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Superheated high temperature to improve size exclusion chromatography separation of polyethylene glycols with chloroform as the mobile phase

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ABSTRACT

In our laboratory, chloroform is increasingly required to be used as the mobile phase for the size exclusion chromatography (SEC) characterization of polyethylene glycol (PEG) derivatives, because some of the derivatives show poor solubility in many other solvents. Four types of SEC columns, all based on highly cross-linked polystyrene–polydivinylbenzene (PS/PDB) and compatible with chloroform, have been tried. However, a problem of using chloroform with all the columns tested is that retention might not be rationalized simply based on the SEC-mechanism even for the PEG standards. It was found that for the PEG standards raising the column temperature can significantly improve the SEC separation. In order to take full advantage of the temperature effect on separation, a system was developed which enables the SEC to be performed at superheated temperatures, i.e., temperatures well above the normal boiling point of the mobile phase. The improved SEC separation at elevated temperatures is most likely due to the combination of reduced adsorption of PEGs by the stationary phase and increased solubility of the solutes in the mobile phase. In this work, the SEC separation operated at temperatures above the normal boiling point of the mobile phase was called "superheated high temperature SEC".

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1. Introduction

Polyethylene glycols (PEGs) are non-toxic polymers that are soluble in water and many types of organic solvents. Because of their attractive properties, they are widely used in many areas including industrial manufacturing, food additives and medical/clinical uses [1–4]. In addition, covalently attaching PEG molecules to pharmaceutical and biological molecules, a strategy termed PEGylation, has increasingly been employed nowadays to improve the chemical and physical behaviors of the target molecules [5–7]. For example, attaching PEG to drug molecules has successfully been applied to improve their pharmaceutical and pharmacological properties [8,9].

In our laboratory, many types of PEGylated products have been synthesized by attaching PEG polymer chains to various kinds of molecules, among which are peptides, proteins, and synthetic molecules such as ureido-pyrimidinone (UPy) and benzene-1,3,5-tricarboxamide (BTA) [10–17]. The PEGylated products, with a broad diversity of molecular structures, were designed to be applied in the fields of bio- and tissue engineering, nano-technology, drug delivery, supramolecular polymers and supramolecular hydrogels [10–17]. In the characterization of a PEGylated product, the average molecular weight (MW) and molecular weight distribution (PD) are important parameters that must be carefully determined. Size exclusion chromatography (SEC) is one of the most useful methods for the determination of MW and PD. In an ideal SEC separation, polymer molecules are separated solely according to their molecular weight/size with the biggest eluted first and the smallest last [18,19].

For (underivatized) PEG samples, excellent SEC separation can be obtained by using water or dimethyl formamide (DMF) as the mobile phase [20]. Unfortunately, some of our PEG derivatives are best soluble in chloroform and only slightly soluble or insoluble in either water or DMF. A problem of using chloroform as the mobile phase, as will be illustrated in this manuscript, is that even the PEG standards might have abnormal peak shapes and show significant deviation of retention from that based on a pure SEC mechanism. Furthermore, the retention behavior was found to be strongly dependent on the type of column used. Perhaps, it is the problem of this complicated non-SEC retention behavior that has limited the use of various organic solvents in the SEC separation of PEGs. To the best of our knowledge, no detailed studies of using chloroform mobile phase for the SEC separation of PEGs have been reported so far. Needless to say, with the increased applications of PEGs in different areas, a lot of new types of PEG derivatives will be synthesized. Due to the possible solubility problem of the new derivatives in water or DMF, different eluents will definitely

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be required for the SEC measurements. Since chloroform is one of the most widely used organic solvents in SEC and shows good solubility for many of our PEG derivatives, in this work we will focus our investigation on this solvent. The aim is to develop a reliable and convenient method for the SEC separation of PEG standards.

In SEC, the non-ideal retention behavior is most likely caused by the interaction of the polymer molecules with the stationary phase and/or poor solubility of the polymer in the mobile phase [18,19]. In principle, the interaction with the stationary phase can be minimized by using a more inert SEC column or by blocking the active sites in the column with a suitable modifier/additive introduced into the mobile phase, while the polymer solubility can be improved by using a better solvent or mixed solvents (including additives). However, a mixed solvent may lead to problems due to preferential solvation of either the stationary phase or the solute by one of the solvents [18]. Practically, therefore, it might be difficult and extremely time consuming in real applications to find the right conditions to completely overcome the non-SEC retention by trying different columns and mobile phase compositions.

Another important parameter affecting the separation in chromatography is temperature. In high performance liquid chromatography (HPLC), raising column temperature has been proved to be a useful way to enhance chromatographic resolution, efficiency and speed [21–24]. Chang et al. [25,26] also reported SEC at high temperatures for fast separations, which is valuable to speed up the two-dimensional HPLC–SEC analysis of synthetic polymers. Theoretically, the selectivity of an ideal SEC separation is only controlled by the entropy change (ΔS) of the solute and is independent of temperature [19]. In many real applications, however, the problem of non-SEC retention cannot be totally ignored. The non-SEC retention is normally caused by interactions between the solute and the stationary phase, which can often be reduced by raising temperature. Unfortunately, the potentials of raising temperature to minimize the non-SEC retention in SEC separations are still remained largely unstudied. Although high temperature (HT) SEC is not a new technique, it generally refers to the separation of polymers (such as polyolefins) that are not soluble in any solvent at room temperature [19]. In this case, a solvent with a high boiling point like o-dichlorobenzene (ODCB) or 1,2,4-trichlorobenzene (TCB), is used as the mobile phase and separation is carried out at elevated temperatures but yet below the normal boiling point of the solvent. The roles of high temperature in these SEC applications are mainly twofold, to improve the polymer solubility and to lower the mobile phase viscosity.

In this contribution, reducing the non-SEC retention as another benefit of raising column temperature was investigated in the SEC separation of PEGs with chloroform as the mobile phase. By using a setup similar to that reported in HT HPLC, chloroform can be operated at temperatures well above its normal boiling point. We name the SEC separation operated at temperatures above the normal boiling point of the mobile phase as 'superheated HT SEC', to distinguish it from the 'conventional HT SEC' that uses high boiling point solvents. It was found that SEC separation of PEG samples using chloroform as the mobile phase, which might be seriously hindered by the non-SEC retention at room temperature, can be improved greatly by using superheated HT-SEC.

2. Experimental

The system used here for the superheated high temperature size exclusion chromatography (HT-SEC) separation is shown in Fig. 1. It consists of a LC-20 AD pump (Shimadzu, Kyoto, Japan), a six-port Valco valve injector (VICI AG International, Schenkon, Switzerland), a FD-53 oven (Binder, Tuttligen, Germany), a UV/vis detector (SPD-20A, Shimadzu), a refractive index detector (RID-10A, Shimadzu),

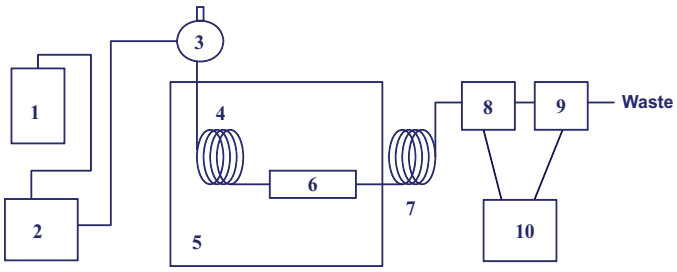

Fig. 1. Schematic diagram of the superheated HT-SEC system. (1) Solvent reservoir; (2) pump; (3) injection valve; (4) heat exchanger; (5) oven; (6) SEC column; (7) cooling tubing; (8) UV/vis detector; (9) RI detector; and (10) data system.

and a Dax data system (PP van Mierlo, Eindhoven, the Netherlands). HPLC grade of chloroform stabilized with amylene (from Biosolve, Valkenswaard, the Netherlands) was used as the eluent throughout this study. The eluent (delivered at 1 mL/min at room temperature) was heated up to the oven temperature by passing through a heat exchanger made from a 1.5 m narrow-bore stainless steel tubing (1/16 in. (o.d.) × 0.004 in. (i.d.), Alltech-Grace, Lokeren, Belgium) located in the oven before it reached the column. Because the UV/vis and RI detectors cannot tolerate high temperature and high pressure, another piece of 1.2 m narrow-bore stainless steel tubing (1/16 in. (o.d.) × 0.004 in. (i.d.), Alltech-Grace) was used to depressurize the superheated eluent and to cool it down to room temperature. The purpose of using narrow-bore tubing for the heating and cooling was to minimize the chromatographic band broadening. Since chloroform was operated above its normal boiling point, the superheated HT-SEC system must be leak-free and was put in an isolated fume hood for obvious safety concerns.

Four columns were tested for the superheated HT-SEC separation of polyethylene glycols (PEGs). The Resipore (RP) column (300 × 7.5 mm i.d., 3 μm particles, linear molecular mass range up to 400,000), the Mixed-C (MC) column (300 × 7.5 mm i.d., 5 μm particles, linear molecular mass range up to 2,000,000) and the Mixed-D (MD) column (300 × 7.5 mm i.d., 5 μm particles, linear molecular mass range up to 400,000) were purchased from Agilent Technologies (Amsterdam, the Netherlands), and the Linear-M (LM) column (300 × 8.0 mm i.d., 3 μm particles, linear molecular mass range up to 1,000,000) from Polymer Standards Service GmbH (Mainz, Germany). The narrow polystyrene (PS) and PEG standards with polydispersity in the range of 1.03–1.11 were obtained from Agilent Technologies.

3. Results and discussion

3.1. SEC of PEGs with chloroform as the mobile phase at room temperature

Method development in size exclusion chromatography (SEC) is usually a straightforward task. For most applications, separation is carried out at room temperature. Solvent optimization is not needed beyond finding a solvent that has good solubility for the sample and is compatible with the stationary phase and the detector. Because of its good solubility for many types of PEG derivatives synthesized in our laboratory, chloroform was intuitively considered to be the solvent of choice for our SEC separations. Unfortunately, though, strange retention behaviors were often observed when chloroform was used as the mobile phase even for the separation of PEG standards.

Fig. 2 shows the SEC chromatograms of PEG standards together with polystyrene (PS) standards on a PL-Resipore (RP) column using chloroform as the mobile phase. According to the specifications, the molecular weight (MW) separation range of this column is

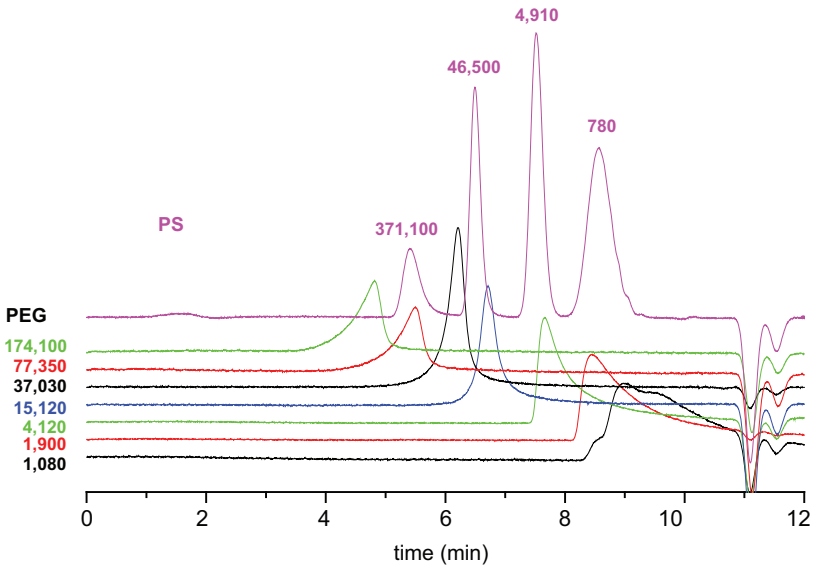

Fig. 2. SEC chromatograms of PEG and PS standards at room temperature with a Resipore column. Column: Resipore, 300 × 7.5 mm, 3 μm particles; mobile phase: chloroform at 1 mL/min; sample concentraion: 1 mg/mL; injection volume: 20 μL; detector: refractive index (RI).

200–400,000 Da (based on PS standards). As expected, PS standards in this MW range eluted nicely based on their molecular sizes. In contrast, however, the PEG standards display a very strange retention behavior. It can be seen clearly from Fig. 2 that only two standards (MW of 15,120 and 37,030) give reasonably good peak shapes. The chromatographic peaks of the other components were found to be either seriously tailing (for smaller MW PEGs) or seriously fronting (for higher MW PEGs). The outcome clearly indicates that using chloroform as the mobile phase with a RP column might give troublesome results in SEC analysis of PEG samples.

In order to study the strange retention behavior of PEG samples in more detail, three more different types of SEC columns were tested. These columns are a Mixed-C (MC, linear MW range 200–2,000,000) and a Mixed-D (MD, linear MW 200–400,000) from Polymer Laboratories, and a Linear M (LM, linear MW range 100–1,000,000) from Polymer Standards Service (PSS). The chromatograms of PEG standards on these columns are shown in Fig. 3. Also in this figure, the relationship between retention time and log(MW) of the standards are plotted. It is interesting to see that on all the three columns the low MW PEG standards (up to MW about 35,000 Da) eluted nicely according to their molecular weights, which indicates that chloroform can still be considered as a suitable mobile phase at least for low MW PEG samples if a right column is chosen. Despite of the good SEC results obtained for the low MW PEGs, the problem for the high MW PEGs remains. Their peaks are broadened and retention times deviate significantly from their corresponding theoretical linear calibration lines. As a result, further efforts are still required to improve the SEC separation. It will be demonstrated in the following section that raising the column temperature (above the normal boiling point of the mobile phase) is an effective way to improve the separation of PEG samples.

3.2. Improving SEC separation of PEGs with chloroform at superheated temperatures

In superheated HT-SEC, a restrictor after the column is needed to provide a sufficient backpressure to keep the mobile phase from boiling at temperatures above its normal boiling point and to cool the solvent down to room temperature before it reaches the detector(s). A piece of 1.2 m narrow-bore stainless steel tubing (1/16 in. (o.d.) × 0.004 in. (i.d.)) was used here for this purpose. Because of their high maximum allowable temperature (T_{max} = 150 °C) and high maximum allowable pressure (P_{max} = 150 bar), the MC and MD columns were chosen in the superheated high temperature experiments. Fig. 4 shows the log(MW) vs. Retention time of PEG and PS standards at different temperatures for these two columns, and Fig. 5 gives the chromatograms at 90 °C.

It can be seen clearly from Fig. 4 that by increasing the column temperature, the problem observed at room temperature, i.e., deviation of retention times from the theoretical linear calibration lines, becomes alleviated. For the MC column, the retention times of the PEG standards up to MW of 442,800 fall nicely on linear calibration lines at temperatures above 70 °C, and for the MD column standards up to MW of 225,300 at temperatures above 90 °C. Standards with higher MW were not tested because they are likely to be outside the exclusion limits of the columns. In addition to the extension of the linearity of the calibration lines to higher MW values at elevated temperatures, the peak shape of the high MW PEGs were also improved significantly, which is evident by comparing the chromatograms shown in Fig. 5 (90 °C) with those in Fig. 3 (room temperature).

It is also obvious from Fig. 4 that all the components, including the PS standards, elute faster at elevated temperatures. In chromatography, retention is controlled by the distribution of an analyte between the mobile phase and the stationary phase, which can be expressed by

$$\ln K = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (1)$$

where K is the distribution coefficient, R is the gas constant, T is the absolute temperature, ΔH is the change of enthalpy and ΔS is the change of entropy. In ideal SEC, separation is only controlled by the entropy change of the molecules ($\Delta H = 0$), and Eq. (1) can be simplified as

$$\ln K_{SEC} = \frac{\Delta S}{R} \quad (2)$$

Thus, K_{SEC} is independent of temperature [19].

A major reason for the fast elution is the expansion of the mobile phase at elevated temperatures. In our SEC setup, the mobile phase is delivered at a given flow rate by a pump operated at room temperature. Because of the expansion, the actual mobile phase flow rate through the column will increase with temperature. By

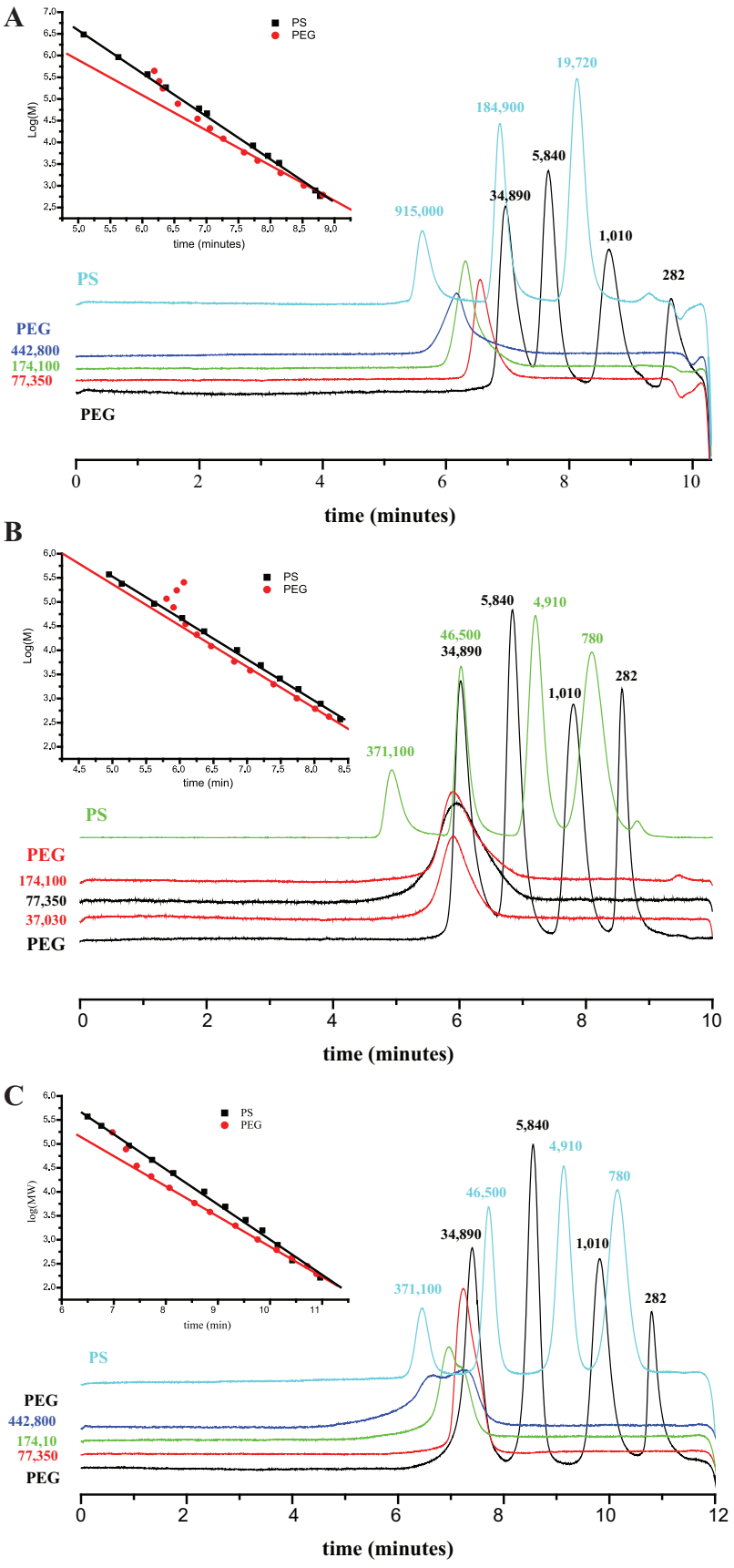

Fig. 3. SEC chromatograms of PEG and PS standards at room temperature. (A) PLgel Mixed-C column, 300 × 7.5 mm, 5 μm particles; (B) PLgel Mixed-D column, 300 × 7.5 mm, 5 μm particles; (C) SDV Linear M, 300 × 8.0 mm, 3 μm particles; other details as in Fig. 2.

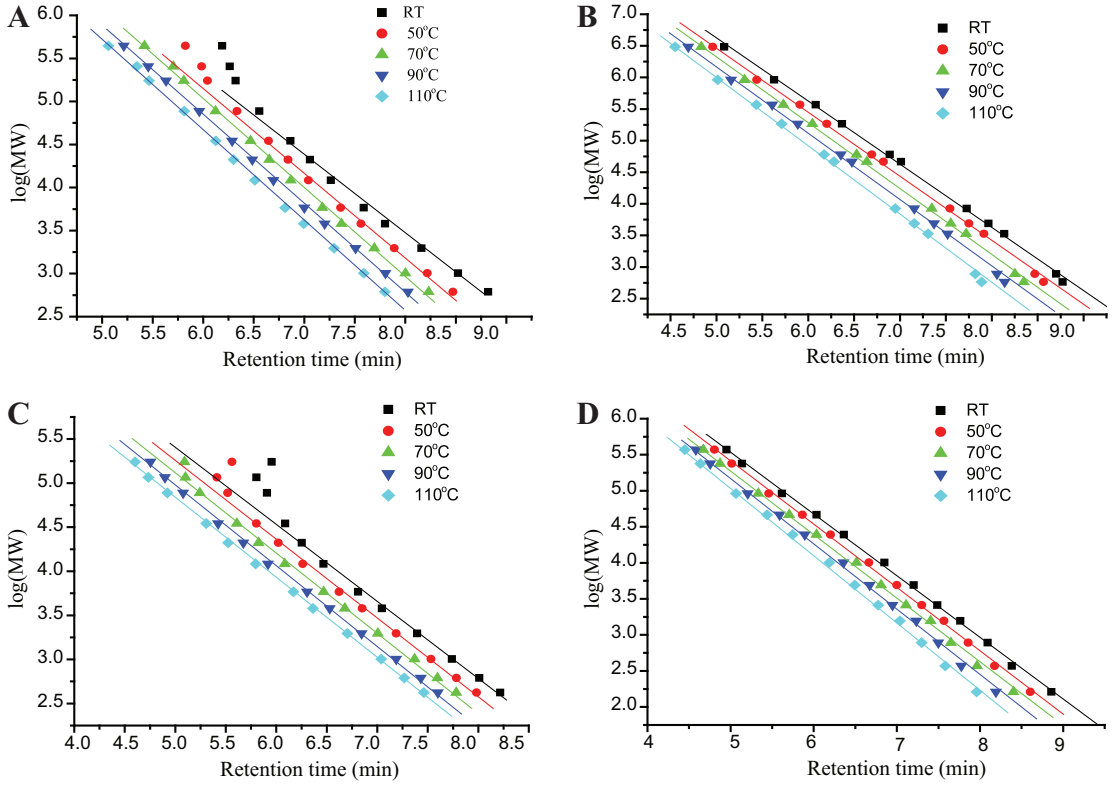

Fig. 4. Log(MW) vs. retention time curves of PEG and PS standards at different temperatures. (A) PEG standards on the PLgel Mixed-C column; (B) PS standards on the PLgel Mixed-C column; (C) PEG standards on the PLgel Mixed-D column; (D) PS standards on the PLgel Mixed-D column; other details as in Figs. 2 and 3.

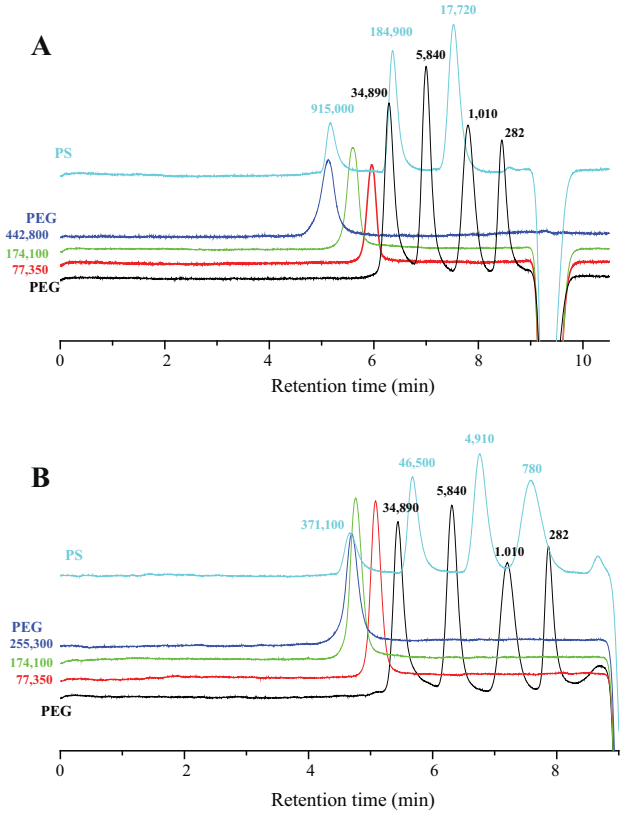

Fig. 5. SEC chromatograms of PEG and PS standards at 90 °C. (A) PLgel Mixed-C column; (B) PLgel Mixed-D column; other details as in Figs. 2 and 3.

normalizing the flow rate based on the retention times of toluene at different temperatures, the log(MW) vs. retention time curves in Fig. 4 can be transformed into log(MW) vs. retention volume curves (see Fig. 6). The adjusted retention volumes in Fig. 6 are calculated according to the following equation

$$V_{adj} = t_T \frac{t_{tol,RT} \times F_{RT}}{t_{tol,T}} \quad (3)$$

where V_{adj} is the adjusted retention volume of an analyte, t_T is the recorded analyte retention time at temperature T, $t_{tol,RT}$ is the retention time of toluene at room temperature, F_{RT} is the mobile phase flow rate at room temperature (1 mL/min) and $t_{tol,T}$ is the retention time of toluene at temperature T.

It can be seen from Fig. 6 that the reconstructed log(MW) vs. retention volume curves for the PS standards at different temperatures are on top of each other for both columns. The concurrence of the curves indicates that for the PS standards the K_{SEC} is indeed independent of temperature and the shorter retention time at higher temperatures is because of the expansion of the mobile phase. Similar results were also observed for the low MW PEG standards. For the high MW PEG samples, however, the situation is slightly different. As expected, at low temperatures the retention volumes of the high MW PEG samples do not fall on theoretically linear calibration lines because of their non-SEC retention behavior. By raising column temperature, the deviations from the linear lines become smaller. For the MC column over 70 °C and the MD column over 90 °C, the reconstructed calibration curves, covering the data points of the high MW PEGs, coincide with the linear lines. The improved fitting can easily be explained by using Eq. (1). For samples showing non-ideal SEC retention mechanism ($\Delta H \neq 0$), raising the column temperature will reduce the influence of ΔH on retention and thus, improving the SEC separation.

In addition to the MD and MC columns, the RP column was also tested with regard to the effects of raising temperature on the SEC

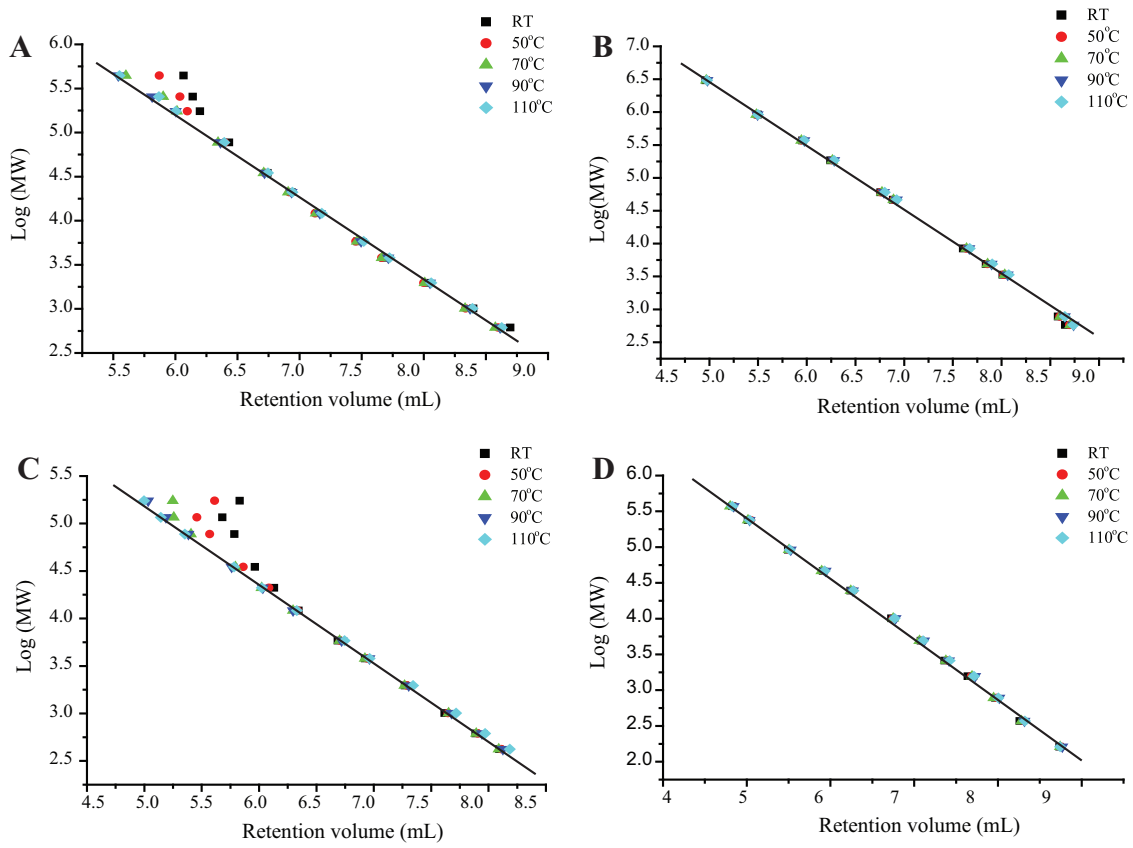

Fig. 6. Reconstructed log(MW) vs. elution volume curves by normalizing the flow rate based on the retention times of toluene at different temperatures. (A) PEG standards on the PLgel Mixed-C column; (B) PS standards on the PLgel Mixed-C column; (C) PEG standards on the PLgel Mixed-D column; (D) PS standards on the PLgel Mixed-D column; other details as in Figs. 2 and 3.

of PEG samples. As shown in Fig. 2, on the RP column high MW PEGs show strongly fronting peaks while the low MW PEGs show strongly tailing peaks. At elevated temperatures, the retention behavior and peak shape of the high MW PEG samples improved significantly, which is similar to what was observed for the MC and MD columns. In contrast, the improvement for the low MW PEGs (e.g., MW of 1080 and of 1900) is not enough, and they still show strongly tailing peaks even at 110 °C (see Fig. 7). According to the column specification, the T_{max} of the RP column is 150 °C. However, it was found that raising the column temperature above 120 °C can cause some changes in the column that are already enough to adversely affect the retention of PEG samples. This limitation of further increasing the column temperature will be discussed in the following section.

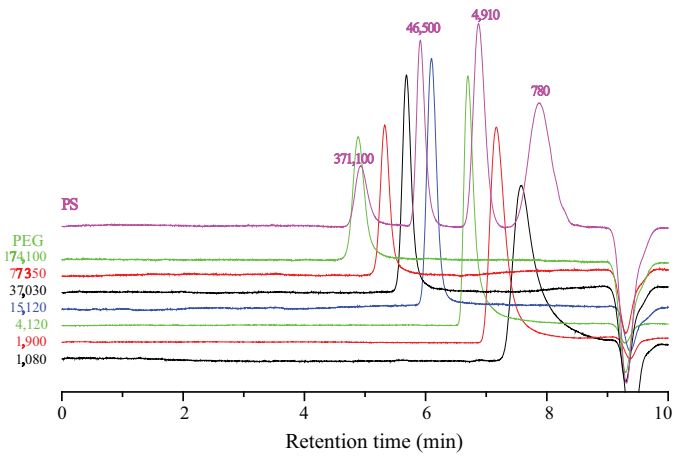

Fig. 7. SEC chromatograms of PEG and PS standards with a Resipore column at 110 °C; other details as in Fig. 2.

3.3. Limitations of superheated HT-SEC for PEG samples

In the previous section it has been illustrated that satisfactory SEC separations of PEGs can be obtained with MC and MD columns at superheated column temperatures. Nevertheless, for the RP column, tailing peaks for low MW PEGs were still remained to be a problem even at 110 °C (see Fig. 7). From the trends of improved peak shapes and improved separations at higher temperatures (by comparing Fig. 7 with Fig. 2 of the RP column), one would expect that further raising the column temperature might solve the problem encountered with the RP column. Unfortunately, the retention of PEG samples was found to be irreproducible and/or their peak shapes worsened significantly by raising the column temperature above 120 °C. In addition, the column performance could not be restored even after the column is cooled down to lower temperatures. Apparently, some irreversible changes have happened in the column at temperatures above 120 °C. Similar results were also observed for the MC and MD columns after being conditioned at temperatures above 120 °C.

According to the column specifications, the T_{max} of all the three columns is 150 °C. Indeed, on all these columns only minor/negligible changes of retention were observed for PS standards even after the columns being conditioned at 150 °C for a few hours. Some typical chromatograms of PEG and PS standards obtained before and after the column conditioning are given in Fig. 8. It must be noted here that the chromatograms of the RP

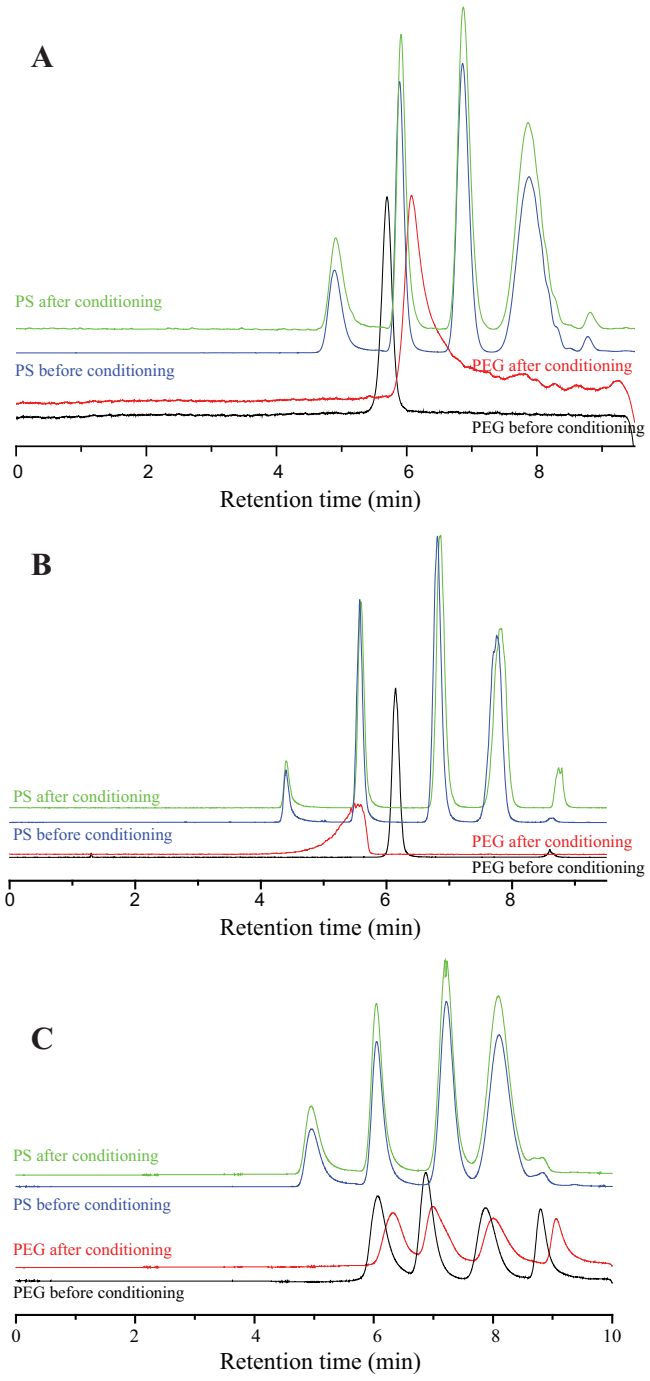

Fig. 8. Comparison of SEC chromatograms of PEG and PS standards obtained before and after conditioning the columns at high temperatures. (A) Resipore column conditioned at 120 °C for 8 h, measured at 90 °C, PS (EasyVial standard mixture), PEG (MW 37,030); (B) PLgel Mixed-C column conditioned at 150 °C for 8 h, measured at 90 °C, PS (EasyVial standard mixture), PEG (MW 37,030); (C) PLgel Mixed-D column conditioned at 120 °C for 8 h, measured at room temperature, PS (EasyVial standard mixture), PEG (EasyVial standard mixture).

and MC columns were recorded at 90 °C because the PEG standards selected for the test, which could earlier be eluted out nicely at room temperature (see Figs. 2 and 3), cannot be eluted out at room temperature after the conditioning. The reason why the column performance deteriorated so dramatically for PEGs but not for PSs is still unclear. Very likely, some small changes in the column packing material might have occurred when being conditioned at high temperatures. Although this small change is insignificant for the SEC of PS samples, it can be enormous for PEGs. In other words, retention of PEGs in SEC can be very sensitive to small changes in the stationary phase. Hence, the inertness and thermal stability of columns need to be further improved for different applications in superheated HT-SEC. The T_{max} values provided by the manufacturer are probably based on PS standards. For the separation of different polymers, these values can sometimes be questionable and should be reevaluated.

In superheated HT-SEC, another important column parameter, in addition to the T_{max}, is the P_{max}. The P_{max} values of most commercially available SEC columns that can be used for high temperature separations are low, normally lie between 15 and 50 bar [20]. Nevertheless, in traditional HT SEC separations the limit of P_{max} is not a big problem. Solvents with very high boiling points are generally used as the mobile phase, and separations are carried out at temperatures below the boiling point of the solvent. In contrast, however, columns with too low P_{max} values cannot be used in superheated HT-SEC because a long narrow bore tubing must be connected after the SEC column to cool down the mobile phase before it reaches the detector(s) and to provide enough backpressure to keep the mobile phase from boiling at temperatures above its normal boiling point. Due to the scarcity of HT columns that can be operated at high pressures (P_{max} > 50 bar), the selection of suitable columns for superheated HT SEC is very much restricted. Further development of thermally stable SEC columns with reasonably high P_{max} values is a topic that surely deserves some more attention.

4. Conclusions

SEC separation using chloroform as the mobile phase often experiences difficulties in the analysis of PEG samples. Even after a careful selection of the SEC column, high MW PEG samples might still give troublesome results. By raising the column temperature well above the normal boiling point of chloroform (superheated HT SEC), satisfactory SEC separation of PEG samples can be achieved with the MC and MD columns. The improved separation for the PEG samples at high temperatures is most likely due to their increased solubility in the mobile phase and reduced interaction with the stationary phase. It was found that PEGs are very sensitive to small changes of the column. Significant variations in retention of PEG standards were observed after the columns were conditioned above 120 °C for a few hours. In contrast, the columns were found to be stable if only based on the PS standards. Therefore, the thermal stability of an SEC column strongly depends on the analyte chosen for the test. Columns that are inert and thermally stable, not only based on PS standards, are required for superheated HT SEC applications. Although in this article only the separation of PEG samples using chloroform as the mobile phase was studied, superheated HT SEC should be a useful and convenient method for polymer characterization that is problematic at room temperature.

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