Journal of Chromatography, 641 (1993) 71–79
Elsevier Science Publishers B.V., Amsterdam

CHROM. 25 112

Analysis and purification of monomethoxy-polyethylene glycol by vesicle and gel permeation chromatography

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(First received December 11th, 1992; revised manuscript received March 18th, 1993)

ABSTRACT

Vesicle chromatography (VC) and gel permeation chromatography (GPC) were used for characterisation and purification of monomethoxy-polyethylene glycol (M-PEG), a reagent for protein modification. Detection of low concentrations of contaminating PEG was facilitated by a very sensitive colourimetric detection method with a detection limit of 1 μg/ml. For analytical purposes GPC on Superose 12 was superior to VC. Molecular masses, polydispersity and percentage of contaminating PEG were estimated. As a comparison ^{1}H NMR spectroscopy was carried out. The results were in good accordance with GPC. A two-step preparative purification with VC of M-PEG containing 22.9% PEG reduced the PEG content to 4.4%.

INTRODUCTION

Proteins can be modified by attaching macromolecules. In general, the modification results in an alteration of the physiological properties and/or an increase of the stability of proteins. The main objectives of the modification of proteins are to use them as therapeutic agents or as biocatalysts in biotechnological processes. Polyethylene glycol (PEG) has been applied to various proteins as an agent for modification [1]. Various coupling methods have been developed so far using mainly monomethoxy-polyethylene glycol (M-PEG) as starting material. In this way a PEG molecule is provided which is activated at only one end of the polymeric chain, thus preventing cross-linking of two proteins or the formation of even larger aggregates. However, M-PEG preparations are often contaminated by PEG with free hydroxyl groups at each end (also called diol-PEG). This contaminant is reported to be formed as a result of simple hydrolysis of some of the ethylene oxide monomers in the starting period of the polymerisation due to the presence of free hydroxyl ions [2]. As a consequence these molecules grow at both ends of the chain and, thus, should have about double the molecular size of M-PEG.

The use of narrow-range M-PEG with a high degree of purity is important to minimise the heterogeneity of the modification products and to prevent the above mentioned formation of aggregates. Thus, the characterisation of the purity and molecular mass distribution of the polymer is necessary. In this study we used gel permeation chromatography (GPC), vesicle chromatography (VC) and ^{1}H NMR spectroscopy.

VC is a type of permeation chromatography

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0021-9673/93/$06.00

using microcapsules made of clusters of extracted higher plant cells from suspension cultures as a separation medium [3–5]. The vesicular packings occur as multicellular complexes of 100–250 μm in diameter. The framework of the primary cell wall remains intact. The cell wall acts as an ultrafiltration membrane which is characterised by a very sharp cut-off or separation limit. Large molecules are excluded and eluted in one fraction with 50% of the bed volume (designated as high-molecular-mass or HMW fraction). Smaller molecules permeate through the thin (less than 1 μm) vesicle membrane into the stationary liquid phase. The permeable fraction (low-molecular-mass or LMW fraction) leaves the column with the total bed volume. We applied VC to fractionate M-PEG 5000 in analytical and preparative scale.

GPC is widely used for determination of the molecular mass and polydispersity of polymers [6,7]. As a complement to VC it was applied in analytical scale to characterise the original and purified material.

^{1}H NMR Spectroscopy was reported to be suitable for characterisation of PEGs [8,9]. Therefore, it was used in this study for determination of molecular mass and purity of the polymer.

EXPERIMENTAL

Materials

Preparations of M-PEG 5000 were purchased from Union Carbide (Versoix, Switzerland) ("Carbowax"), Union Carbide (South Charleston, USA) (low-diol "Carbowax") and Aldrich (Milwaukee, WI, USA). M-PEG 2000 was obtained from Aldrich (Gillingham, UK). Trifluoroethanesulphonyl-methoxy-polyethylene glycol (TM-PEG) was prepared using standard M-PEG 5000 according to ref. 10. PEG standards for calibration of the Superose 12 column with molecular masses given by the manufacturer of 4100, 7100 and 8650 were purchased from Polymer Labs. (Church Stretton, UK).

The preparation of the vesicular packing material as well as the estimation of the separation limit was done in the laboratory of Professor Ehwald at the Department of Biology of the Humboldt-University (Berlin, Germany) as described for VP_1 (plant cell material with unaltered separation limit) in refs. 11 and 12.

Methods

Vesicle chromatography. Dry vesicular packing material was suspended in 0.05 *M* sodium phosphate buffer, pH 5.5. The packed bed was washed with one volume of the appropriate elution buffer. Samples of given size (about 0.9 to 7.8% of the bed volume) and concentration were eluted with an elution rate of about 0.5 mm/min. The amount of PEG was estimated in all fractions by a colourimetric method according to Childs [13] or by using a Hewlett-Packard refractive index detector 78977A (first preparative fractionation). The preparative separations were carried out under the following conditions: first fractionation —bed volume: 6.5 l (13 cm × 25.2 cm I.D.), sample: 500 ml of 3% (w/v) M-PEG, elution buffer: 0.01 *M* sodium phosphate, pH 5.5, elution rate: 0.41 mm/min; second fractionation —bed volume: 220 ml (7.8 cm × 6 cm I.D.), sample: 5 ml of 5% (w/v) M-PEG, elution buffer: 0.05 *M* sodium phosphate, pH 5.5, elution rate: 0.51 mm/min. After preparative purification the HMW fractions (first fractionation: 2700–4100 ml) and LMW fractions (first fractionation: 5400–7400 ml, second fractionation: 180–260 ml) were evaporated under reduced pressure and re-dissolved in ethanol, the insoluble phosphate coming from the elution buffer was removed by filtration. The ethanol was evaporated under reduced pressure and the dried samples were dissolved in acetone and precipitated with *n*-hexane, the solvents were finally removed under vacuum.

Gel permeation chromatography. M-PEG samples (1 mg/200 μl) were analysed on a Pharmacia fast protein liquid chromatography (FPLC) system using a Superose 12 HR 10/30 column previously equilibrated with phosphate-buffered saline (PBS), pH 7.0. The samples were eluted with the same buffer at a flow-rate of 0.3 ml/min (3.8 mm/min); 0.25-ml fractions were collected. The PEG concentrations were estimated colourimetrically in all fractions according to Childs [13]. The column was calibrated using

narrow-range polydisperse PEG standards from Polymer Labs. The estimated linear calibration curve was log $M_r = -0.1394\ V_e + 5.919$ ($R = 0.998$), where V_e is the elution volume. The corresponding hydrodynamic diameter (d) of the polymer in nm can be calculated using the following equation given by Hagel [14]: d (nm) = $0.051\ M_r^{0.517}$. The mass-average molecular mass (M_w) and number-average molecular mass (M_n) were calculated using the equations $M_w = \Sigma(w_i M_i)$ and $M_n = 1/\Sigma(w_i/M_i)$, where w_i is the mass fraction and M_i the molecular mass of fraction i. The polydispersities of preparations were determined as the ratio M_w/M_n.

Detection of PEG using the method of Childs [13]. Fractions of PEG were diluted to the appropriate concentration of 1 to 10 μg/ml to measure absorbances within the linear range. A 200-μl volume of each was pipetted in microtiterplates and mixed with 50 μl of 5% $BaCl_2$ in 1 *M* HCl and 50 μl of 0.05 *M* iodine solution (12.69 g I_2 + 20 g K/l). After shaking the plates for 3 to 5 min the absorbance was read at 540 nm against water in a Titertek Multiscan Plus MK II (Flow Labs., Switzerland). Fig. 1 shows the standard curves for M-PEG 5000 (Union Carbide), TM-PEG 5000 and standard PEGs of molecular masses of 4100 and 8650. The detection limit of the method was estimated to be 1.6 μg/ml for PEG 4100 and 1.7 μg/ml for PEG 8650.

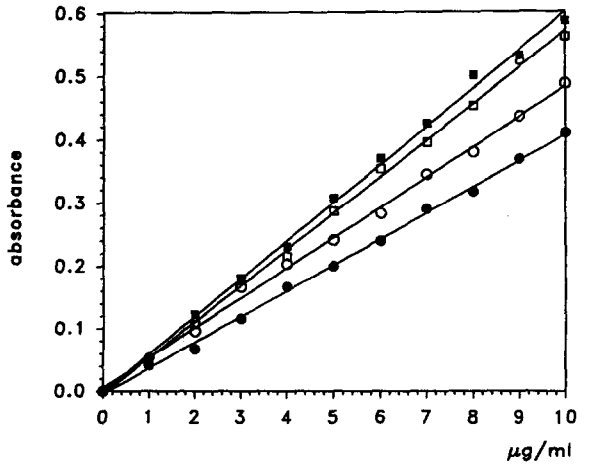

Fig. 1. Standard curves of PEG detection by Childs' method (see Experimental). ■ = PEG 4100; □ = PEG 8650; ○ = M-PEG 5000; ● = TM-PEG 5000.

NMR Experiments. Preparations of M-PEG were dissolved in dry [$^2H_6$]dimethyl sulphoxide (DMSO-d_6) (samples contained 50 to 150 mg/ml) and kept overnight over molecular sieve 3A (Aldrich, Gillingham, UK) to remove traces of water. The samples were then injected into standard NMR tubes, previously swept with nitrogen, through the septum cap. ^1H Fourier transform NMR spectra were recorded within a spectral width of 3760 Hz (15 ppm) on a Bruker spectrometer WM 250 operating at 250 MHz. The reference standard was DMSO-d_6 itself at 2.5 ppm.

The characteristic signals are as follows: carbon satellite bands of the polymer backbone (due to the natural occurrence of 1.108% of ^{13}C): m, 3.22 ppm and 3.78 ppm, CH_3O–: s, 3.25 ppm, H_2O: s, 3.28 ppm, CH_2CH_2O– polymer backbone: m, 3.40 ppm–3.60 ppm, OH–: t, 4.56 ppm.

The percentage of PEG in M-PEG was determined by comparing the integrals of the hydroxyl and methoxy end groups of the polymer. As the first step, the apparent integral of the hydroxyl end groups coming from PEG was calculated as $n_{OH} = (OH - CH_3O/3)$. There the integral of the CH_3O– group divided by three represents the OH groups in M-PEG. The ratio of PEG in M-PEG in mol% is then: % PEG = $[n_{OH}/(CH_3O/3 + OH)] \times 100$.

The M_n value of the polymer was determined on the basis of the average number of (CH_2CH_2O–) units in the polymer backbone. The latter is given by the number of protons in the backbone (the integral for the polymer backbone compared to the integrals of the end group signals which were normalised to a single proton) divided by four: $n = [(CH_2CH_2O-)_n/(CH_3O-/3 + OH)/2]/4$. The integral of the backbone signal was calculated using the carbon satellite signal at 3.78 ppm which represents 0.554% of the backbone protons because they are in the same range of intensity as the end group signals.The M_n value of the polymer can then be calculated according to $M_n = 44n + 32 \times$ molar ratio of M-PEG + 18 × molar ratio of PEG.

RESULTS

Chromatographic analysis of M-PEG 5000

Fig. 2 shows the fractionation of M-PEG by VC and by GPC. VC of M-PEG 5000 (Union Carbide) using vesicular packing material with a separation limit of 5.4 nm results in a profile consisting of two peaks, the HMW fraction and the LMW fraction. The HMW fraction was estimated to be 8.2% (w/w) of the applied sample. The fractionation of M-PEG 5000 by GPC on Superose 12 resulted also in a separation in two peaks corresponding to molecular masses (M_r) of 9280 and 5970. In contrast to VC, the HMW fraction represented 22.9% of the total sample. Since there was a discrepancy in the percentage of material eluting with the HMW fraction between GPC and VC, we studied whether the identities of the HMW and LMW fractions obtained by both techniques were comparable. The analysis of the HMW fraction from VC by GPC showed a main peak co-eluting with the peak corresponding to a molecular mass of 9280 and some contamination with the LMW component (see below in Fig. 6). Similarly, the LMW fraction from VC showed a main peak which co-eluted with the 5970 peak in the GPC chromatogram of the original M-PEG 5000 and some contamination with the HMW fraction (see below in Fig. 6). Given the identity of the HMW and LMW fractions of GPC and VC, it is clear that GPC outperforms VC regarding the efficiency of the fractionation of the two components.

As a pre-step of the preparative purification the influence of sample concentration on the resolution of the fractionation of M-PEG on Superose 12 was studied. Increasing the sample concentration tenfold causes a strong right-shift, which is accompanied by an almost complete loss of resolution (Fig. 3). In Fig. 4 the influence of sample concentration on VC of M-PEG 5000 and tresylated M-PEG (trifluoroethanesulphonyl-methoxy-polyethylene glycol or TM-PEG) is shown. Despite an increase in the sample concentration from 0.2 to 6%, the HMW and LMW fractions appear in the same place, there is no

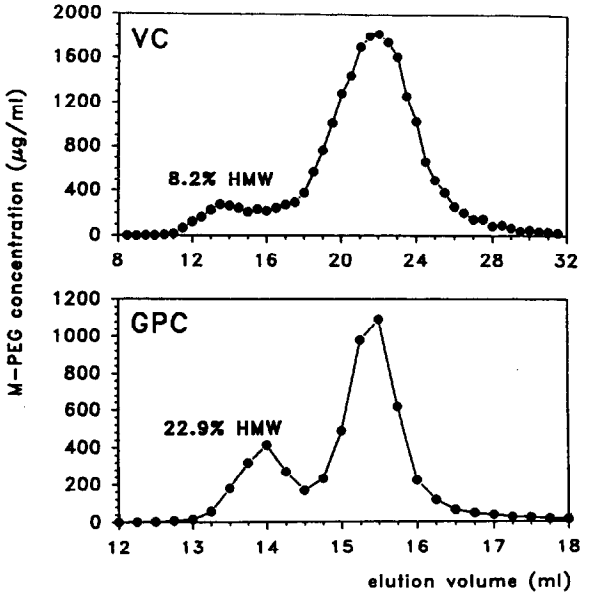

Fig. 2. Vesicle chromatography (VC) and gel permeation chromatography (GPC) of M-PEG 5000. VC: column dimensions 12.4 cm × 1.5 cm I.D., sample 0.5 ml of 2% (w/v) M-PEG, elution buffer 0.05 *M* sodium phosphate, pH 5.5, elution rate 0.49 mm/min. GPC: Superose 12 HR 10/30, sample 0.2 ml of 0.5% (w/v) M-PEG, elution buffer: PBS, pH 7.0, elution rate 3.8 mm/min. The M-PEG concentration of all fractions was estimated, the given chromatograms show the part of the profiles where M-PEG was eluted, the ratio of the high-molecular-mass (HMW) fraction is given in percent (w/w).

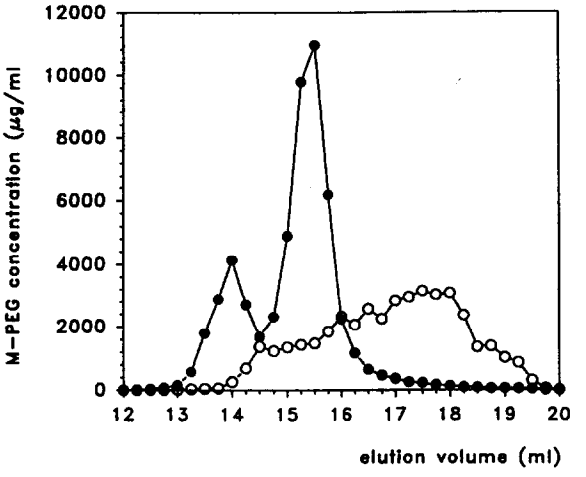

Fig. 3. Influence of sample concentration on GPC of M-PEG 5000. Column: Superose 12 HR 10/30; sample 0.2 ml; sample concentration in % (w/v): (●) 0.5, (○) 5; elution rate: 0.3 ml/min. FPLC profile of M-PEG with a sample concentration of 0.5% was upscaled by factor 10.

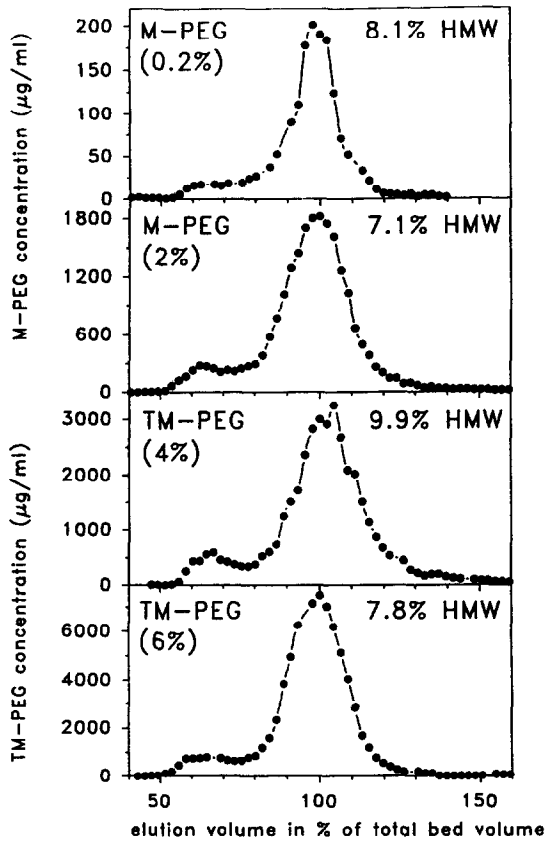

Fig. 4. Influence of sample concentration on VC of M-PEG and TM-PEG 5000. Column dimensions 12.4 cm × 1.5 cm I.D., sample volume 0.5 ml, sample concentrations in % (w/v) are given in parentheses, elution buffer 0.05 *M* sodium phosphate, pH 5.5 (M-PEG 0.2%) or 0.08 *M* PBS, pH 7.0. % HMW gives the percentage (w/w) of the high-molecular-mass fraction.

right-shifting. Furthermore, the resolution of the separation remains almost unchanged with only slight decreases in the percentage of the HMW fraction (up to 2.1) despite increases in the sample concentration applied of up to tenfold (Fig. 4).

In view of the possibility to apply higher sample concentrations VC was selected for a large-scale preparative fractionation. Fig. 5 gives the chromatogram of the first preparative fractionation of M-PEG 5000 (Union Carbide) by VC. The scale-up from around 25 to 6500 ml bed volume causes an obvious loss in resolution. The indicated fractions from 2700 to 4100 ml (HMW) and 5400 to 7400 (LMW) were collected and

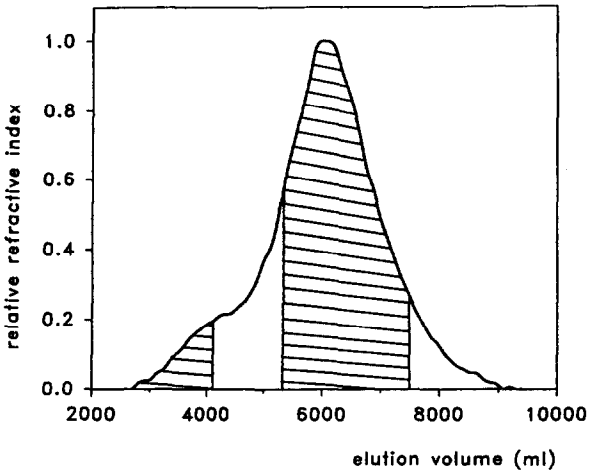

Fig. 5. Preparative fractionation of M-PEG 5000 by VC. Column dimensions 13.0 cm × 25.2 cm I.D., sample 500 ml of 3% (w/v) M-PEG, elution buffer 0.01 *M* sodium phosphate, pH 5.5, elution rate 0.41 mm/min. Indicated fractions were collected and prepared to give purified HMW and LMW preparations.

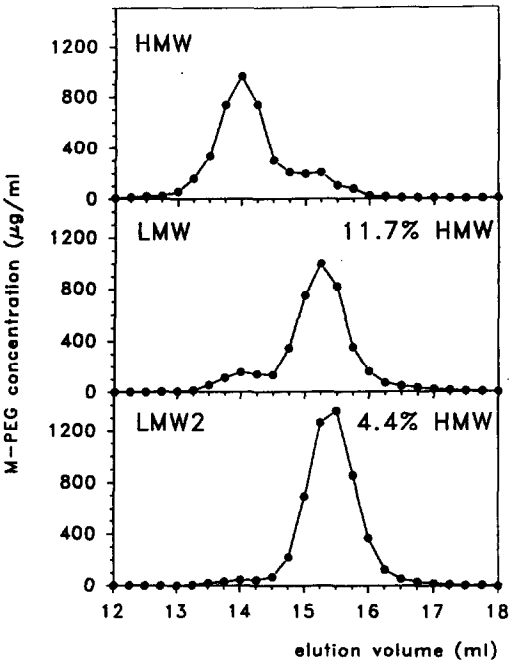

Fig. 6. GPC of low-molecular-mass (LMW) and high-molecular-mass (HMW) fractions coming from preparative VC of M-PEG 5000. Sample: 1 mg of M-PEG in 200 μl PBS, pH 7.0, column: Superose 12 HR 10/30, elution rate: 3.8 mm/min. LMW2 = Purified M-PEG obtained by a second preparative VC of LMW, the ratio of HMW in LMW and LMW2 is given top right in percent (w/w).

treated as described in Experimental. To characterise the purified preparations GPC was carried out (Fig. 6). The LMW preparation was still contaminated with 11.7% of HMW component and therefore further purification was required. A second preparative fractionation with VC provides a LMW fraction (LMW2) which contains only 4.4% HMW component as analysed by GPC (Fig.6).

To further characterise the M-PEG preparations (original M-PEG 5000, LMW preparation from first purification step and LMW2 from second purification), their molecular masses and polydispersities were estimated. In order to do this the GPC column was calibrated with narrow-range polydisperse PEG standards of known molecular masses. Table I summarises the results. With each preparative purification step carried out using VC the average molecular mass as well as the polydispersity is reduced as expected after the removal of the high-molecular-mass contamination.

Estimation of purity and molecular mass of M-PEGs by NMR

As a reference method the ^{1}H NMR spectra of the different preparations were recorded. Fig. 7 shows the NMR spectrum of the original M-PEG 5000 in DMSO-d_6. The CH_3 signal at 3.25 ppm was overlapped by one of the ^{13}C carbon satellite signals, each of which equals 0.554% of the backbone signal (according to the natural occurrence of ^{13}C of 1.108%). Thus, for quantitative calculations the integral of the CH_3O– signal was corrected by subtracting from it the integral of the carbon satellite at 3.87 ppm, which was free of contributions from other signals. Based on the intensities of the signals of the polymer backbone and the methoxy and hydroxyl groups the molecular mass of the polymer and the percen-

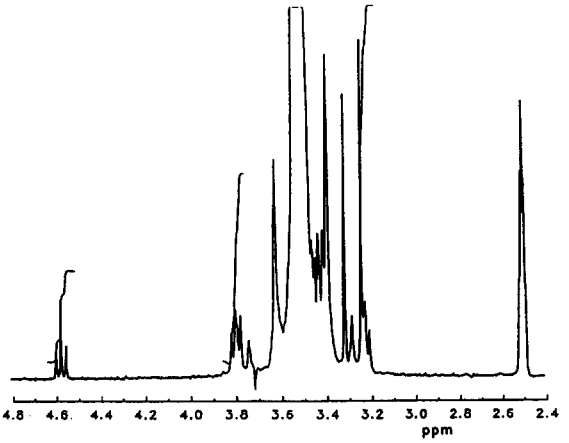

Fig. 7. 250 MHz ^{1}H NMR spectrum of M-PEG 5000 (containing 23.9% PEG). Peaks: 50 mg/ml in DMSO-d_6, 2.5 ppm: DMSO; 3.22 and 3.78 ppm: carbon satellite bands; 3.25 ppm: CH_3O–; 3.28 ppm: H_2O; 3.29, 3.42, 3.64 and 3.74 ppm: spinning side bands of the backbone signal; 3.40–3.60 ppm: CH_2CH_2O– (polymer backbone); 4.56 ppm: OH–.

TABLE I

MOLECULAR MASS AND POLYDISPERSITY OF ORIGINAL M-PEG 5000 (UNION CARBIDE) AND PURIFIED PREPARATIONS ESTIMATED BY GPC

Sample: 1 mg of M-PEG in 200 μl PBS, pH 7.0, column: Superose 12 HR 10/30, elution rate: 0.3 ml/min, for calculation see Experimental.

PEG-preparation	M_w	M_n	Polydispersity M_w/M_n
M-PEG 5000 original	6550	6059	1.081
M-PEG LMW	6500	6230	1.043
M-PEG LMW2	5970	5820	1.026

TABLE II

MOLECULAR MASS AND PEG CONTENT OF VARIOUS M-PEG PREPARATIONS ESTIMATED BY ^{1}H NMR SPECTROSCOPY

250 MHz, 50–150 mg/ml in DMSO-d_6, calculation of M_n and % PEG see *Methods*.

Sample	M_n[a]	% PEG[a]
M-PEG 5000 (Union Carbide)	6040 (12.9%, 6)	23.9 (2.8%, 6)
LMW	6372	11.1
	6084	5.1
LMW2	5195	0
M-PEG 5000 low-diol (Union Carbide)	5390 (9.9%, 3)	0.6

[a] Standard deviation (M-PEG 5000 from Union Carbide) in percent of the mean value and number of independent measurements are given in brackets, otherwise single estimations or duplicates (LMW).

tage of PEG were estimated as described in Experimental. Table II shows the results of the calculations. The percentage of PEG in the LMW preparation was reduced compared to the original M-PEG. However, ^{1}H NMR spectroscopy failed to show any PEG content in the LMW2 preparation which according to GPC contained 4.4%. In order to test this apparent discrepancy we subjected a commercial low-diol M-PEG 5000 from Union Carbide to the same analysis. ^{1}H NMR spectroscopy showed a content of 0.6% PEG while GPC combined with the applied detection method indicated a PEG contamination of 2.15%.

DISCUSSION

The fractionation of M-PEG by VC and GPC results in a profile showing two fractions. For original M-PEG 5000 (Union Carbide) the M_r values of the HMW and the LMW fraction were estimated to be 9280 and 5970, respectively. The percentage of the HMW fraction was 8.2 and 22.9%, estimated by VC and GPC, respectively. The reason for this discrepancy lies in the separation limit of 5.4 nm of the vesicular packing material used. It allows part of the HMW fraction, the molecules which are smaller than 5.4 nm, to permeate through the cell wall. These molecules are eluted with the LMW fraction. Figs. 2 and 6 show that the trough between the two peaks in the GPC elution profile corresponds to an elution volume of 14.7 ml. The corresponding molecular size of PEG is 5.1 nm. The efficiency of the separation by VC could therefore be increased by using a vesicle packing material with this separation limit. For that purpose plant cell material with smaller natural pore size has to be found.

Experiments concerning the influence of sample concentration on the resolution showed right shifting and a severe loss in resolution for M-PEG on Superose 12 at a sample concentration of 5% (w/v). In contrast, sample concentrations up to 6% (w/v) had little influence on the resolution of VC. Mori [15,16] reported a concentration effect when fractionating 0.1 to 0.4% polystyrene on porous glass packing materials due to a decrease in the hydrodynamic volume of the polymer with increasing polymer concentration. They found a slight right shifting and increase in the slope of the calibration curve resulting in reduced separation efficiency. A decrease in the hydrodynamic volume of the polymer could be the reason for the slight decrease in the percentage of the HMW component in VC we have observed when increasing the concentration of M-PEG and TM-PEG in the sample. However, a reduction in the hydrodynamic volume cannot explain the magnitude of the observed loss of resolution and right shifting of M-PEG on Superose 12. As possible explanations we consider an overload effect [17,18] which might be more likely to occur in the gel structure of Superose 12 than in the vesicular structure of the packing material for VC, and the occurrence of phase separation on the surface of the agarose gel. The latter is worth being taken into account because the phenomenon of phase separation has been reported for a wide variety of polysaccharides [19], in addition to the well-studied phase system of dextran and polyethylene glycol [20–22].

For the detection of PEG we used a very sensitive colourimetric method developed by Childs [13]. So far, this method has been used to measure PEG concentrations in solutions of proteins precipitated by the polymer. Although more laborious than standard methods such as refractive index and direct UV detection, it is superior in its detection limit, which was estimated to be 1.6 μg/ml for PEG 4100 and 1.7 μg ml for PEG 8650, *i.e.* 0.4 and 0.2 nmol/ml, respectively. In contrast, detection limits of other methods are reported to be in the submilligram range for refractive index detection or to be in the range of 5 to 10 nmol/ml, which would equal 25 μg/ml for PEG 5000, for UV detection of derivatised PEG (dibenzoates) at 254 nm [23]. For the direct UV detection of PEG 200 at 185 nm a molar extinction coefficient ε of 148 l mol^{-1} cm^{-1} has been estimated [24], whereas ε of PEG 4100 using Childs' method is $4.4 \cdot 10^5$ l mol^{-1} cm^{-1}. The limit of detection of an indirect UV detection method at 210 nm for GPC of oligomers of ethylene glycol is given as 0.6 nmol/ml [25]. It was found that the detection method of Childs is influenced by the molecular mass of

the sample (Fig. 1). Therefore, for quantitative measurements it is advisable to use standard curves of PEGs with similar molecular mass.

The results of ^{1}H NMR spectroscopy showed that the removal of the HMW fraction by VC is accompanied by a decrease in the amount of diol-PEG in the preparation. Thus, ^{1}H NMR spectroscopy confirmed the fact that the HMW fraction contains largely diol-PEG. The resolution of the OH signal was very much dependent on the pH of the solution from which the sample was freeze dried prior to dissolving it in DMSO-d_6. Adjusting the pH of the solutions from a pH of around 5.5 to a pH of 6.0 improved the appearance of the OH peak dramatically. The calculated percentages of PEG determined by ^{1}H NMR spectroscopy correspond very well to the GPC results for the original preparation and the LMW fraction from the first purification. However, the standard deviation of the data are relatively high. In the case of the low-diol M-PEG from the second purification step (LMW2) the percentage of PEG was zero in contrast to the 4.4% detected by GPC. The analysis of a commercial low-diol M-PEG 5000 from Union Carbide showed a similar discrepancy between ^{1}H NMR spectroscopy and GPC. Thus, a contamination of less than 4.4% PEG in M-PEG 5000 cannot be detected by ^{1}H NMR spectroscopy. The molecular masses of the original M-PEG, the LMW fractions (LMW and LMW2) and low-diol M-PEG 5000 from Union Carbide are in good accordance to the GPC data. The relatively high standard deviations were not unexpected as similar findings had been reported by Dust *et al.* [9] for PEG samples with molecular masses around 5000. For the HMW fraction (with a molecular mass of *ca.* 10 000 as estimated by GPC) ^{1}H NMR spectroscopy could not be used to estimate the molecular mass and the PEG content because the signals for the end groups were marginal in comparison to the backbone signal as well as to the ^{13}C carbon satellite signal. Especially the signal for the hydroxyl end groups was negligible although the pH was adjusted to 6.0 as for the other samples. The basis for this observation still remains unclear.

CONCLUSIONS

GPC on Superose 12 in combination with the detection method of Childs is a good method to analyse the purity and molecular mass distribution of M-PEG preparations. In its accuracy and reliability it is superior to ^{1}H NMR spectroscopy especially in the high-molecular-mass range above 5000. Vesicle chromatography is very useful for preparative fractionation applying high sample concentrations, *e.g.* for the removal of an excess of polymer from the reaction mixture of a protein modification process. However, a material with the appropriate separation limit for every particular fractionation problem has to be selected. It was already shown that the cut-off limit of vesicular packing material can be increased by depolymerisation of the polysaccharides in the cell wall [11]. The degree of enlargement is determined by the specific conditions of treatment (pH, temperature, time), as a result a variety of material with different separation limit ($\geq$5.4 nm) is available. For smaller separation limits other types of plant cell material have to be found.

ACKNOWLEDGEMENTS

We thank Mrs Jane Hawkes and Mr Jonathan Cobb of ULIRS, Kings College, London, UK for providing the ^{1}H NMR spectra and for their advice. B.S. is supported by the German Academic Exchange Service.

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