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Research Article

Synthesis of Functional Polyester Based on Polylactic Acid and Its Effect on PC12 Cells after Coupling with Small Peptides

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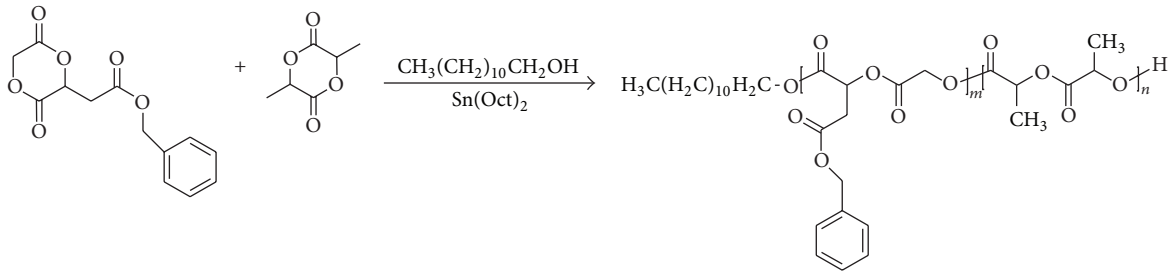
Polyesters containing functional groups are a suitable candidate matrix for cell culture in tissue engineering. Three types of semicrystalline copolymer poly(L-lactide-*co*-*β*-malic acid) [P(LA-*co*-BMD)] with pendent carboxyl groups were synthesized in this study. The functional monomer 3(S)-[(benzyloxycarbonyl)methyl]-1,4-dioxane-2,5-dione (BMD) was synthesized using L-aspartic acid. The copolymer P(LA-*co*-BMD) was then synthesized through ring-opening copolymerization of L-LA and BMD, with dodecanol as initiator and stannous octoate as catalyst. Copolymer structure was characterized by ^{1}H nuclear magnetic resonance (^{1}H NMR), gel permeation chromatography (GPC), and differential scanning calorimetry (DSC) analyses. Results of ^{1}H NMR and GPC analyses showed that the copolymers were synthesized successfully. DSC curves showed that the crystal melting peak and enthalpy decreased with increased BMD. The crystallinity of the copolymer was destroyed by the presence of the functional monomer. After deprotection, carboxyl groups were coupled with the isoleucine-lysine-valine-alanine-valine peptide through *N*-hydroxysuccinimide/dicyclohexylcarbodiimide method. The small peptide was beneficial to the axon growth of PC12 cells.

1. Introduction

Multiple synthetic biopolymers including polyglycolic acid, polylactic acid, poly(lactic-co-glycolic acid) (PLGA), and poly(caprolactone) are used as biomedical polymeric materials in tissue engineering and drug delivery because of their good biocompatibility and biodegradability [1–5]. However, these biodegradable polymers are hydrophobic. Therefore, the wettability of these materials must be enhanced through functionalization. Synthetic polyesters are also unsuitable for polymer-protein interaction because of their insufficient chemical functionalities. Functional polymers have been synthesized using hydrophilic groups to satisfy the design criteria for advanced applications. These studies promoted the coupling reaction to covalently immobilize biologically active peptides onto the scaffolds. Peptide-modified surfaces have gained increased attention because surface-immobilized biologically active cues can lead to cell adhesion, migration, or differentiation. Laminin-derived peptides, such as isoleucine-lysine-valine-alanine-valine (IKVAV), can promote cell adhesion and induce neurite outgrowth for neural progenitor cells [6–11].

Copolymerization between functional monomer and polyester matrix introduces functional groups, through which a small peptide can be bonded onto the polymer. This method can promote cell growth and differentiation [12–16]. Polymaleic acid (PMA) is a water-soluble polyester compound, whose side chain contains the carboxyl group. PMA is metabolized in vivo and generates malic acid, which is an intermediate of the Krebs tricarboxylic acid cycle [17]. Malic acid polymers with bacterial polyesters constitute an attractive material for tissue engineering. These copolymers with lateral groups can be used to couple with small peptides [18–21].

In this study, the cyclic monomer 3(S)-[(benzyloxycarbonyl)methyl]-1,4-dioxane-2,5-dione (BMD) was used for ring-opening polymerization with L-lactide (L-LA). BMD was synthesized through a four-step reaction, with L-aspartic acid as raw material. The copolymerization behavior of the functional monomer BMD and L-LA was investigated and



Scheme 1: $Sn(Oct)_2$-catalyzed ring-opening polymerization of BMD with L-LA.

characterized by ^{1}H nuclear magnetic resonance (^{1}H NMR), gel permeation chromatography (GPC), and differential scanning calorimetry (DSC) analyses. Moreover, the short IKVAV peptide was chemically bonded using functional monomers on the copolymer film containing carboxyl group in the side chain. The effect of short IKVAV peptide on PC12 cell behavior was also determined.

2. Materials and Methods

2.1. Materials. Benzyl alcohol ($C_6H_5CH_2OH$), ethyl alcohol (CH_3CH_2OH), ether ($CH_3CH_2OCH_3CH_2$), ethyl acetate ($CH_3COOC_2H_5$), dodecanol ($C_{12}H_{25}OH$), pyridine (C_5H_5N), sodium bicarbonate ($NaHCO_3$), and sodium nitrite ($NaNO_2$) were purchased from Guangzhou Chemical Reagent Factory (China). L-Aspartic acid was obtained from Shanghai GiAo Biochemical Co., Ltd. (China). Bromoacetyl chloride, *N*-hydroxysuccinimide (NHS), and *N*,*N*′-dicyclohexylcarbodiimide (DCC) were purchased from Acros Organics. Triethylamine from Guangzhou Chemical Reagent Factory was distilled from CaH_2. *N*,*N*-Dimethylformamide (DMF) was distilled from 4 Å molecular sieve. L-LA was obtained from Huizhou Foryou Medical Device Co., Ltd. (Guangdong, China) and recrystallized three times in ethyl acetate. Stannous octoate [$Sn(Oct)_2$] was obtained from Alfa Aesar. Trifluoroacetic acid was purchased from Aldrich. Hydrogen bromide (HBr) in acetic acid (33%) was purchased from Acros Organics. IKVAV peptides were synthesized by GL Biochem Co., Ltd.

2.2. Characterization. The mole ratio of L-LA to BMD was analyzed by ^{1}H NMR spectroscopy, with $CDCl_3$ as solvent and tetramethylsilane as internal standard. Fourier transform infrared (FTIR) spectra were obtained by Nicolet/Nexus 670. Number-average (M_n) molecular weights were determined using GPC (Waters 410, Milford, MA) equipped with organic GPC columns. Chloroform was used as mobile phase at a flow rate of 1.0 mL/min at 35°C. Thermal properties were examined by DSC, and the samples were heated from 0°C to 200°C at a heating rate of 10°C/min under nitrogen atmosphere. Cell morphology was studied using scanning electron microscopy (SEM, JSM-6380LA Analytical, JEOL Ltd., Tokyo, Japan), equipped with vacuum mode and at 15 kV imaging.

2.3. Copolymer Synthesis

2.3.1. Copolymerization of L-LA with BMD. BMD was synthesized using L-aspartic acid through a four-step reaction. Different molar ratios of L-LA and BMD (98/2, 95/5, and 92/8) were added to a dry Schlenk tube under dry Ar atmosphere. Dodecanol was added as initiator ($[M]/[I] = 700/1$), with $Sn(Oct)_2$ as catalyst ($[M]/[I] = 1000/1$). The tube was evacuated for 2 h at ambient temperature, sealed, immersed in an oil bath, and heated at 110°C for 48 h. The three types of copolymers were dissolved in chloroform and precipitated with methanol (Scheme 1).

2.3.2. Deprotection of Copolymers to Obtain Carboxyl-Substituted Polymers. Copolymers with protecting groups (2 g) were dissolved in trifluoroacetic acid (20 mL). Briefly, 33% HBr/CH_3COOH solution (8 mL) was added dropwise under Ar atmosphere after the complete dissolution of the copolymer. The product was poured into anhydrous ether (200 mL) after 5 h at ambient temperature. The precipitates were dissolved in chloroform and purified by ethanol.

2.4. Modification of Copolymer Films by Short Peptides. Copolymers after deprotection (2 g) were dissolved in chloroform (30 mL). NHS (3 mmol, 0.6 g) and DCC (3 mmol, 0.34 g) were added dropwise. The compounds were reacted for 2 h in an ice bath and for 24 h at room temperature. The product was dissolved in ethanol and precipitated with chloroform.

The polymer films were prepared by solution pouring and solvent evaporation. The samples were then immersed in 1.0 mg/mL short-peptide solution after drying. The film was then soaked for 12 h with mild agitation and washed three times (once per hour) with phosphate-buffered saline (PBS) solution to remove unreacted short peptides.

2.5. Cell Culture and Morphology Observation. The polymer film functionalized with peptide on 15 mm cover slip was placed in a 24-well plate and pressed with ring to ensure complete contact of the scaffolds to the wells. The specimens were sterilized under UV light, washed three times with PBS, and immersed in Dulbecco's modified Eagle's medium (DMEM) overnight before cell seeding. Rat pheochromocytoma-derived cell line (PC12) was seeded on the film at a density of 1.0×10^4 cells/well and cultured in DMEM containing

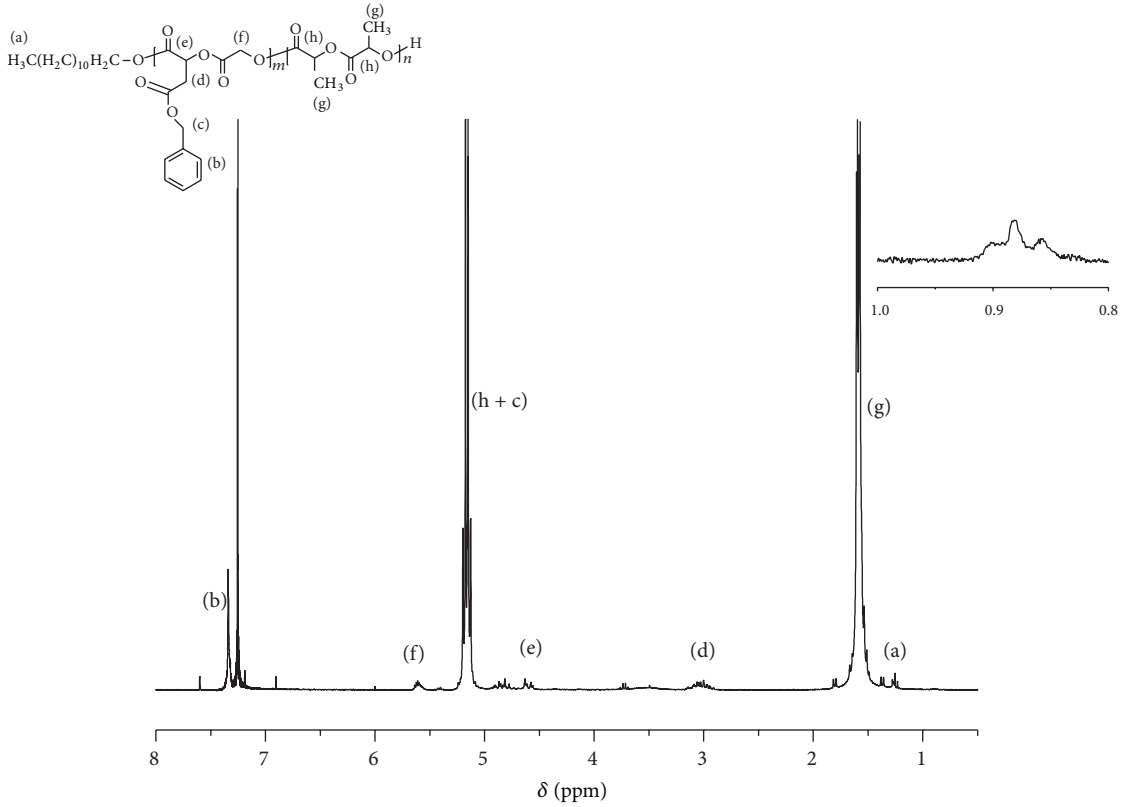


Figure 1: ^{1}H NMR spectrum of P(LA_{92}-*co*-BMD_8) random copolymers ($CDCl_3$).

Table 1: Polymerization of L-LA with BMD initiated by dodecanol and catalyzed with stannous octoate[a].

L-LA_{theo}/BMD	L-$LA_{H\,NMR}$[b]/BMD	M_n[c] (g/mol)	M_n[c] (g/mol)	M_w/M_n[c]	T_g[d] (°C)	T_m[d] (°C)	ΔH_m[d] (J/g)
100/0	100/0	—	—	—	60.8	177.2	93.6
98/2	98.0/2.0	1.0×10^5	1.6×10^5	1.03	55.9	162.4	55.6
95/5	95.4/4.6	1.0×10^5	1.1×10^5	1.12	53.7	154.2	54.8
92/8	93.6/6.4	1.0×10^5	1.0×10^5	1.19	53.4	147.4	9.4

[a]Polymerization of BMD with L-LA at 100°C for 48 h.
[b]Obtained from ^{1}H NMR analysis.
[c]Number-average molar mass (M_n) and polydispersity index (M_w/M_n) obtained from gel permeation chromatography in THF using polystyrene standards.
[d]The thermal properties were determined using DSC at 10°C/min heating rate under N_2.

10% FBS, heat-inactivated fetal bovine serum, and 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The culture medium was replaced every 3 days. Tissue culture polystyrene was used as control.

The cell-cultured films were processed for SEM studies 5 days after cell proliferation. The scaffolds were washed twice with PBS and fixed in 3% glutaraldehyde for 3 h. The scaffolds were then washed with deionized water and ethanol (50%, 70%, 90%, and 100% concentrations) twice for 15 min each. Finally, the films were coated with gold and observed by SEM analyses.

3. Results and Discussions

3.1. Characterization of the Copolymers. The results of the ^{1}H NMR spectrum and GPC test showed the successful synthesis of the copolymer poly(L-lactide-co-β-malic acid) [P(LA-*co*-BMD)]. The ^{1}HNMR spectrum of the copolymer P(LA-*co*-BMD) is shown in Figure 1. The resonance absorption assigned to the PLLA segment was observed at δ1.6 ppm (g, 3H, CH_3) and δ5.1 ppm (h, 1H, OCH) after the ring-opening polymerization of L-LA. The peaks at δ4.6, δ5.1, δ5.5, and δ7.3 ppm could be attributed to the resonance absorption of the PBMD segment. The proportion of the functional monomers in the copolymer was calculated using the ratio of relative integral intensity of resonance peak in the ^{1}H NMR spectrum. In particular, the integral intensity ratio of the characteristic peak (g) was calculated after ring-opening polymerization of L-LA to the peak (b) for BMD unit, that is, $(I_g/6)/(I_b/5)$. The molar ratios of PLLA to PBMD were in agreement with the feed ratios (Table 1). However, the actual ratios of L-LA/BMD in the copolymer to the feed ratios increased with the increase in feed ratio of BMD.

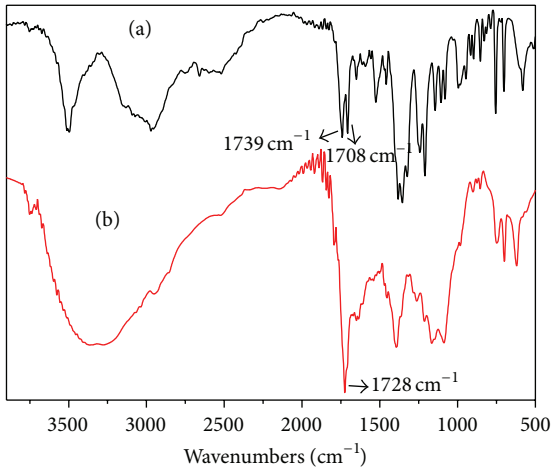


Figure 2: Infrared spectra of (a) BMD and (b) after ring-opening polymerization.

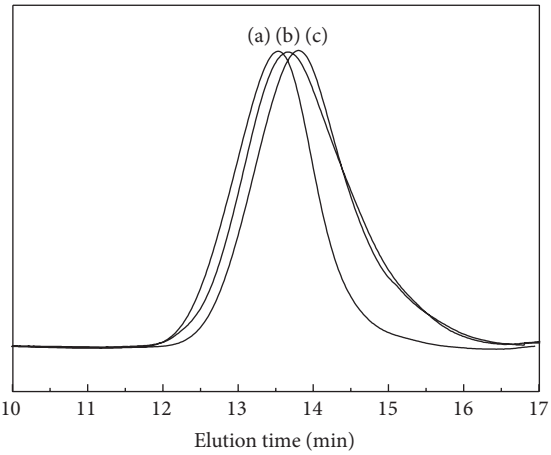


Figure 3: Gel permeation chromatography traces of the obtained P(LA-*co*-BMD). (a) P(LA_{98}-*co*-BMD_2), (b) P(LA_{95}-*co*-BMD_5), and (c) P(LA_{92}-*co*-BMD_8).

Figure 2 shows the FTIR spectra over the range 4000–500 cm^{-1} for the BMD and ring-opening of BMD. The spectra (Figure 2(a)) showed the carboxyl and hydroxyl band for BMD at 2968 and 3504 cm^{-1}, respectively. The characteristic bands at 1739 and 1708 cm^{-1} were the carbonyl bands of BMD. After ring-opening polymerization (Figure 2(b)), the carbonyl band of PBMD on the main chain appeared at 1728 cm^{-1}. The carbonyl bands of BMD (1739 and 1708 cm^{-1}) decreased. The results indicated the ring-opening polymerization of BMD.

The M_n values of polymers P(LA_{98}-*co*-BMD_2), P(LA_{95}-*co*-BMD_5), and P(LA_{92}-*co*-BMD_8) were 1.6×10^5, 1.1×10^5, and 1.0×10^5, respectively. The polymer distributions of molecular weights were 1.03, 1.12, and 1.19. M_n decreased and the distribution of the molecular weights widened (Table 1). This result could probably be due to the interaction between benzyl groups and stannous octoate [22]. The GPC curves of the copolymer with different ratios are shown in Figure 3.

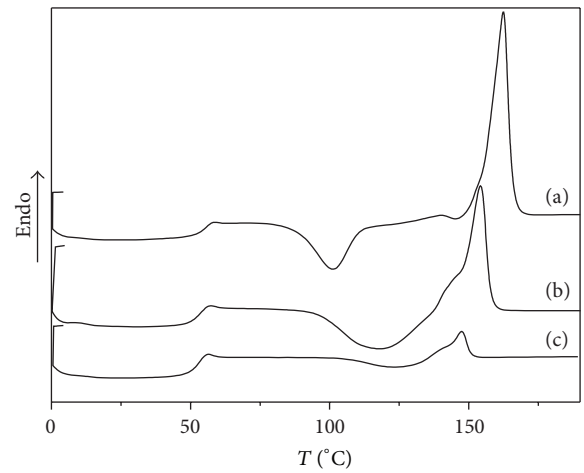


Figure 4: Differential scanning calorimetry thermograms of the obtained P(LA-*co*-BMD). (a) P(LA_{98}-*co*-BMD_2), (b) P(LA_{95}-*co*-BMD_5), and (c) P(LA_{92}-*co*-BMD_8).

3.2. Thermal Properties of the Copolymers. The thermal properties of the copolymers were investigated by DSC measurement (Figure 4). The endothermic peaks between 147°C and 162°C could be attributed to the melting transition of the crystalline phase in the second-heating runs. The endothermic peaks between 53°C and 55°C could be ascribed to the glass transition temperature of the polymer. T_m, T_g, and ΔH of the polymer were lower than those of pure PLLA and decreased with the increase in molar ratio of BMD. The reason could be that the crystallization was significantly disturbed by the functional BMD.

3.3. Adhesion and Morphology of Cells on Films Modified by Short Peptides. The SEM images of the PC12 cells after 5 days of seeding are shown in Figure 5. PC12 cells showed better adhesion and proliferation because of the film modification by small peptide. PC12 cells showed spheroid body at P PLLA film. At 5 days after cell seeding, PC12 cells over the film modified with small peptide (IKVAV) spread out. However, the effect of different polymer materials on cell behavior was different. The functional group on the three types of copolymers was different, and thus the coupling with peptide was different. The axon length of the cells on P(LA_{98}-*co*-BMD_2) film was less than that of the cells on P(LA_{95}-*co*-BMD_5) and P(LA_{92}-*co*-BMD_8). However, no significant difference between the axon lengths of the cells on P(LA_{95}-*co*-BMD_5) and P(LA_{92}-*co*-BMD_8) was observed. Therefore, the effect of short-peptide density on cell behavior was significant in a certain density range. The results are consistent with the results found in literature [23].

4. Conclusions

In this study, a series of PLLA-based functional copolymers containing different density of functional monomer (BMD) were synthesized and were characterized. The structure of BMD with pendent carboxyl groups was used for coupling

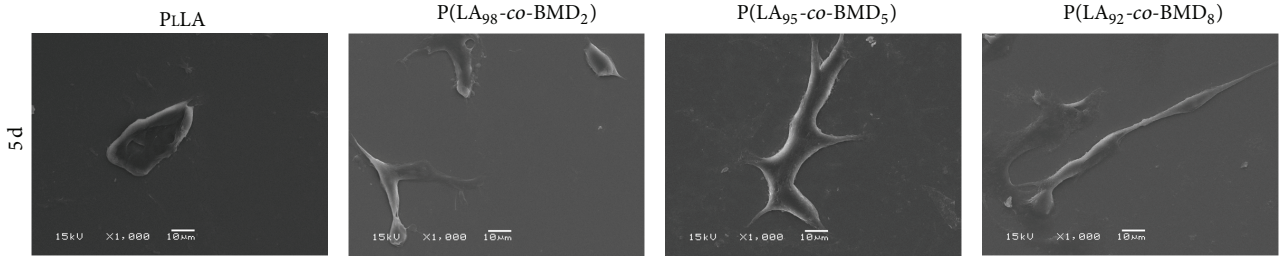


Figure 5: Scanning electron micrographs of PC12 cells seeded on the copolymeric films 5 days after isoleucine-lysine-valine-alanine-valine linking.

with small peptide IKVAV. The effects of the density of peptide IKVAV on PC12 cells axon growth were explored. The axon length of PC12 cells on the polymeric surface of P(LA_{95}-*co*-BMD_5) and P(LA_{92}-*co*-BMD_8) was significantly longer than that of P(LA_{98}-*co*-BMD_2). These results demonstrated higher density of peptide IKVAV can promote axon growth of PC12 cells and the biomimetic polymers have potential application as biomaterials in tissue engineering.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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