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Characterization of Poly(ethylene oxide)-*block*-poly(L-lactide) by HPLC and MALDI-TOF Mass Spectrometry

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Introduction

Poly(ethylene oxide)-*b*-poly(L-lactide) (PEO−PLLA), a nonionic water-soluble block copolymer with one hydrophilic poly(ethylene oxide) (PEO) component and the other a hydrophobic poly(L-lactide) (PLLA) component, are of growing interest in the field of pharmaceutical and biomedical applications because of its biodegradability and biocompatibility.[1,2] Various physical properties, such as the sol−gel transition, micellization, and degradation, need to be considered to optimize the functional characteristics of the block copolymer systems, and they are mainly determined by the molecular structure of the block copolymers. In particular, the balance of hydrophilic and hydrophobic components is crucial.[1−3] Consequently, the accurate molecular characterization of block copolymer is highly desired, even though synthetic copolymers are usually inhomogeneous in chemical composition as well as in molar mass, making accurate characterization a demanding task.

High-performance liquid chromatography (HPLC) is the most widely used method in characterizing the heterogeneity of copolymers.[4−17] In a two-dimensional approach, a compositional and/or molecular weight contour map of copolymers can be made by coupling two different kinds of liquid chromatography.[11−17] Among a number of different approaches in the characterization of copolymers, HPLC analysis at the transition point of size exclusion and interaction offers a possibility to characterize individual blocks of block copolymers separately.[8−13,18] For example, Pasch et al. and Gorshkov et al. have examined poly(ethylene oxide-*b*-propylene oxide) according to the length of poly(propylene oxide) by HPLC at the critical condition of PEO.[8,10,11] The critical condition of liquid chromatography is the transition point of the separation mode between size exclusion and adsorption, in which the retention of a particular polymer species becomes independent of the length of the polymer chain. Under this condition, for example, end-functional polymers are separated by the number and the type of the functional groups, while block copolymers can be separated in terms of the molecular weight of the other block. The critical condition can be achieved by controlling the eluent composition and/or temperature.

Recent advent of the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) method has brought about a new promising methodology in the analysis of copolymers.[10,19−22] In principle, thorough analysis of a high-resolution mass spectrum alone could provide the copolymer distribution function, but it still remains as a distant goal. MALDI-TOF MS detection combined with HPLC separation gives an opportunity to analyze chromatographic fractions very efficiently. For example, the characterization of each block of block copolymers to the molecular level is possible.[10] In this paper we report the characterization of individual blocks of a diblock copolymer PEO−PLLA: a high-resolution separation by HPLC in terms of PLLA block length under the critical condition of PEO and a precise characterization of PEO blocks by MALDI-TOF-MS analysis of the HPLC fractionated samples.

Experimental Section

The PEO−PLLA diblock copolymer was synthesized by ring-opening polymerization of L-lactide (Boehringer Ingelheim) on monomethoxypoly(ethylene oxide) (MPEO, Aldrich) catalyzed by tin(II) bis(2-ethylhexanoate) (stannous octoate, Sigma). The M_n and M_w/M_n of MPEO (where M_n is the number-average molecular weight and M_w is the weight-average molecular weight) were determined as 2000 and 1.10, respectively, by size exclusion chromatography (SEC) calibrated with poly(ethylene glycol) (PEG) standards. M_n of the diblock copolymer was estimated by ^{1}H NMR (Bruker, DPX 300 MHz). The peak from the methoxy end group of the PEO block at 3.38 ppm and the peak from the methine proton of the PLLA block at 5.18 ppm were used in determining the number-average degree of polymerization of the PLLA block relative to that of the PEO block. M_n of the diblock copolymer was determined as 2680, indicating that the average degree of polymerization of PLLA is less than 10.

The HPLC system consists of an HPLC pump (LDC, constaMetric 3200), a reversed phase column (Phenomenex, Luna C18, 100 Å pore size, 250 × 4.6 mm), a refractive index detector (Shodex, RI 71), and a UV−vis detector (LDC, spectroMonitor 3200). A mixture of acetonitrile (HPLC grade) and water (deionized and filtered with 0.45 μm membrane filter) at the volume ratio of 60/40 was used as the eluent. The temperature of the column was maintained within 0.1 °C by circulating a fluid through a column jacket.

All the HPLC fractionated polymer samples were dried under vacuum and then diluted with 200 μL of THF (Fisher) which was distilled over sodium metal before use. A 2 μL aliquot of this solution was mixed with a 5 μL volume of the matrix solution which was 0.1 M 2,5-dihydroxybenzoic acid (Aldrich) in THF. A 1 μL aliquot of the above solution was pipetted on the MALDI sample plate, and the THF was allowed to evaporate in air. A Voyager Elite MALDI-TOF MS (Perkin-Elmer PerSeptive Biosystem) equipped with a 337 nm N_2 laser was used. All the spectra were obtained using an accelerating voltage of 25 kV and a laser intensity of approximately 10% greater than threshold. The grid voltage, guide wire voltage, and delay time were chosen for each spectrum such as to achieve a signal-to-noise ratio as high as possible. Mass calibration was performed using protein standards from a Sequazyme Peptide Mass Standard Kit (PerSeptive Biosystems) and a two-point calibration method.

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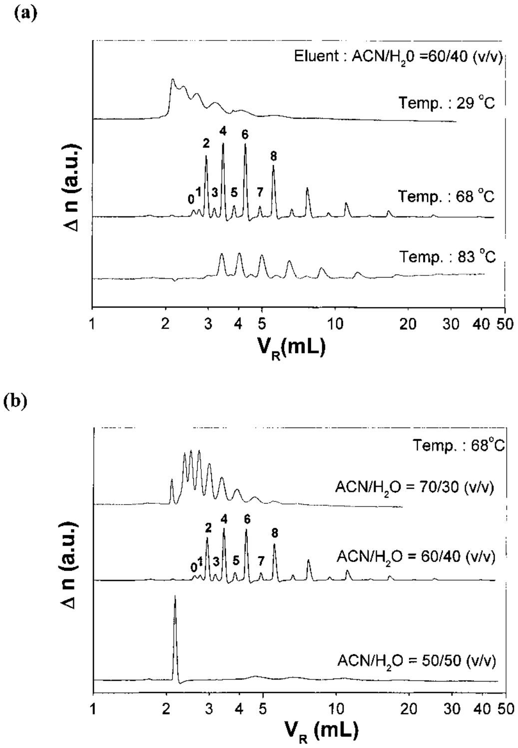

Figure 1. Change of HPLC chromatograms of the PEO−PLLA diblock copolymer at the off-critical conditions of PEG: (a) temperature effect, (b) solvent composition effect. The middle HPLC chromatograms in (a) and (b) are the same as the chromatogram of PEO−PLLA diblock copolymer at the critical condition of PEG.

Results and Discussion

HPLC Chromatograms under the Critical Condition of PEG. Figure 1 displays the chromatograms of the PEO−PLLA diblock copolymer at different temperatures (a) and at different eluent compositions (b). The middle chromatograms in Figure 1a,b display the separation at the critical condition of PEG, the mobile phase of acetonitrile/water (60/40, v/v), and the column temperature of 68 °C. The critical condition of PEG is obtained empirically by monitoring the retention volume (V_R) of three different molecular weight PEG standards (M_n: 1500, 3400, and 8000).[23] At this condition, the retention of PEG is independent of molecular weight and eluted at the injection solvent peak position, the PEO block is “invisible” in principle,[6,18] and the PEO−PLLA block copolymer behaves like a PLLA homopolymer of the molecular weight equivalent to the PLLA block as far as the HPLC retention behavior is concerned.

If the HPLC separation condition is changed from the critical condition by altering either eluent composition or temperature, the retention behavior of PEO−PLLA was affected by both the interaction mechanism with the stationary phase and the size exclusion mechanism of both PLLA and PEO blocks. This is well illustrated in Figure 1. At a temperature higher than 68 °C or with the water-rich mobile phase, we observed a longer retention of the block copolymers and broadening of individual peaks. This indicates that the PEO block becomes more interactive with the stationary phase, and the retention of the block copolymer is influenced by the presence of the PEO block at this off-critical condition. On the other hand, at temperatures lower than 68 °C or with the acetonitrile-rich mobile phase, the peaks are shifted to lower retention volume and begin to overlap each other. This is an indication that the interaction with the stationary phase becomes weaker, and the size exclusion mechanism starts to dominate for the PEO block. These trends are consistent with that the sorption of PEO to the stationary phase is endothermic and acetonitrile is a better solvent than water for PEO.[23]

In the middle chromatograms shown in Figure 1, the number of lactide residues is labeled for each peak. The identification of the peaks was done by MALDI-TOF MS analysis to be detailed later. The small peak labeled 0 prior to one lactide residue peak is the MPEO precursor without lactide residue, and the injection solvent peak appears around V_R of 2.2 mL. We confirmed the position of the MPEO peak by injection of the precursor MPEO. The fact that the MPEO peak does not appear at the solvent peak position indicates that the critical condition of MPEO is slightly different from PEG due to the end effect. PEG has a hydroxyl group at both ends of a chain while MPEO has a hydroxyl group at one end and a methoxy group at the other end, which is a large enough difference to exhibit a measurable change in the critical condition.[8,23] We have not tried to repeat the experiments at the precise critical condition of MPEO since the separation of the PEO−PLLA block copolymers at the critical condition of PEG is satisfactory for our purpose, and we can also identify the unreacted MPEO precursor at this separation condition.

In Figure 1 we use the log scale abscissa for the retention volume in order to show all the peaks in a reasonable space. According to a well-known empirical relation, the so-called Martin’s rule, the interaction strength of a homologue chemical species with the stationary phase is proportional to the degree of polymerization, which leads to the logarithmic dependence of the retention volume on the degree of polymerization.[24] If Martin’s rule works well and the retention of the block copolymers is governed by the number of lactide units only, all the peaks would be equally spaced in this plot. Despite the fact that the peaks are not exactly equally spaced, likely due to the off-critical condition of MPEO in part, the log scale abscissa is effective for displaying the chromatogram. It is apparent that the block copolymer species with even numbers of lactide residues have much higher abundance than that with an odd number of residues. This even−odd effect is expected if we consider the fact that the cyclic lactide dimers were used in the polymerization of the PLLA block. If all the reaction proceeds ideally so that two units of lactide moiety are added in each growing step and side reactions are absent, only the even-numbered lactide units would be found. We think that a small amount of lactic acid hydrolyzed from the cyclic dimers was involved in the polymerization to result in the odd number of repeat units. In the recent report on the HPLC analysis of PLLA oligomers of de Jong et al., no such even−odd effect was observed.[25] They speculated that the intermolecular transesterification during the polymerization would erase the even−odd effect in their PLLA oligomers.[26] The different polymerization conditions, their bulk polymerization vs solution polymerization in this work, may explain the difference.

In principle, a quantitative analysis of the molecular weight distribution of the PLLA block is possible from the middle chromatogram shown in Figure 1. It requires detailed information on the refractive index increment (dn/dc) of PLLA and PEO blocks since the response of

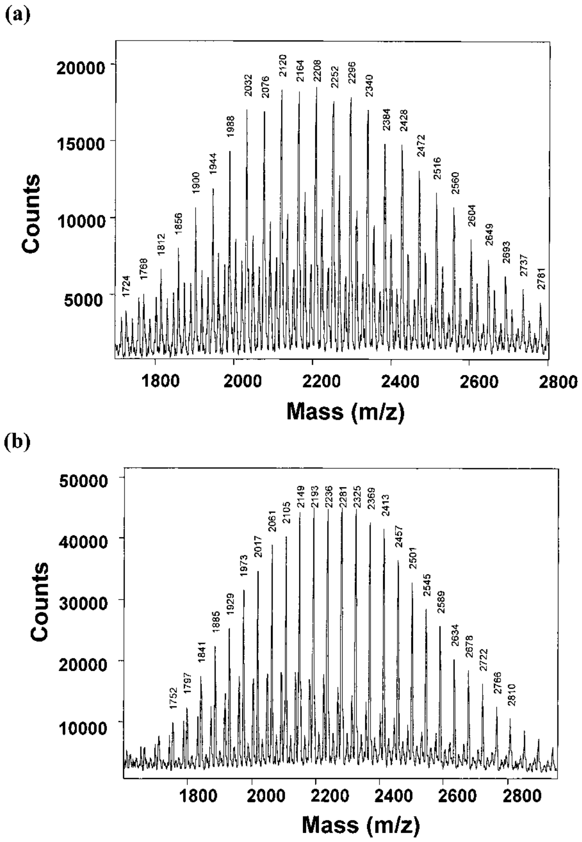

Figure 2. MALDI-TOF MS spectra of PEO−PLLA fractionated at the critical condition of PEG: (a) fraction 3 and (b) fraction 4 of the middle chromatograms in Figure 1.

the refractive index detector depends on the d*n*/d*c* of the individual block. Furthermore, the d*n*/d*c* of polymers depends on their degree of polymerization, especially for low molecular weight oligomers. Therefore, we did not attempt such an analysis in this work.

MALDI-TOF MS Analysis of HPLC Fractions. To further characterize the individual HPLC peaks, the peak fractions were collected and analyzed by MALDI-TOF MS. Parts a and b of Figure 2 show MALDI-TOF MS spectra of fractions 3 and 4 in Figure 1, respectively. These spectra appear as a series of groups of triplet peaks, each group being made up of a large peak (m) and two small peaks appearing at mass numbers of ($m + 16$) and ($m + 32$). Figures on the top of each large peak represent the molar mass rounded to the nearest whole number. The mass difference between the corresponding large (m) and small ($m + 16$ and $m + 32$) peaks in each group is 44, which indicates that each of these peaks represents masses that differ by one EO group. We will first interpret the pattern of large peaks, and the origin of the small peaks will be discussed later.

All polymer species consist of x ethylene oxide (EO) units and y lactide (LLA) units plus end groups of CH_3O and H ($CH_3O{-}(EO)_x(LLA)_yH$). Therefore, the major peak labeled 2208 in Figure 2a is made up of 44 EO groups ($m = 44.05$), 3 LLA groups ($m = 72.06$), and end groups of CH_3O and H ($m = 32.04$). The additional mass ($m = 23$) is accounted for by one sodium ion that was present in the sample as an impurity. The next large peak at $m = 2252$ contains one additional EO group. Thus, the large peak in each triplet differs by one ethylene oxide group from the large peak in a neighboring triplet but has the same number of lactide groups ($CH_3O{-}(EO)_x(LLA)_3H$).

Table 1. Molecular Weight Characterization Result of PEO−PLLA Block Copolymer by MALDI-TOF MS

peak no.	M_n of PEO−PLLA	M_n of PEO block[a]	M_w/M_n of PEO block
1	2072	1977	1.026
2	2195	2028	1.027
3	2202	1963	1.026
4	2264	1953	1.027
5	2395	2012	1.019
6	2472	2017	1.023
7	2574	2047	1.021
8	2669	2070	1.014

[a] M_n(PEO−PLLA) − {72.06 × number of LLA units} − 22.99 (Na).

A similar triplet pattern is found in the MALDI-TOF MS spectrum of fraction 4. Here the major peak in each triplet has four lactide groups. For instance, the major peak labeled 2281 differs from the major peak discussed in fraction 3 (2208) by 73 additional mass units, which indicates that it has the same number of EO groups but one more lactide group. Therefore, this peak (2281) corresponds to $CH_3O{-}(EO)_{44}(LLA)_4H$. The cationizing agent again was sodium ion. It confirms the HPLC peak assignment that the fraction Y corresponds to the diblock copolymer with Y lactide residues, and the corresponding MALDI-TOF MS spectrum represents the molecular weight distribution of the PEO block. Then the shapes of the mass spectra for all fractions have to be identical. We calculated M_n and M_w/M_n of the PEO block from the mass spectrum of each fraction and tabulated the results in Table 1. Thus determined M_n's of the PEO block in HPLC fractions are somewhat scattered. We believe the deviation is from the uncertainty of the MALDI-TOF MS analysis since the M_n values do not show a noticeable trend among different HPLC fractions. The average value of the eight fractions in Table 1 is 2008 ± 41, which is in excellent agreement with our SEC analysis of the precursor MPEO.

Regarding the minor peaks, the presence of the $m + 16$ and $m + 32$ peaks found in each triplet can be explained by degradation of the polymer by loss of lactide units during the transport and storage of the fractionated samples after the separation had taken place. As mentioned in the Introduction, PLLA is easily degradable. The minor peak representing ($m + 32$) can be identified as ($CH_3O{-}(EO)_{x+4}(LLA)_{y-2}H$). The envelop of ($m + 32$) peaks clearly shifts to lower molecular weight by about 150 mass units, indicating the loss of two lactide units. The $m + 16$ peaks seem to represent either $CH_3O{-}(EO)_{x+2}(LLA)_{y-1}H$ or potassium ion attached species. To clarify this question, an excess of potassium ion was added to a sample to ensure that all species were cationized with potassium. It was observed in these spectra that the large peaks and small peaks were all shifted by 16 mass units and that the original pattern of triplets (large peak followed by two small peaks) remained. This indicates that all species in the original spectrum were cationized with sodium and that the $m + 16$ peaks are in fact caused by the presence of $CH_3O{-}(EO)_{x+2}(LLA)_{y-1}H$.

In general, we have found in the MALDI-TOF MS spectra of the remaining fractions this same pattern of peaks with the peaks of major intensities having the same number of LLA groups as the fraction number although the relative intensities of the triplet fluctuate likely due to the different extent of sample degradation. The $m + 32$ peak was not seen in fraction 1 as would be expected. Additional degradation was observed in these samples when spectra were obtained after a few months storage, resulting in larger $m + 16$ and $m + 32$ peaks. Combining these observations, it is quite convincing that the minor peaks stemmed from the degradation of the PLLA block. Such an analysis can be used to monitor the degradation mechanism and/or kinetics of the PLLA block.

In summary, we have analyzed individual blocks in a PEO−PLLA diblock copolymer to the molecular level combining the critical condition HPLC and MALDI-TOF MS. At the critical condition of PEO block, each oligomeric PLLA block species was resolved by reversed-phase HPLC. It was found that the population with even numbers of LLA residues was far more abundant than the odd-numbered ones, which is consistent with the synthetic scheme of using cyclic lactide dimers as the monomer for the polymerization of PLLA block. From the analysis of each fractionated peak by MALDI-TOF MS, it was confirmed that the molecular weight distributions of PEO blocks with different numbers of LLA residues are identical within experimental precision and that the M_n of PEO blocks are in good agreement with the SEC analysis result of the precursor MPEO. The loss of lactide units by degradation after the fractionation was also clearly observed.

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