injector to detector. Separation is achieved by means of a force field applied perpendicular to the channel flow. When the samples reach the inner of the channel, the channel flow is bypassed. This allows the sample to equilibrate by the influence of the cross-flow, which forces particles to the bottom of the channel (accumulation wall). Due to the Brownian motion smaller particles may diffuse faster and therefore move more towards the middle of the channel, according to the diffusion coefficients. Because of the thinness of the channel, the channel flow displays a strong parabolic profile, so that fractions closer to the accumulation wall will be transported much slower than those higher up in the channel. The larger particles will then elute later in the separation. The conditions of elution can be controlled and optimized by the operator to improve resolution.

The coupling system is the same as described for SEC (detection with MALLS and RI, data collection and handling with ASTRA software). It is possible to switch from SEC to F^4.

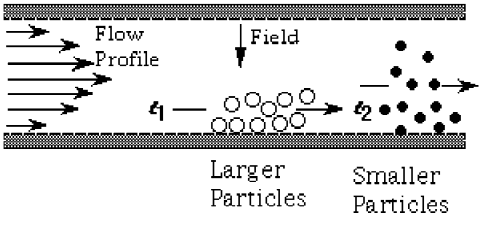

Fig. 2. Illustration of the F^4 separation process.

The F^4 is a Universal Fractionator model F-1000 from FFFractionation, LLC. The linear channel flow stream is regulated with an intelligent pump HPLC 301 (FLOM) while a P-500 (Pharmacia Biotech) dual-piston syringe pump controlled by FF Universal Fractionator Flow software (version 1.60, FFFractionation, LLC) generates the cross-flow.

The samples were dried in vacuum at 40°C overnight, then dispersed at about 8 g l^{-1} in the eluent. After stirring at room temperature for a few hours they were filtered through a 0.45 μm Millex-HV type membrane (Millipore) and 0.1 ml was injected. Eluent was $LiNO_3$ 0.1 *M* for comparison of analysis of CMP with SEC and F^4. For all other experiments, eluent was Tris–HCl 10 m*M*, pH 7.4, since this buffer will be used in further applications of the amphiphilic pullulans.

The following elution conditions were used:

- channel flow-rate: 0.3 ml min^{-1};
- cross flow-rate in $LiNO_3$: 1 ml min^{-1} for 3 min, then exponential decrease down to 0.03 ml min^{-1} for 30 min;
- cross flow-rate in Tris–HCl: 1 ml min^{-1} for 3 min, then exponential decrease for 10 min down to 0.1 ml min^{-1}, then exponential decrease for 10 min down to 0.03 ml min^{-1}.

As an example, the conditions used for analysis in Tris–HCl are presented in Fig. 3.

3. Results and discussion

3.1. Analysis of unmodified hydrophilic CMP

An unmodified CMP was analyzed by SEC and F^4. The chromatograms are shown in Fig. 4. As expected, the orders of elution are in the reverse order for F^4 and SEC.

The molecular masses ($\overline{M_n}$ and $\overline{M_w}$) and polydispersity indexes (I_p) are listed in Table 1. In both cases $\overline{M_n}$ are identical within the uncertainty. The program of cross-flow can account for the difference in $\overline{M_w}$. Indeed, the light scattering signal indicates the presence of large species at large elution times (15–25 min), which are not taken into account for

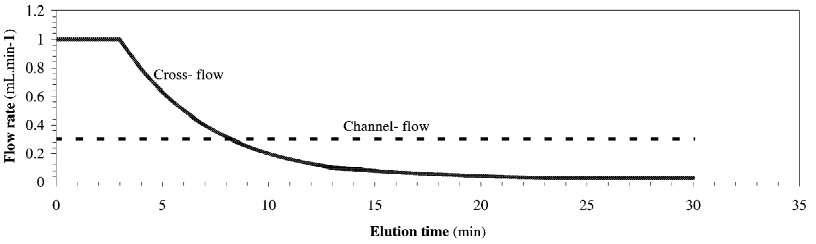

Fig. 3. F^4/MALLS: evolution of cross-flow and channel flow-rates with elution time for analysis in Tris–HCl.

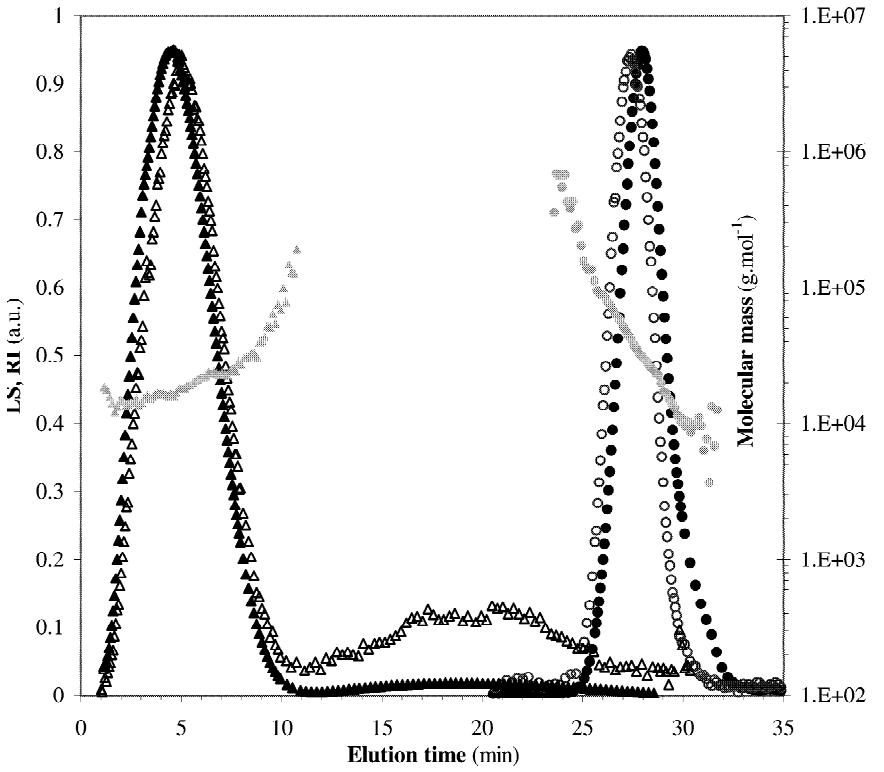

Fig. 4. SEC/MALLS (○) and F^4/MALLS (△): molecular mass distribution (grey symbols), RI (black symbols) and LS (empty symbols) chromatograms versus elution time of unmodified CMP in $LiNO_3$ 0.1 *M*.

Table 1
Analysis of the unmodified CMP with SEC/MALLS and F^4/MALLS

	SEC	F^4
$\overline{M_n}$ (g mol^{-1})	24 000±5000	18 000±3000
$\overline{M_w}$ (g mol^{-1})	36 000±4000	19 000±3000
I_p	1.5±0.4	1.0±0.2

the calculation of the molecular mass because of a too low RI response. This leads to lower $\overline{M_w}$ and I_p in F^4. The protocol used certainly could be improved: using a lower cross-flow those species would elute earlier, but the small ones would be badly separated. This shows the difficulty in finding accurate separation conditions in F^4. For SEC, mass

distribution indicates a good separation.

This experiment confirms that SEC is the best method for the analysis of unmodified CMP, and it requires no optimization of elution conditions, contrary to F^4.

3.2. Analysis of hydrophobically modified CMP

When analyzing amphiphilic biopolymers with SEC, various problems were encountered. Using a polyether gel with a high hydroxyl groups content as stationary phase, only 30% of the injected mass of a pullulan derivative bearing hexadecyl groups was detected by refractometry [12].

Cholesterol-bearing pullulans (CHP) have been successfully characterized using SEC columns packed with a hydrophilic siliceous matrix, used for globular protein analysis [19]. However, using the same columns, 60% of the injected mass was detected by refractometry for the pullulan derivative bearing hexadecyl groups [12]. For a fluorocarbon-containing pullulan, non-linear mass distribution was noticed, with only 60% of mass detected [14]. The successful analysis of CHP may be due to the fact that they form compact nanoparticles with a hydrophobic core and a hydrophilic shell so that the hydrophobic cholesteryl chains are poorly exposed to the stationary phase.

The pullulan derivatives will then be characterized with F^4/MALLS, using conditions such that interactions between the samples and the system are reduced. F^4 requires the optimization of elution conditions for each sample studied. To analyze our pullulan derivatives in Tris–HCl, those conditions were determined for CMP-18C_{10}. All the samples were then injected using the same protocol.

Six injections of CMP-18C_{10} at different concentrations were performed, and the average mass detected by refractometry was 85±5% of the injected mass. This result is much better than those obtained with SEC in the studies mentioned above [12,14].

The elution profile reported in Fig. 5 indicates the presence of two populations. The major fraction (2–11 min), which represents 95% of the detected mass, is rather monodisperse with $\overline{M}_n = 40\,000$

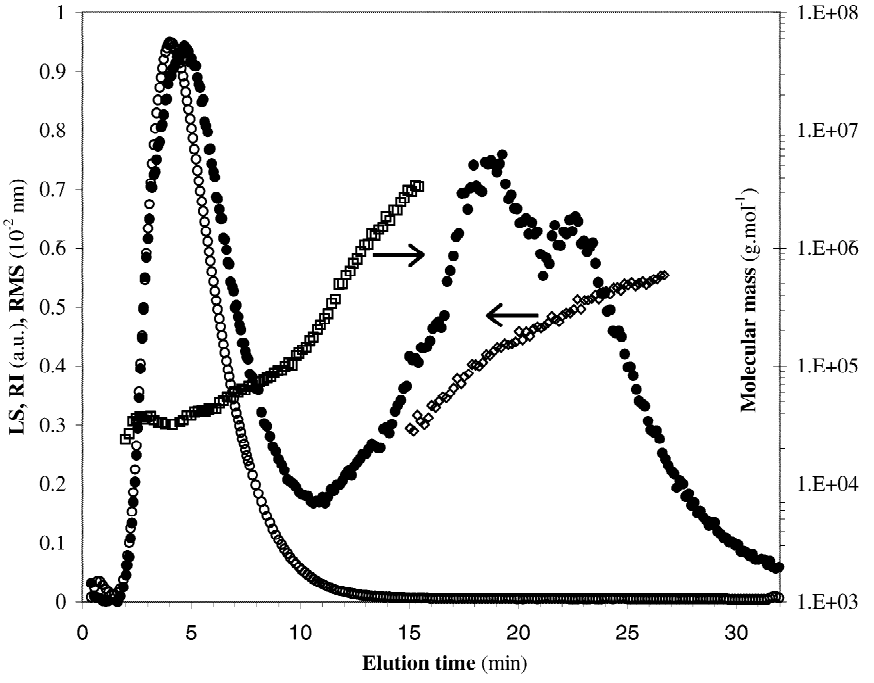

Fig. 5. F^4/MALLS: molecular mass (□) and gyration radius (◇) distributions, RI (○) and LS (●) chromatograms versus elution time of CMP-18C_{10} in Tris–HCl 10 m*M*.

g mol^{-1} and $I_p = 1,1$. The mass distribution indicates a correct separation. Owing to low molecular mass of the pullulan derivatives, the gyration radii were too small to be measured according to the wavelength of the photometer.

The second fraction (11–32 min, 5% of detected mass) displays a very low RI signal, preventing the determination of an accurate molecular mass. This fraction is eluted later and scatters the light with a high intensity, corresponding to a low fraction of species with high hydrodynamic radii. They are aggregates, or high-molecular mass species that were not eluted in the major peak because of the protocol used. The root mean-square radius distribution indicates a good separation of aggregated species.

Finally, F^4 allows the characterization of amphiphilic biopolymers, with a good recovery of the sample and correct separation, but the elution conditions have to be optimized.

3.3. Comparison of CMP-47C_8 and precursor CMP

The protocol described for CMP-18C_{10} was used for all the samples studied.

Fig. 6 represents the elution profiles of the major fractions for unmodified CMP and for CMP-47C_8. The hydrophobically modified sample is eluted after the precursor, indicating the presence of species with larger hydrodynamic radii. At similar concentrations, CMP-47C_8 scatters the light with a much higher intensity than CMP, revealing self-assembly of polymer chains. If polymer chains were isolated, we should obtain a $\overline{M}_n$ near 22 000 g mol^{-1} (calculated from $\overline{M}_n$ of unmodified CMP and the grafting extent of CMP-47C_8). Here we obtain an apparent measured $\overline{M}_n$ about 150 000 g mol^{-1}. It is thus possible to conclude that the introduction of octyl moieties causes aggregation of the major fraction of CMP-47C_8.

3.4. Influence of grafting ratio

Two CMP bearing different extents of decyl moieties were compared (Fig. 7). Comparison with unmodified CMP is impossible since those samples were submitted to ultrasonic degradation after hydrophobic modification.

CMP-18C_{10} has been described above (the LS signal of CMP-18C_{10} seems lower than in Fig. 5,

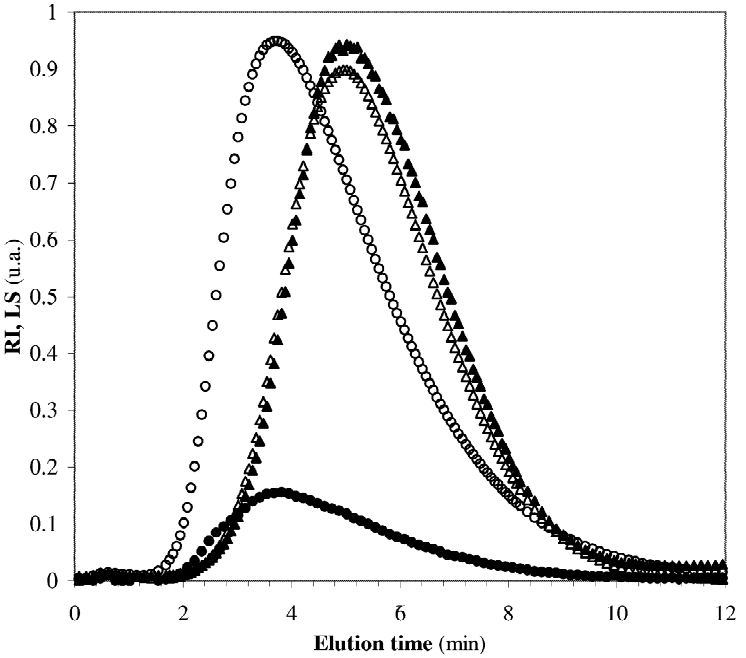

Fig. 6. F^4/MALLS: RI (empty symbols) and LS (filled symbols) chromatograms versus elution time of unmodified CMP (○) and CMP-47C_8 (△) in Tris–HCl 10 m*M*.

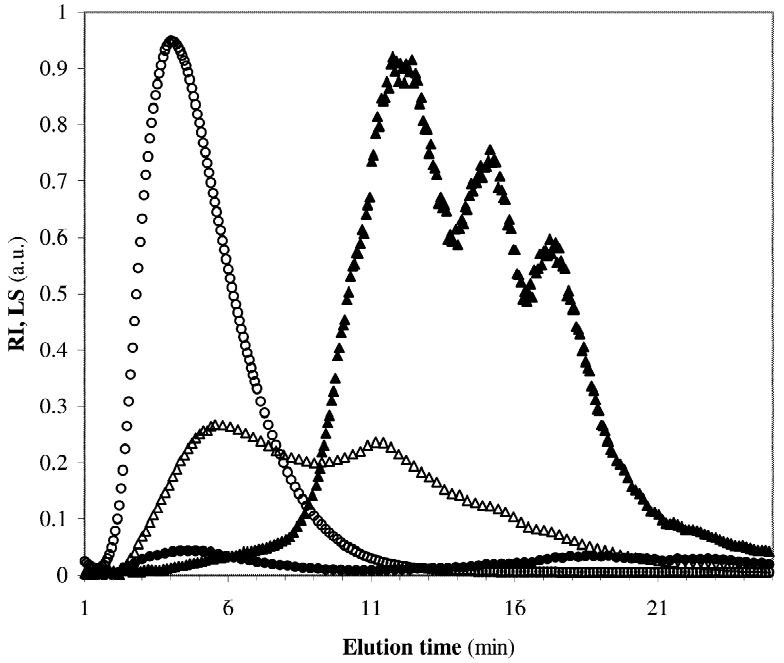

Fig. 7. F^4/MALLS: RI (empty symbols) and LS (filled symbols) chromatograms versus elution time of CMP-$18C_{10}$ (○) and CMP-$43C_{10}$ (△) in Tris–HC1 10 m*M*.

because here it is normalized in accordance with the LS signal of CMP-$43C_{10}$). CMP-$43C_{10}$ presents a larger RI distribution with species eluting at higher hydrodynamic radii. This can be explained by an increase of aggregation with increasing the hydrophobic extent [24]. However, this large distribution may also be due to an incorrect degradation of CMP-$43C_{10}$. The comparison of the elution profiles of this sample and a newly synthesized CMP-$43C_{10}$ should allow us to conclude about this assumption.

In addition, the protocol optimized for CMP-$18C_{10}$ is obviously not suitable for CMP-$43C_{10}$. This underlines the difficulty to compare different samples using flow-field flow fractionation: the comparison can only be reliable if the same protocol is used, but this protocol may not suit each sample.

4. Conclusion

Flow-field flow fractionation with on-line coupling multi-angle laser light scattering was used to characterize hydrophobically modified pullulans in aqueous solution. Information concerning the mass distribution and the presence of aggregates was obtained, with a good recovery of the samples and a correct mass distribution. Flow-field flow fractionation is a suitable method for the analysis of amphiphilic biopolymers in aqueous solution.

However, more samples must be investigated to obtain real conclusions concerning the influence of the number and the length of the alkyl chains on the formation of aggregates.

In conclusion, F^4 is a good alternative for the analysis of amphiphilic biopolymers when SEC fails. Elution conditions may be modulated to improve resolution. However, those conditions are different from one sample to another one and problems may arise when various samples have to be compared. On the other hand, even in the case of an unmodified complex polysaccharide (gum arabic), F^4 has been shown to give more information than SEC [25]. It is thus very useful to combine the two techniques coupled on-line with MALLS for a more complete analysis of complex systems.

Acknowledgements

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