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Application of Gel Permeation Chromatograph–Low Angle Laser Light Scattering System to Kinetic Study on Degradation of Bioerodible Polymers

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Data analysis software for gel permeation chromatography coupled with low-angle laser light-scattering photometry (GPC-LALLS) was developed which was especially suitable for kinetic studies of polymer degradation, where the molecular weight of the polymer decreases with time. The GPC-LALLS system developed was applied to a kinetic study on enzymatic degradation of poly-L-glutamic acid. The molecular weight distribution (MWD) could be quickly and easily followed as a function of time by this system. A difference in the MWD time course was clearly shown for the degradations induced by α-chymotrypsin and carboxypeptidase B. The MWD time courses observed in the two cases were interpreted by computer simulation and the degradation mechanism was discussed. This system was found to provide valuable data on the degradation mechanism, and to be useful for evaluation of the degradability of biodegradable polymers.

Keywords——biodegradable polymer; molecular weight distribution; low-angle laser light-scattering photometry; gel permeation chromatography; poly-L-glutamic acid; enzymatic hydrolysis; degradation kinetics

Biodegradable polymers such as polyesters and poly(amino acid)s have been investigated as matrices for controlled-release drug-delivery systems.[2] Biodegradability of these polymers determines not only the elimination profile of the matrices but also the drug release profile. Therefore, degradation profiles should be fully understood before such polymers are applied to drug-delivery systems. Several papers have dealt with quantitative investigation on polymer degradation, observing molecular weight averages or molecular weight distribution (MWD) as a function of time. Measurement of viscosity has been carried out during degradation of poly(D,L-lactide)[3] and poly(L-amino acid)s.[4] This procedure, however, provides only semiquantitative information on the molecular weight. Even if viscosity data can be quantitatively converted to molecular weight by establishing the relationship between the intrinsic viscosity and the molecular weight, this method estimates just a molecular weight average, but provides no information on the MWD. On the other hand, gel permeation chromatography (GPC) has been used for kinetic studies on degradation of biodegradable polymers including polyglutamines,[5] polylactides[6,7] and poly(lactide-co-glycolide).[8] In order to extract molecular weight information from the GPC data, it is necessary to establish a calibration relating the retention volume to the molecular weight of the polymer sample. Calibration with narrow-MWD standards is subject to large errors, because the molecular shape of the polymers used as calibration standards may be different from that of the polymer sample whose molecular weight is being determined. It has been reported that more accurate calibration curves can be obtained by the universal calibration method.[9] This method, however, involves tedious steps—measurement of the intrinsic viscosity and establishment of the Mark-Houwink relationship for the standards and the polymer sample, respectively.

GPC coupled with low-angle laser light-scattering photometry (GPC-LALLS) has been successfully applied to the measurement of the MWD of macromolecules.[10-13] The GPC-LALLS method has a great advantage in that much information on the MWD can be quickly and easily obtained without calibration of the GPC column, since the low-angle laser light-scattering photometry (LALLS) gives absolute molecular weight data.

This paper describes work carried out to develop data analysis software for the GPC-LALLS method, which is particularly suitable for kinetic studies, and to apply the GPC-LALLS system developed to a kinetic study on degradation of bioerodible polymers. Enzymatic degradation of polyglutamate, which is a model of biodegradable polymers, was studied, and the degradation mechanism was considered on the basis of the observed change of the MWD.

Experimental

GPC-LALLS System——A low-angle laser light-scattering photometer (LS-8000, Tosoh) and a refractometer (RI-8011, Tosoh) were coupled to a gel permeation chromatograph. GPC was carried out with TSK gel columns ($G4000PW_{XL}+G3000PW_{XL}+G2500PW_{XL}$ (300 × 7.8 mm i.d. × 3) maintained at 40 °C in a column oven (RE-8000, Tosoh). The mobile phase was a 50 mM phosphate buffer solution containing 0.005% NaN_3 (pH 7.4), which was filtered through a 0.22 μm membrane filter (FM-22, Fuji Photo Film Co., Ltd.), degassed by a type 880-51 apparatus (Japan Spectroscopic Co., Ltd.) to prevent the formation of gas bubbles in the refractive index (RI) cell, and delivered at a rate of 0.8 ml/min by a pump (CCPD, Tosoh).

The analog signals from the RI and LALLS detectors were amplified (Type 3131 amplifier, Yokogawa), and collected by an A/D converter with 12-bit resolution (EC-2325, Elmec). The low-pass filter was 10 Hz, and the sampling time was 30 ms. The digitized data point readings for the RI and LALLS detectors were separately averaged in real-time to obtain a series of mean value data points for each detector. The averaging process was conducted over 10 data point readings. The digitized data were then transferred to the RAM (640 kbyte) of a personal computer (PC-9801 VMII, NEC). Programs for data acquisition were written in BASIC and compiled.

Data Analysis for GPC-LALLS System——The raw data of the LALLS and RI were stored on disk files (PC-9801, VMII, NEC) for manipulation. The spike noise observed in the LALLS signals was eliminated by neglecting the signals whose intensity increments were more than 10 times larger than that observed for the ordinary peak of the chromatogram.[14] Since the signal-to-noise (S/N) ratio was relatively low, the LALLS signals were smoothed using a fast algorithm for the Fourier transform.[15]

For each sample chromatogram of the LALLS and RI stored on the disk file, the molecular weight (M_i) and the concentration (C_i) at a distinct time of the chromatogram were calculated from the LALLS and RI signals using Eqs. 1 and 2,[9,10]

$$C_i = k_1(x_i/\sum x_i) \tag{1}$$

$$M_i = \frac{1}{\frac{k_2 C_i}{y_i} - 2A_2 C_i} \tag{2}$$

where x_i and y_i are the RI and LALLS data point readings, respectively, and A_2 is the second virial coefficient. The instrumental constants, k_1 and k_2, were determined by obtaining the LALLS and RI chromatograms with a standard polymer whose molecular weight average was known, and by measuring the refractive index increment for the standard polymer and the sample polymer, respectively. Poly(ethylene oxide) (SE-8, Tosoh), was dissolved in the GPC mobile phase (0.1%), and used as the standard polymer solution. The value of A_2 was computed from the slope in the plots of k_2C_i/y_i against C_i according to Eq. 3, which follows from Eq. 2. The LALLS data for calculation of A_2 were obtained at various concentrations (0.02—0.1%) of the sample polymer in an off-line analysis. Dependence of the refractive index and A_2 on the molecular weight was considered to be negligible.

$$\frac{k_2 C_i}{y_i} = \frac{1}{M_i} + 2A_2 C_i \tag{3}$$

Before the calculation of C_i and M_i using Eqs. 1 and 2, the baseline for each of the LALLS and RI chromatograms was corrected and pairs of LALLS and RI data at a distinct time were obtained by correcting the difference in the observation time between the LALLS and RI detectors, which were connected in series. The time difference was

measured with poly(ethylene oxide) with a narrow MWD (SE-8, Tosoh). Since calculation of molecular weight from the small signals at both sides of the chromatograms can lead to misestimation, molecular weight at this range was computed by extrapolation of the molecular weight–retention time relationship (a polynomial relationship represented by Eq. 4), which was established from LALLS signals large enough to calculate the molecular weight (*i.e.*, larger than one-fifth of the highest signal in the chromatogram).

$$\ln M = a_0 + a_1 t + a_2 t^2 \tag{4}$$

Molecular weight distribution was determined from the molecular weight and concentration data calculated over the time range where RI signals were detected. Number average, M_n, and weight average, M_w, molecular weights were calculated without band-broadening correction. Programs for the data analysis (for the Fourier transform and calculations described above) were written in BASIC and compiled (PC-9801 VM II, NEC).

Kinetic Studies on Enzymatic Degradation of Poly-L-glutamic Acid——Poly-L-glutamic acid (degree of polymerization = 700, Ajinomoto Co.) was dissolved in an acetate buffer solution (pH 4.9) to make 0.2%, and incubated in the presence of α-chymotrypsin (0.2 mg/ml) (Boehringer Mannheim Yamanouchi, BMY) at 37 °C. At appropriate intervals, 500 μl of the sample solution was injected into the GPC through a 0.45 μm filter (HV, Millipore). Degradation of poly-L-glutamic acid by carboxypeptidase B (42 μg/ml) (BMY) was also studied in an acetate buffer solution (pH 6.5).

Computer Simulation of Enzymatic Hydrolysis——The MWD of biodegradable polymers subjected to enzymatic degradation was simulated as a function of time with a personal computer (PC-9801, VM II). A polymer with the same MWD as the poly-L-glutamic acid sample studied above was assumed to be subjected to random cleavage. A particular bond to be cleaved was successively determined by selecting a random number equal to or less than the total number of the bonds remaining.

Change of the MWD resulting from enzymatic hydrolysis which proceeded from the molecular ends of the polymer molecules was also simulated by selecting a random number equal to or less than the total number of molecules remaining. The program for the simulation was written in BASIC and compiled.

Results and Discussion

Data Analysis

Figure 1 shows a typical chromatogram of poly-L-glutamic acid obtained by the GPC-LALLS system. The LALLS signals include the spike noise which was caused by particles. In addition, the S/N ratio of the LALLS signals was much smaller than that of the RI signals. It was necessary to eliminate the spike noise and smooth the signal before the data analysis. The LALLS chromatogram obtained after elimination of the spike noise and smoothing by Fourier transform (see the experimental section) is shown in Fig. 2, indicating that these data processings are effective. The molecular weight and the concentration of the polymer at a distinct retention time were calculated from the RI data and the smoothed LALLS data. The

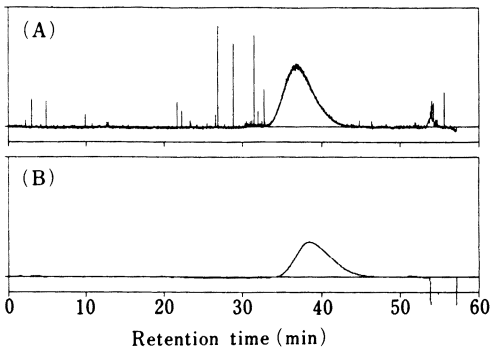

Fig. 1. Typical LALLS (A) and RI (B) Chromatograms of Poly-L-glutamic Acid

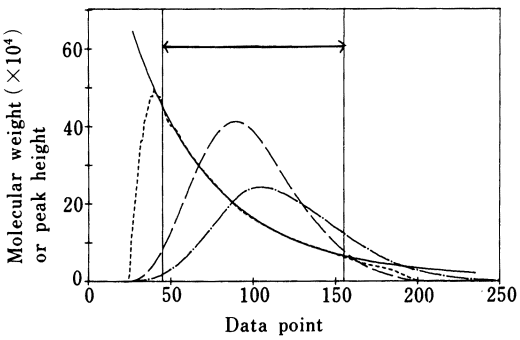

Fig. 2. Typical Calibration Curve for Molecular Weight of Poly-L-glutamic Acid

----, molecular weight calculated from LALLS (——) and RI (—·—) chromatograms; ——, molecular weight regression curve calculated from the data range indicated by ↔.

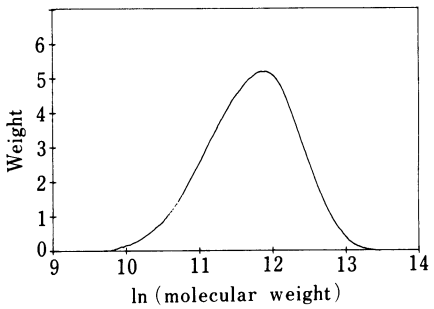

Fig. 3. Molecular Weight Distribution Determined by the GPC-LALLS System

The y-axis is in arbitrary units.

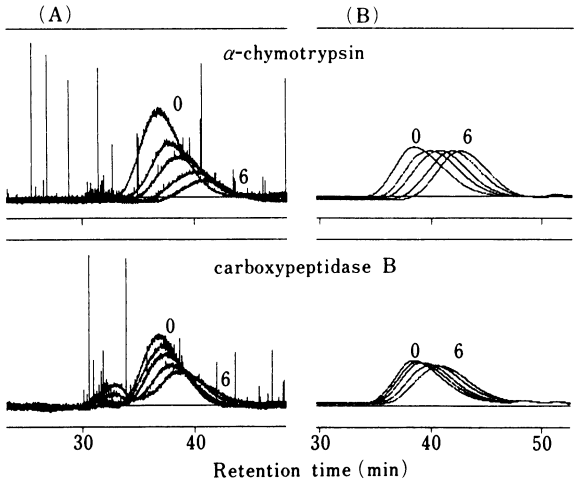

Fig. 4. LALLS (A) and RI (B) Chromatograms of Poly-L-glutamic Acid Dissolved in Enzyme Solutions Observed at Different Times (0, 1, 2, 4 and 6 h of Incubation)

molecular weights calculated are shown in Fig. 2, indicating that unreasonable values of molecular weight were obtained from the small signals at both sides of the chromatograms. Therefore, molecular weight was recalculated using a calibration curve which relates the retention time to the molecular weight. The calibration curve was established from the LALLS signals larger than a prescribed limit. The RI signals corresponding to these LALLS signals were large enough to provide reliable data. The range of the LALLS data used for establishment of the calibration curve is also shown in Fig. 2. The MWD was determined by using the calibration curve, and is shown in Fig. 3. The GPC-LALLS system coupled with the data analysis software seemed to provide a reasonable MWD. The reliability of the MWD obtained by this system was examined for degradation of a model biodegradable polymer as follows.

Enzymatic Degradation of Poly-L-glutamic Acid

Enzymatic degradation of poly-L-glutamic acid was studied by the GPC-LALLS system. Figure 4 shows the LALLS and RI chromatograms of the polymer incubated with an endopeptidase, α-chymotrypsin and an exopeptidase, carboxypeptidase B. Neither the enzymes nor the buffer components used interfered with the polymer peak. The chromatograms indicate that the polymer was degraded to smaller-molecular-weight molecules with time by each of the enzymes in different ways. The difference in the pattern of the chromatograms suggests a different mechanism of degradation for each of the enzymes. Further information on the degradation mechanism, however, is provided by determining the MWD.

As the polymer was degraded to smaller-molecular-weight molecules, the intensity of the LALLS signals decreased remarkably, as did the number of the LALLS signals which could be used for establishment of a calibration curve. This may result in poor reliability of the calibration curve established especially for the samples taken out at the later stage of degradation. Therefore, a calibration method was developed which was suitable for kinetic studies of polymer degradation where the molecular weight of the polymer decreased with time. A single calibration curve was established from all the chromatograms observed during the degradation process. The lowest limit of the LALLS signals to be used for calibration was fixed to be one-fifth of the largest signal in the chromatogram of the polymer sample which

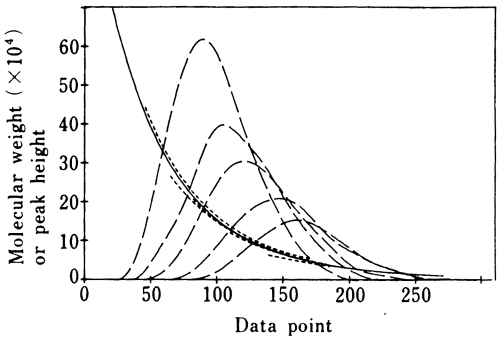

Fig. 5. Calibration

----, calibration curves calculated from each of the LALLS chromatograms (— — —); ——, calibration curve calculated from all of them.

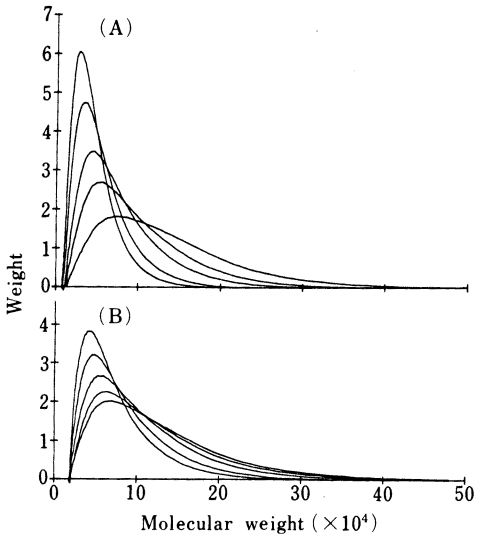

Fig. 6. Molecular Weight Distribution of Poly-L-glutamic Acid in α-Chymotrypsin (A) and Carboxypeptidase B (B) Solutions as a Function of Time

The y-axis is in arbitrary units.

TABLE I. Molecular Weight Averages of Poly-L-glutamic Acid Incubated in Enzyme Solutions

Incubation time (h)	α-Chymotrypsin			Carboxypeptidase B		
	$\bar{M}_n$	$\bar{M}_w$ ($\times 10^4$)	$\bar{M}_w/\bar{M}_n$	$\bar{M}_n$	$\bar{M}_w$ ($\times 10^4$)	$\bar{M}_w/\bar{M}_n$
0	91.6	136.1	1.49	91.6	136.1	1.49
1	67.8	99.5	1.47	85.4	123.0	1.44
2	55.1	79.2	1.44	75.8	107.7	1.42
4	41.4	57.4	1.39	64.6	88.4	1.37
6	34.2	46.4	1.36	56.3	75.4	1.34

was taken out at the time zero. All signals observed in the experiment higher than the lowest limit were used to establish the polynomial relationship between the retention time and the molecular weight. The calibration curve thus obtained seems to be more reliable than the one calculated from the small number of signals in each chromatogram, as shown in Fig. 5.

Figure 6 shows the MWD of poly-L-glutamic acid determined by the GPC-LALLS system according to the calibration curve established above. A rapid decrease of higher-molecular-weight polymer molecules was observed in the initial stage of the degradation by α-chymotrypsin. On the other hand, the decrease of higher-molecular-weight molecules was relatively slow in the degradation by carboxypeptidase B. A difference in the degradation mechanism was clearly shown by the MWD data obtained by the GPC-LALLS system. The molecular weight averages of poly-L-glutamic acid were determined as a function of time, and are shown in Table I.

Computer simulation of enzymatic degradation of polymers provided a clear interpretation of the degradation mechanisms. Figure 7 shows the MWD simulated on the

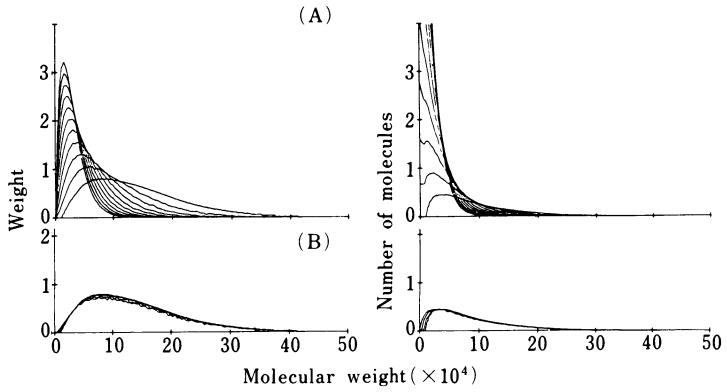

Fig. 7. Computer Simulation of MWD of Poly-L-glutamic Acid for Enzymatic Degradation

(A) Every 100000 random cleavages. (B) After 500000, 6000000 and 12000000 end cleavages. The y-axis is in arbitrary units.

assumption that the polymer sample of poly-L-glutamic acid used in this study is subjected to two types of hydrolysis: one is the hydrolysis by an endopeptidase which brings about random cleavage. The other is the hydrolysis induced by an ideal exopeptidase, which cleaves only the end peptide bond. Figure 7-A shows the remarkable change of the MWD after every 100000 random cleavages of the polymer, the total number of molecules of which at the time zero is 200000. This time course of the MWD is similar to that observed in the degradation of the poly-L-glutamic acid samples by α-chymotrypsin. On the other hand, even 2000000 end cleavages caused no major change in the MWD, as shown in Fig. 7-B. This explains the slow decrease of higher-molecular-weight molecules observed in the degradation of poly-L-glutamic acid by carboxypeptidase B. The MWD of poly-L-glutamic acid observed in the degradation induced by the enzymes was consistent with that obtained by computer simulation, indicating the high reliability of the GPC-LALLS system.

In conclusion, the GPC-LALLS system developed in this study can provide useful information on the MWD in kinetic studies on degradation of polymers. The MWD time courses of poly-L-glutamic acid in the enzymatic degradation were quickly and easily obtained by this system. A difference in the MWD time courses was clearly shown for the degradations induced by α-chymotrypsin and carboxypeptidase B. The reliability of this system was confirmed by computer simulation. This GPC-LALLS system was found to provide useful data on degradation mechanisms, and to be applicable for evaluation of the degradability of biodegradable polymers.

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