

Structural Study of PLGA [Copoly (DL-Lactic/Glycolic Acid)], a Biodegradable Polymer for Parenteral Sustained Release Preparations, by Tandem Mass Spectrometry

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A copoly (DL-lactic/glycolic acid) (PLGA), with a weight-average molecular weight of about 8400, has been characterized using fast atom bombardment (FAB)-tandem mass spectrometry in order to determine the sequence. Because of the large molecular size, PLGA was partially hydrolyzed and the terminal hydroxyl groups in the resulting oligomer mixture acetylated as the indicator. The FAB spectrum of this sample showed a complex ion signal pattern containing monomer to octamer. Diagnostic product ions containing useful information for sequence determination were observed in collision-induced dissociation-MS/MS and MS/MS/MS of these oligomer ions. The results of analysis for dimers through pentamers showed that they have random sequences of lactic and glycolic acid, suggesting that the whole structure of PLGA also has a random sequence.

Key words copoly (DL-lactic/glycolic acid); sequence; tandem mass spectrometry; FAB spectrum; collision-induced dissociation-MS/MS; MS/MS/MS

PLGA [copoly (DL-lactic/glycolic acid), molar ratio of lactic to glycolic acid *ca.* 3 : 1]¹⁻³ is a biodegradable polymer for parenteral sustained release preparation, and has been applied to Leuprorelin [Leuprolide, D-Leu⁶-(des-Gly¹⁰-NH₂)-luteinizing hormone-releasing hormone (LH-RH) ethylamide] acetate,⁴ a superactive agonist of LH-RH developed by Takeda Chemical Industries. PLGA is arranged around the drug like a micelle and forms microspheres, which can release Leuprorelin with an apparent zero-order rate over one month by subcutaneous injection.^{1,2} For analysis of PLGA, gel permeation chromatography, giving the rough molecular mass distribution and NMR spectroscopy, showing the copolymer molar ratio (lactic/glycolic acid), have been used.^{1,2} However, it is impossible to determine the sequence of lactic acid and glycolic acid in PLGA by using these methods.

Mass spectrometry gives absolute molecular masses with superior resolution and is a useful method to determine molecular mass distribution and to characterize the copolymer, as well as the homopolymer. To study the chemical structure of polymers, many studies using pyrolysis MS,^{5,6} FAB-MS,^{7,8} fourier-transform MS⁹ and matrix-assisted laser desorption ionization time-of-flight MS^{10,11} have been reported. The MS spectra of homopolymers are relatively simple, displaying a pattern of peaks separated by the mass number of the monomer. However, in the case of copolymers consisting of two or more components, the spectra are more complicated, and compositional information is much more difficult to extract. Thus, only a few studies to determine directly the sequence for a copolymer by MS have been reported.

The purpose of this work was to investigate sequence analysis of PLGA using tandem MS with collision-induced dissociation (CID) analysis. Because of the large molecular size, sequence determination of PLGA was considered to be quite difficult, and it was thus partially hydrolyzed to yield an oligomer mixture. When this oligomer mixture was applied

to CID directly, it could not be determined where the generated product ions come from in the precursor ions. However, by acetylation of the terminal hydroxyl group in each oligomer as an indicator, the terminal components could be clarified. This method was very useful for determination of the sequence of PLGA.

Experimental

Materials and Reagents PLGA (copolymer ratio of DL-lactic/glycolic acid *ca.* 3 : 1, weight-average molecular weight about 8400) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other reagents and solvents were of reagent grade and also from Wako Pure Chemical Industries.

Hydrolysis of PLGA A suspension of 100 mg of PLGA in 5 ml of water was stored for 3 d in a thermostat (Advantec CV-700 constant temperature incubator, Toyo Seisakusho Co., Ltd., Japan) controlled at 60 °C. The mixture was filtered through a membrane filter (Ekicordisc 13, Gelman Science Japan Ltd., Japan) with a pore size of 0.45 μm. The filtrate was lyophilized, and the residue dissolved in 5 ml of methanol for sample preparation.

Acetylation of Oligomer Mixture 1 ml of the filtrate obtained by hydrolysis of PLGA as mentioned above was lyophilized. The residue was dissolved in 1 ml of a mixture of acetic anhydride and pyridine (2 : 1) and allowed to stand at ambient temperature for 2 h. The solution was concentrated to dryness under a stream of N₂ gas and the residue dissolved in 1 ml of methanol for sample preparation.

Instruments and Conditions All mass spectra were obtained on a JEOL JMS-HX/HX 110A tandem mass spectrometer equipped with a FAB gun that produces a 6 keV xenon beam. Measurements were made in the negative-ion mode with glycerol as a matrix. The ion accelerating voltage was 10 kV, and argon was used as a collision gas. MS/MS spectra were obtained by performing CID in the third field-free region (3rd FFR) between MS-1 and MS-2. In the case of MS/MS/MS (MS³), 1st generation product ion generated from the precursor ion by CID in the first FFR was introduced to the 3rd FFR where CID was further performed. To the collision cell located in the 3rd FFR, voltage corresponding to 30% of the kinetic energy of the selected ion was passed through MS-1.

Results and Discussion

FAB Spectra of Water Soluble Hydrolyzed (Depolymerized) PLGA Figure 1 shows the FAB spectrum of the

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water-soluble fraction obtained by hydrolysis of PLGA in water at 60 °C for 3 d. The yield of oligomer mixture calculated from the weight of the lyophilized water-soluble fraction of PLGA was 70% of the initial amount. Table 1 shows the expected and detected values of $[M-H]^-$ for given oligomers. The spectrum showed a series of peaks separated by 72 u, based on homooligomers of lactic acid, and a series of peaks separated by 14 u from each homooligomer peak. This is because the molar weight of glycolic acid is 14 units less than that of lactic acid. In the case of pentamer as magnified in Fig. 1, m/z 377 (5L), 363 (4L+G), 349 (3L+2G) and 335 (2L+3G) were observed, wherein L and G represent lactic acid and glycolic acid, respectively. However, no information on the sequence of lactic and glycolic acid was obtained.

Figure 2 shows the FAB spectrum of acetylated oligomer mixture obtained by acetylation of the water soluble fraction from PLGA. By comparison with the non-acetylated oligomer mixture, each ion peak was shifted by 42 u to

higher mass, corresponding to the mass gain by acetylation (Table 1). It was noteworthy that while the distribution pattern of non-acetylated oligomers from PLGA had a valley (Fig. 1) owing to weak intensity around the trimer and tetramer peaks, an increment in their intensity was observed by acetylation. Table 1 includes the expected and detected values of $[M-H]^-$ given by acetylation.

Structural Study of PLGA by CID-MS/MS and MS/MS (MS^3) The structures of homooligomers consisting of either lactic acid or glycolic acid were easily determined by mass number, but those of heterooligomers containing both components could not be determined. Therefore, acetylated dimers to pentamers derived from PLGA were analyzed by CID-MS/MS and MS^3 as described below.

Dimer The precursor ion at m/z 189, as shown in Fig. 2, was identified as Ac-(L+G) and has two possible sequences, Ac-L-G and Ac-G-L (Chart 1), wherein Ac represents an acetyl group. Figure 3 shows the MS/MS product ion spectrum at m/z 189. The observed product ions at m/z 131 and 117 were assigned to $[M-H]^-$ of Ac-L and Ac-G, respectively, indicating the existence of both Ac-L-G and Ac-G-L. In this way, sequences of Ac-L-G and Ac-G-L which can not be distinguished without acetylation were distinctively detected. Also note that the spectrum was very simple since electron charge was left in the fragment with the acetyl group selectively.

Trimer The precursor ion at m/z 261 as shown in Fig. 2 was identified as Ac-(2L+G) and has three possible sequences, Ac-L-L-G, Ac-L-G-L and Ac-G-L-L (Chart 2). Figure 4A shows the MS/MS product ion spectrum at m/z 261. The observed product ion at m/z 203 was assigned to $[M-H]^-$ of Ac-L-L, which means the existence of Ac-L-L-G. Additionally, product ion at m/z 189 has two possible sequences, Ac-L-G and Ac-G-L as mentioned above. Therefore, CID of the 1st generation product ion at m/z 189 from precursor ion at m/z 261 was performed. From the results as shown in Fig. 4B, 2nd generation product ions at m/z 131 and 117 were observed and assigned to $[M-H]^-$ of Ac-L and Ac-G, respectively. These results indicate the existence of Ac-L-L-G.

Table 1. Calculated Values for Molecular-Related Ions of Oligomers Obtained from PLGA

$$H \left[\begin{array}{c} O \\ | \\ -C- \\ | \\ CH_3 \end{array} \right]_m \left[\begin{array}{c} O \\ || \\ -C- \\ | \\ OH \end{array} \right]_n$$

$m^{(a)}$	$n^{(a)}$				
	0	1	2	3	4
0	—	75 (117)	133 (175)	191 (233)	249 (291)
1	89 (131)	147 (189)	205 (247)	263 (305)	321 (363)
2	161 (203)	219 (261)	277 (319)	335 (377)	393 (435)
3	233 (275)	291 (333)	349 (391)	407 (449)	465 (507)
4	305 (347)	363 (405)	421 (463)	479 (521)	537 (579)
5	377 (419)	435 (477)	493 (535)	551 (593)	609 (651)
6	449 (491)	507 (549)	565 (607)	623 (665)	681 (723)
7	521 (563)	579 (621)	637 (679)	695 (737)	753 (795)
8	593 (635)	651 (693)	709 (751)	767 (809)	825 (867)
9	665 (707)	723 (765)	781 (823)	839 (881)	897 (939)
10	737 (779)	795 (837)	853 (895)	911 (953)	969 (1011)

a) m and n are the numbers of lactic acid and glycolic acid, respectively. () : Acetylated. \square : Observed ions in the FAB spectra.

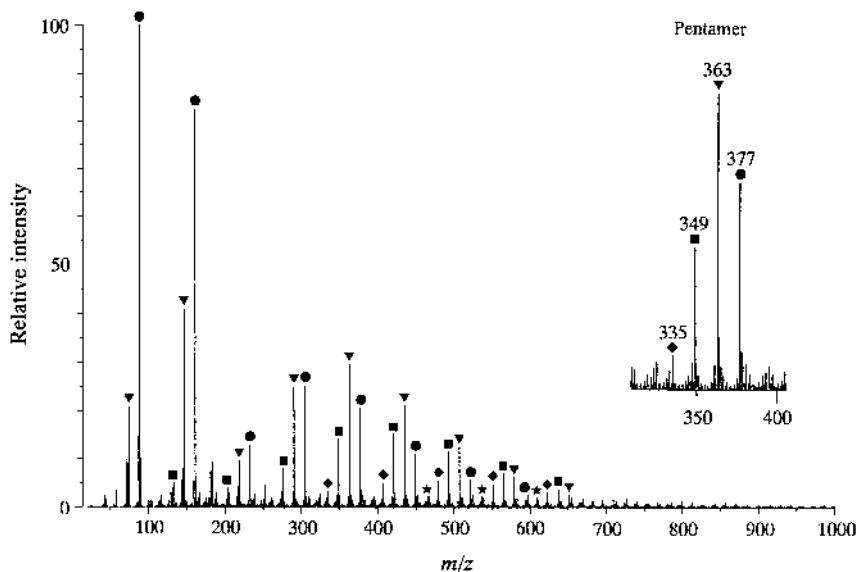


Fig. 1. FAB Spectrum of Water-Soluble Fraction Obtained by Hydrolysis of PLGA Suspension at 60 °C for 3 d
Labels designate copolymer compositions for ions with the empirical formula $[L_mG_n-H]^-$, where n =the number of associated glycolic acids \bullet =0, ∇ =1, \blacksquare =2, \blacklozenge =3 or \star =4.

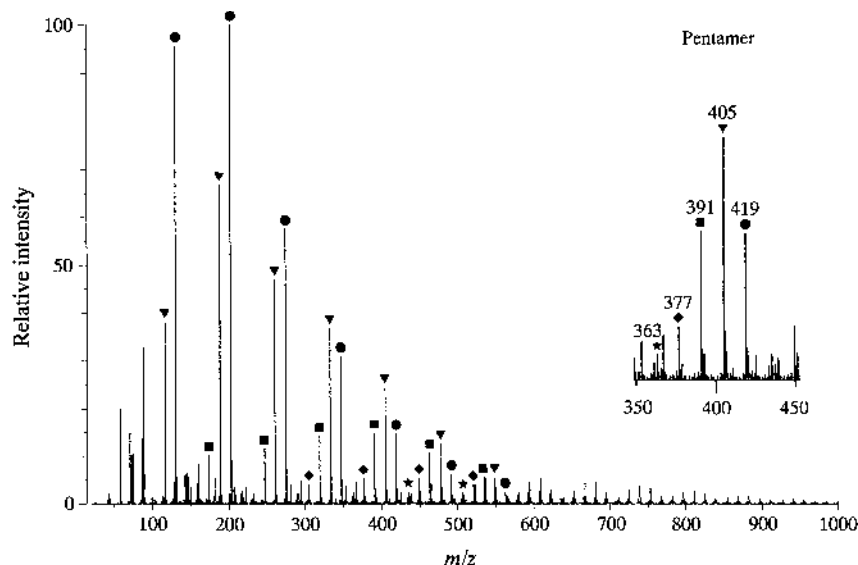


Fig. 2. FAB Spectrum of Acetylated Water-Soluble Fraction Obtained by Hydrolysis of PLGA Suspension at 60 °C for 3 d
 Labels designate copolymer compositions for ions with the empirical formula $[L_mG_n-H]^-$, where n =the number of associated glycolic acids ●=0, ▼=1, ■=2, ◆=3 or ★=4.



Chart 1. CID Analysis of m/z 189

Chart 2. CID Analysis of m/z 261

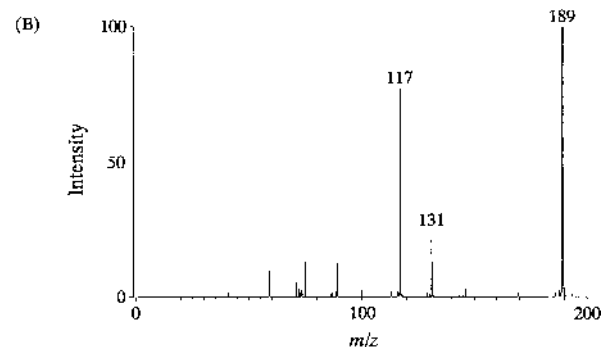
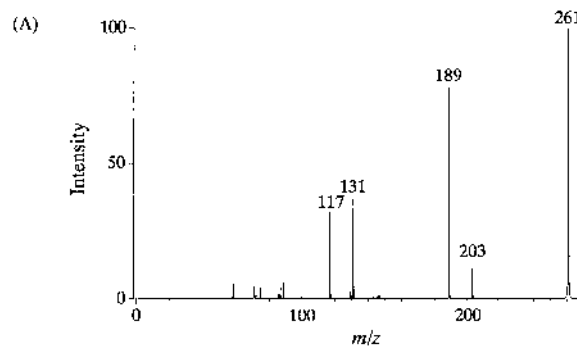
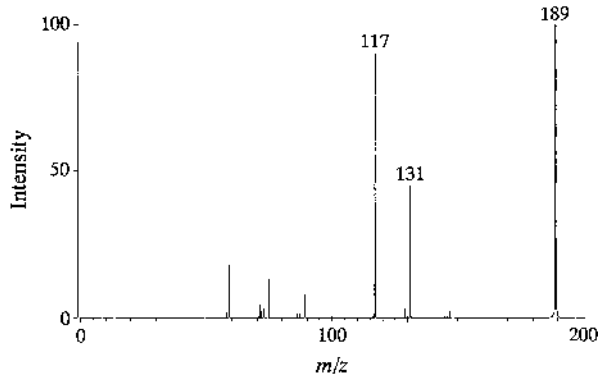


Fig. 3. CID-MS/MS Spectrum of m/z 189

Fig. 4. CID Spectra of m/z 261
 (A): MS/MS Spectrum, (B): MS³ Spectrum (Precursor ion: 261, 1st generation product ion: 189).

tence of Ac-L-G and Ac-G-L, namely Ac-L-G-L and Ac-G-L-L. In this manner, all of the three possible sequences of precursor ion at m/z 261 were confirmed.

In the same manner as described above, the precursor ion at m/z 247 as shown in Fig. 2 was also confirmed to contain three possible sequences, Ac-L-G-G, Ac-G-L-G and Ac-G-G-L.

Tetramer The precursor ion at m/z 333 as shown in Fig. 2 was identified as Ac-(3L+G) and has four possible sequences, Ac-L-L-L-G, Ac-L-L-G-L, Ac-L-G-L-L and Ac-G-L-L-L (Chart 3). The MS/MS product ion spectrum of this peak gives product ions at m/z 275 and 117 (Fig. 5A), which were assigned to $[M-H]^-$ of Ac-L-L-L and Ac-G, respectively, indicating the existence of Ac-L-L-L-G and Ac-G-L-L-L.

The 1st generation product ion at m/z 261 has three possi-

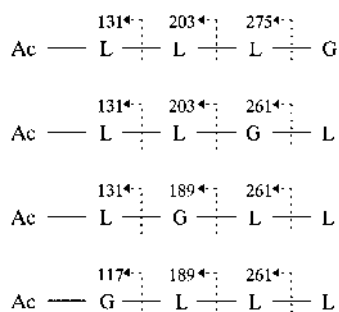


Chart 3. CID Analysis of m/z 333

Table 2. Sequence Determination of Pentamer

m/z	Sequence	Method for sequencing analysis		
		MS	MS/MS	MS ³
349	Ac-G-G-G-G-G	×		
363	Ac-L-G-G-G-G		○	
	Ac-G-L-G-G-G			×
	Ac-G-G-L-G-G			×
	Ac-G-G-G-L-G			×
	Ac-G-G-G-G-L		○	
	Ac-L-L-G-G-G		○	
	Ac-L-G-L-G-G			
	Ac-L-G-G-L-G			
	Ac-L-G-G-G-L			○
	Ac-G-L-L-G-G			○
	Ac-G-L-G-L-G			
	Ac-G-G-L-L-G			○
391	Ac-G-G-G-L-L		○	
	Ac-L-L-L-G-G		○	
	Ac-L-L-G-L-G			
	Ac-L-L-G-G-L			○
	Ac-L-G-L-L-G			
	Ac-L-G-L-G-L			
	Ac-L-G-G-L-L			○
	Ac-G-L-L-L-G			○
	Ac-G-L-L-G-L			
	Ac-G-L-G-L-L			
	Ac-G-G-L-L-L		○	
	405	Ac-L-L-L-L-G		○
Ac-L-L-L-G-L				○
Ac-L-L-G-L-L				○
Ac-L-G-L-L-L				○
419	Ac-G-L-L-L-L		○	
	Ac-L-L-L-L-L	○		

○: Sequence determined, ×: Sequence could not be determined because of weak ion intensity. Vacant columns mean no possibility for sequence determination by FAB-MS, MS/MS and MS³.

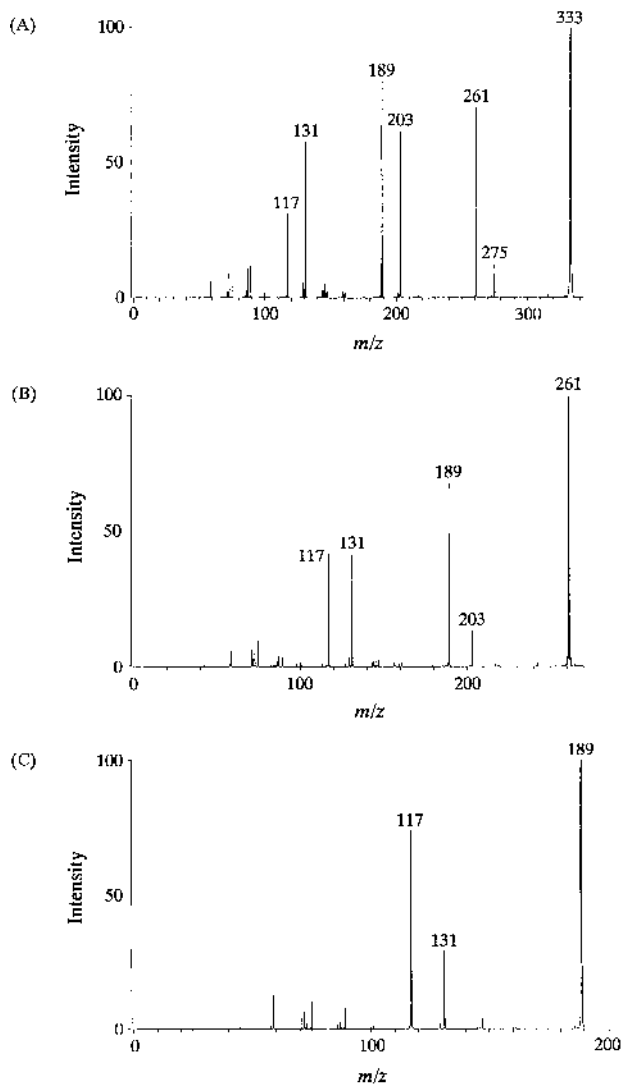


Fig. 5. CID Spectra of m/z 333
 (A): MS/MS Spectrum, (B): MS³ Spectrum (Precursor ion: 333, 1st generation product ion: 261), (C): MS³ Spectrum (Precursor ion: 333, 1st generation product ion: 189).

ble sequences, Ac-L-L-G, Ac-L-G-L and Ac-G-L-L as mentioned for the trimer. CID of the product ion at m/z 261 generated from the precursor ion at m/z 333, gave a 2nd generation product ion at m/z 203 (Fig. 5B), which was assigned to $[M-H]^-$ of Ac-L-L. This result indicates the existence of Ac-L-L-G, namely Ac-L-L-G-L.

The 1st generation product ion at m/z 189 has two possible

sequences, Ac-L-G and Ac-G-L as mentioned for the dimer. CID of the product ion at m/z 189 generated from the precursor ion at m/z 333, showed a 2nd generation product ion at m/z 131 (Fig. 5C), which was assigned to $[M-H]^-$ of Ac-L, indicating the existence of Ac-L-G, namely Ac-L-G-L-L. In this manner, all of the four possible sequences of precursor ion at m/z 333 were confirmed.

In a similar manner, the precursor ion at m/z 319 as shown in Fig. 2 was also confirmed to contain all of the six possible sequences, Ac-L-L-G-G, Ac-L-G-L-G, Ac-L-G-G-L, Ac-G-L-L-G, Ac-G-L-G-L and Ac-G-G-L-L. On the other hand, for the precursor ion at m/z 305 (Fig. 2) identified as Ac-(L+3G), the existence of Ac-L-G-G-G and Ac-G-G-G-L were confirmed by CID-MS/MS. However, since the 1st generation product ion did not give high enough intensity to detect 2nd generation product ions, the existence of Ac-G-L-G-G and Ac-G-G-L-G could not be confirmed. This is because the probability of a tetramer containing three moles of glycolic acid is extremely low (molar ratio of lactic acid to glycolic acid contained in the intact PLGA is 3 : 1).

Pentamer The precursor ions at m/z 405, 391, 377, 363, 349 and 419, as shown in Fig. 2, have 32 possible sequences as shown in Table 2. The results of analysis by FAB-MS, MS/MS and MS³ clarified the existence of 18 sequences, marked (○) in the table. However, pentamers containing

more than three moles of glycolic acid, *i.e.* Ac-G-G-G-G-G, Ac-G-L-G-G-G, Ac-G-G-L-G-G and Ac-G-G-G-L-G, as marked (×) in the table, could not be confirmed, since the intensity of the precursor ions decreased with increasing moles of glycolic acid in the oligomer. Other possible sequence not mentioned above (vacant columns in the table) could not be theoretically determined by FAB-MS, MS/MS and MS³, and additional CID (MS⁴) would be required for the determination of these sequences.

Further sequence analysis for oligomers greater than pentamer was not attempted because the number of sequences which must be determined is very great and also because the intensity of precursor ions was very weak.

Conclusion This study demonstrated the possibility of sequence determination of copolymer by tandem mass spectrometry. PLGA, a copolymer of lactic acid and glycolic acid, was partially hydrolyzed and the terminal hydroxyl group in the obtained oligomers acetylated. CID analysis (MS/MS, MS³) of these acetylated oligomers gave 1st and 2nd generation product ions, which give useful information on sequence. For example, although the MS/MS product ion spectrum of a dimer mixture of L-G and G-L shows product ions corresponding to L and G, the sequences can not be confirmed. In the case of acetylated dimer mixture, Ac-L-G and Ac-G-L, characteristic product ions such as Ac-L and Ac-G are observed. Finally, the results of analysis for dimers

through pentamers showed that they all have random sequences of lactic and glycolic acid, suggesting that the whole structure of PLGA also has a random sequence.

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