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Preparation, characterization, and blood compatibility of polylactide-based phospholipid polymer

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Abstract L-α-glycerophosphorylcholine (GPC) was obtained by hydrolysis of lecithin extracted from eggs. FT-IR and ^{1}H-NMR analysis indicated a successful preparation of GPC. Polylactide-based phospholipid polymer (PLLA-PC) was synthesized by ring-opening polymerization of L-lactide in the presence of GPC to improve the cell/material interfacial reaction of PLLA for tissue engineering applications. The yield of the reaction strongly depended on the reaction time. Values above 80% were obtained which are much higher than those reported in literature. Copolymers with the largest molecular weights were obtained at 122 °C for 48 h. The properties and biocompatibility of the PLLA-PC copolymers were characterized. Surface rearrangement was detected due to the dynamic molecular motion according to X-ray photoelectron spectroscopy data. Besides, increase in hydrophilicity and decreases in fibrinogen adsorption and platelet adhesion were observed due to the hydrophilic phosphorylcholine moieties in the copolymer.

Introduction

Tissue engineering is a multidisciplinary science that combines the principles of engineering and life sciences to create new tissues from their cellular components. Langer et al. defined the strategy of tissue engineering as the creation of new tissue from isolated cells, bioactive molecules, and material scaffold [1–3]. The scaffold should be biodegradable and nontoxic. Furthermore, the surface is expected to be cytocompatible, i.e., to initiate proper cell responses which are beneficial to the development of new tissue [4]. Numerous polymers have been tested as tissue engineering scaffolds. Among them, poly(lactic acid) (PLA) is the most attractive candidate due to its hydrolytic degradability in physiological environment. The final degradation product is lactic acid, which can be easily eliminated from the body by incorporation into the tricarboxylic acid cycle. Unfortunately, PLA exhibits poor cell/material interactions. In fact, the highly hydrophobic surface of PLA may result in non-specific protein adsorption and subsequently non-specific cell type adhesion [5], which largely limits its further applications in tissue engineering.

A variety of surface modification methods have been developed to improve the adhesion and function of cells on PLA scaffold. Biomembrane is regarded as the ideal surface for smooth interaction with proteins and cells. And the phosphorylcholine (PC) layer is believed to provide an inert surface for biological reactions of proteins and glycoproteins to occur smoothly on the membrane. This inspired scientists to develop novel biocompatible materials by introducing PC into PLA chains [6–12]. Watanabe and Ishihara [13] firstly reported a novel copolymer (PLA–BMA–MPC) composed of poly(D,L-lactic acid) (PDLLA) macromonomer, *n*-butyl methacrylate (BMA), and 2-methacryloyloxyethyl phosphorylcholine (MPC). Cell culture results suggested that the adhesion and morphology of fibroblasts on the copolymer film depend on the content of MPC units. Similar results were obtained in the case of porous scaffold of PLLA and PDLA stereocomplex containing MPC and BMA [14]. It is assumed

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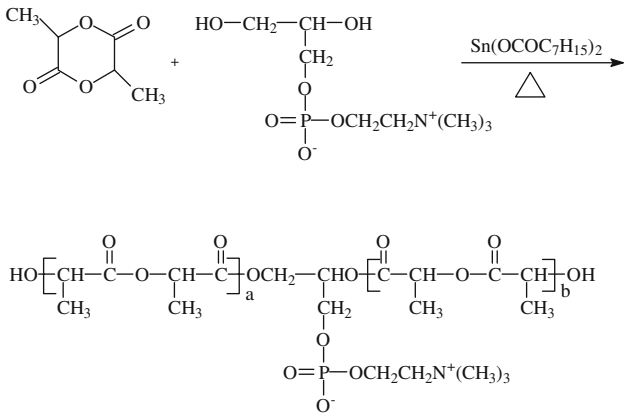

Scheme 1 Synthetic route of PLLA–PC copolymers by ring-opening polymerization of L-lactide with GPC

that the most favorable characteristics of the copolymer surface were of dual functions of cytocompatibility by the phospholipid polymer and cell adhesion property by the oligo(lactic acid).

PLA units preserve the degradability in PLA–BMA–MPC copolymers, but the main chain consisting of C–C bonds is not easily degradable in biological environment. Later on, PLLA–PC, another PC-containing copolymer was synthesized by copolymerizing L-lactide (LLA) with glycerophosphorylcholine (GPC) (Scheme 1) [15]. Desired degradability was obtained by changing the composition of copolymers. However, these copolymers have not been extensively investigated probably due to the high cost of commercial GPC (about 200 USD/100 mg) and the low yield of copolymerization (34–69%).

In this study, GPC was prepared by direct hydrolysis of lecithin from eggs instead of commercial purchase, which cost too much. The resulting GPC was then used to initiate ring-opening polymerization of L-lactide, with stannous octoate as catalyst. The yield was much higher than previous study reported. The obtained PLLA–PC copolymers were characterized by NMR, FT-IR, GPC, XPS, etc. Hydrophilicity was evaluated by water absorption tests. Meanwhile, fibrinogen adsorption and platelet adhesion were examined on the PLLA–PC, in comparison with PLLA.

Experimental

LLA (Fushun Tianyuan Bioabsorbable Materials Co. Ltd) was recrystallized in ethyl acetate and toluene. Stannous octoate (Aldrich) was distilled before use. Tetrabutylammonium hydroxide (~20%) was purchased from Ke Long Co. Sichuan. All the other reagents and solvents were of analytical grade. Diethyl ether and toluene were dried with sodium and distilled before use. Dichloromethane was used without further purification.

Glycerophosphorylcholine was prepared according to the method reported by Regen [16]. Briefly, the combined egg yolks from 12 eggs were separated and washed thoroughly with acetone until the washings became colorless. The resulting off-white crystalline solid was extracted twice with a mixed solvent ($CHCl_3$:CH_3OH = 2:1). Then the extracts were concentrated under reduced pressure to yield a waxy product. Chromatographic purification was then performed on neutral alumina, successively with $CHCl_3$, $CHCl_3$:CH_3OH (9:1), and $CHCl_3$:CH_3OH (1:1) as eluting solvent. Hydrolysis of the resulting lecithin was carried out according to the following procedure: dissolve lecithin in diethyl ether; remove the suspended particles by centrifugation, and add 0.1 M tetrabutylammonium hydroxide methanol solution. After precipitation of GPC, the liquid phase was decanted. The products were washed with diethyl ether, dried in vacuum at room temperature, and finally stored in CH_3OH at −15 °C. The structure of GPC was confirmed based on ^{1}H-NMR (Bruker DMX-300, Bruker, Switzerland) and FT-IR (Nicolet 560, Thermo Nicolet, USA). ^{1}H-NMR spectra were recorded at 400 MHz, using deuterated H_2O as solvent.

Prescribed amounts of LLA, GPC, and stannous octoate were introduced in a polymerization round-bottomed flask. The mixture was degassed for 5 h. Polymerization was then carried out under vacuum in an oil bath for designed time periods. The resulting PLLA–PC copolymer was recovered by dissolution in chloroform and precipitation in an excess of diethyl ether. The white precipitates were dried in vacuum at room temperature for 2 days. PLLA homopolymer was prepared according to the same procedure and conditions, using 1,2-propanediol as an initiator.

Size exclusion chromatography (SEC) was performed by using a setting composed of a Waters 510 HPLC pump, a Waters 410 differential refractometer, and a PLgel 5 mm mixed-C 60 cm column, the mobile phase being THF and the flow rate 1 mL/min. The number-average (M_n) and weight-average (M_w) molecular weights were expressed with respect to polystyrene standards from Polysciences.

The surface properties of PLLA–PC copolymers were characterized in terms of dynamic molecular motion at the surface. The X-ray photoelectron spectroscopy (XPS) was performed using ESCALAB 250 instrument (Thermo, USA). A monochromatic Al X-ray source was used.

Water absorption was used to evaluate the hydrophilicity, which was defined as the weight percentage of water in wet PLLA–PC films. The films were prepared by casting 1 wt% polymer solution in chloroform onto PET substrates (1×1 cm^2). After vacuum drying for 2 days, the films were weighed (W_0) and placed in distilled water. They were recovered at designed time intervals, wiped with filter paper, and weighed (W_1) again. The water absorption was calculated with the following equation:

Water uptake $(\%) = (W_1 - W_0)/W_0 \times 100\%$

Fibrinogen, labeled with 125I (China Isotope Corporation, CIC) according to the ICl method, was passed through an AG 1-X4 column (Bio-Rad Laboratories, Hercules, CA, USA) to remove free iodide. For tests of fibrinogen adsorption from buffer, labeled fibrinogen was mixed with unlabeled fibrinogen (1:19, labeled:unlabeled) at a total concentration of 1 mg/mL and serially diluted with Tris–HCl buffer solution (TBS, 50 mM, pH = 7.4).

The PLLA–PC copolymer films on PET substrates were incubated with fibrinogen in TBS buffer at room temperature. And 3 h later, they were rinsed three times with TBS (10 min/each) and dried on filter paper. The films were finally put into clean tubes for radioactivity determination by gamma counting. To measure the desorption of fibrinogen from the copolymer film, samples were put into 1 mL of 2% sodium dodecyl sulfate (SDS, Sigma) and incubated overnight before the radioactivity determination.

Fresh human blood was obtained from healthy volunteer and mixed with anticoagulant (citrate, 0.129 mol/L, anticoagulant to blood ratio 1:9). The mixture was centrifuged at 1,200 rpm/min for 15 min to obtain platelet-rich plasma (PRP).

PLLA and PLLA–PC films were prepared by casting 1 wt% chloroform solution on a glass wafer (7 × 9 mm^2) and evaporating the solvent in air. After vacuum drying for 2 days and sterilization with ultraviolet radiation for 2 h, they were placed in a 24-well culture plate (Corning, US) and were soaked in distilled water for 30 min. Then, distilled water was removed, and PRP was added to the culture plate with a volume 200 μL for each well. One hour later, samples were washed with phosphate buffer solution (PBS, pH = 7.4) three times to remove non-adhered platelets. Samples were then fixed with 2.5% glutaraldehyde for 1 h, dehydrated in an ethanol-graded series (30, 50, 70, 80, 90, 95, and 100%) for 30 min each, and allowed to dry at room temperature. Finally, the samples were sputter-coated with gold in vacuum and observed by using a scanning electron microscope (SEM, JSM-5900 LV, JEOL Ltd, Japan).

Results and discussion

Synthesis of PLLA–PC

PLLA–PC, a novel biodegradable polymer with a phospholipid polar group, was firstly synthesized with GPC as ring-opening initiator for LLA by Iwasaki et al. However, the GPC used in Iwasaki's study was from commercial origin. In this study, lecithin was hydrolyzed from eggs according to a classical method. The structure of the obtained GPC was confirmed by FT-IR and ^{1}H-NMR analysis.

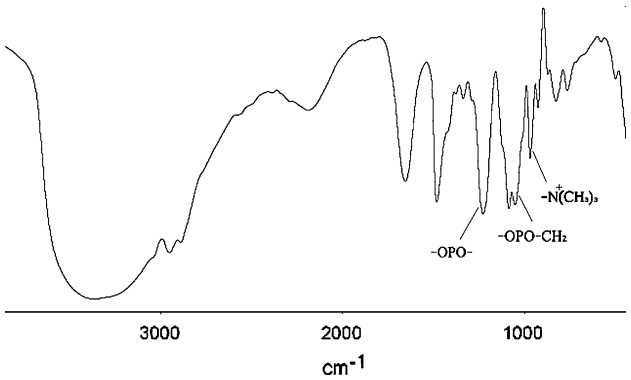

Fig. 1 FT-IR spectrum of GPC obtained by hydrolysis of lecithin extracted from eggs

Figure 1 shows the FT-IR spectrum of GPC obtained by extraction from egg lecithin. The peak at 970 cm^{-1} is assigned to the trimethyl ammonium group ($–N^+(CH_3)_3$), the most characteristic group of GPC. The peaks at 1,220 and 1,087 cm^{-1} correspond to –OPO– and $–P–OCH_2$, respectively.

Figure 2 shows the ^{1}H-NMR spectrum of GPC obtained by hydrolysis of lecithin extracted from eggs. The methyl protons in $–N^+(CH_3)_3$ appear at 3.12 ppm. Signals at 3.58, 3.80, and 4.21 ppm correspond to the methylene protons in $–CH_2N^+(CH_3)_3$ and $–CH_2–OH$, methylene and methine protons in $–OPO–CH_2–CH–OH$ and the methylene protons between $–N^+(CH_3)_3$ and –OPO–, respectively.

Iwasaki previously reported successful polymerization of LLA with GPC. However, the yield was only 34 and 37% when the LLA/PC molar ratio was 39/0.4 and 39/1.2, respectively. In this study, different reaction conditions were used to improve the yield with the same LLA/PC ratio of 39/1.

The copolymerization under different conditions was examined and the results are summarized in Table 1. The yield was very low when the reaction time was 14 h or 17 h. When the reaction time was prolonged to 48 h, the yield increased to 83%, i.e., much higher than the reported values. Beyond 48 h, no obvious changes were detected while molecular weight decreased slightly which might result from oxidative degradation. This suggests that the reaction time is an important factor in the copolymerization, and the optimal reaction time is 48 h. The low yields reported in literature could be due to the short reaction time (2 h). On the other hand, the yield seemed not to be very sensitive to the reaction temperature in the range from 110 to 130 °C. The highest molecular weight was obtained at 122 °C. Therefore, 122 °C was selected as the reaction temperature in the following synthesis.

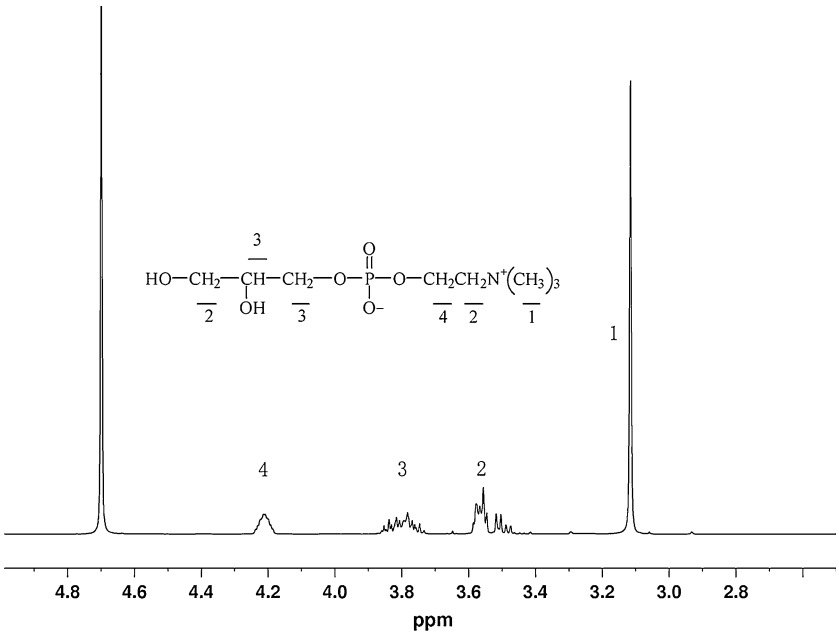

Fig. 2 ^{1}H-NMR spectrum of GPC obtained by hydrolysis of lecithin extracted from eggs in deuterated water

Table 1 Copolymerization of LLA and GPC under different conditions

No.	LLA/PC in feeding	Temperature (°C)	Time (h)	Yield (%)	M_n[a]
1	39:1	122	14	25	2,270
2	39:1	122	17	30	4,580
3	39:1	122	48	83	8,180
4	39:1	122	72	84	7,460
5	39:1	110	48	89	6,880
6	39:1	130	48	84	6,160

[a] Obtained by SEC with polystyrene standards

Table 2 Molecular weights of PLLA and PLLA–PC copolymers

Polymers	LLA/initiator in products	M_n[c]	M_w[c]	I_p[c]
PLLA 50	50/1[a]	9,730	13,720	1.4
PLLA–PC 55/1	55/1[b]	3,170	5,310	1.7
PLLA–PC 46/1	46/1[b]	2,940	5,020	1.7
PLLA–PC 30/1	30/1[b]	2,670	4,550	1.7

[a] The LLA/initiator molar ratio in the copolymers is determined from the integrations of signals at 5.15 ppm from methine protons of LLA and at 4.35 ppm from the methylene of 1,2-propanediol

[b] The LLA/initiator molar ratio in the copolymers is determined from the integrations of signals at 5.15 ppm from methine protons of LLA and at 3.25 ppm from the $-N^+(CH_3)_3$ of PC units

[c] Obtained by SEC with polystyrene standards

A series of PLLA–PC copolymers were then synthesized at 122 °C for 48 h, together with a PLLA homopolymer. The SEC results are summarized in Table 2. The molecular weights of PLLA–PC 55/1 are lower than those of PLLA 50 despite longer LLA chain length. This could be assigned to the different hydrodynamic volumes of both components; PLLA–PC was amphiphilic polymer. On the other hand, the molecular weights of the copolymers depend on the LLA/PC ratio. The higher the LLA/PC ratio, the longer the PLLA chain length, and the higher the molecular weights. Meanwhile, the polydispersity index ($I_p = M_w/M_n$) significantly increases for the copolymers as compared to PLLA homopolymer.

Figure 3 shows the FT-IR spectra of PLLA and PLLA–PC 30/1. In both spectra, the typical absorption of lactide monomer at 935 cm^{-1} was not detected. The broad absorption in the 3,200–3,600 cm^{-1} region is assigned to the terminal hydroxyl groups. The adsorption bands near 2,995 and 2,945 cm^{-1} correspond to $-CH_3-$, $-CH-$ in PLLA, and to $-CH_2-$ in PC, respectively. The absorption band at 969 cm^{-1} is assigned to $-N^+(CH_3)_3$ group of PC units, and those at 1,084 and 1,230 cm^{-1} to $-OPO-CH_2-$ and $-OPO-$, respectively, which are not found on the spectrum of PLLA. Furthermore, the absorption intensity at 969 cm^{-1} and the peak width near 1,200 cm^{-1} both increased with an increase in PC content (data not shown).

The ^{1}H-NMR spectrum of PLLA–PC copolymer is shown in Fig. 4. Peaks for methyl and methine protons in PLLA main chain are observed at 1.56 and 5.15 ppm, respectively. The methyl protons in $-N^+(CH_3)_3$ groups appear at 3.25 ppm, suggesting successful introduction of PC into PLLA chains. Signals at 3.75 and 4.04 ppm are assigned to the methylene protons in $-CH_2-N^+(CH_3)_3$ and $-OPO-CH_2-CHO$, respectively. This was an additional evidence for the successful copolymerization.

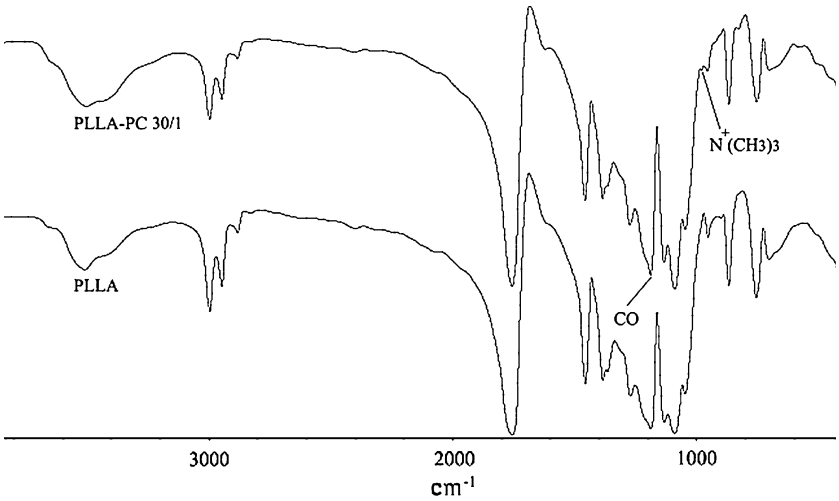

Fig. 3 FT-IR spectra of synthesized PLLA and PLLA–PC 30/1

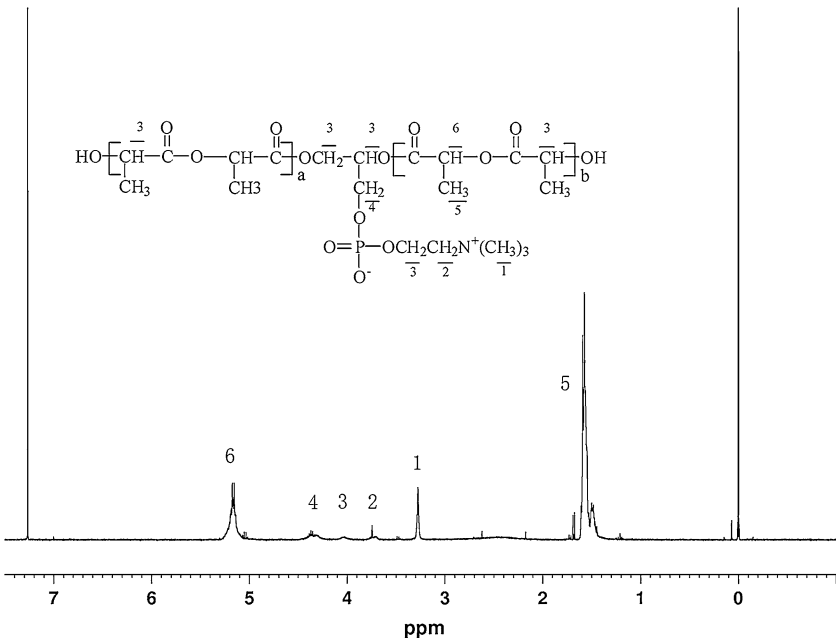

Fig. 4 ^{1}H-NMR spectrum of synthesized PLLA–PC 30/1

Additional information about the structure of the copolymers was obtained from ^{13}C-NMR (Fig. 5). Signals at 16.7, 69.3, and 169.6 ppm are assigned to the *–CH$_3$*, *–CH–*, and *–CO–* moieties in LLA chain, respectively. Signals at 20 and 66.6 ppm are attributed to *–CH$_3$*, *–CH–* at the end of the LLA chain, respectively. The characteristic signal at 55 ppm is assigned to *–N$^+$(CH$_3$)$_3$*.

Phosphorylcholine is highly hydrophilic and is likely to migrate to the polymer surface to minimize the interfacial energy under aqueous condition. In this study, surface rearrangement was examined in the case of PLLA–PC 30/1.

The copolymer films were prepared by coating on polyethylene terephthalate (PET) substrates. XPS was employed to monitor the elemental composition on the film surface. Films under dry and wet conditions were both analyzed. The dry film surface was directly analyzed after vacuum drying, while the wet film was examined after immersion in distilled water at room temperature for 24 h, followed by freeze-drying. XPS measurement was carried out with releasing angles of 30, 45, 75, and 90°, respectively. The N_{1s} and P_{2p} contents are summarized in Table 3. The XPS spectra of dry samples exhibit peaks at 285, 283, and 281 eV which are attributed to –CH_2–, –CH_3, –CH (CH_3)O–, and –COO– in LLA units, respectively (data not shown), while peaks N_{1s} and P_{2p} are not observed with a releasing angle of 30°. In contrast, a peak at 400 eV appears under wet condition, indicating existence of N_{1s} at the outmost surface with an atomic percentage of 0.39. It is noted that the intensity of phosphorus is zero. This might be attributed to the small testing depth at 30°. When the film is placed under aqueous condition, the –$N^+(CH_3)_3$ groups are likely to migrate to the outmost

Fig. 5 ^{13}C-NMR spectrum of synthesized PLLA–PC 30/1

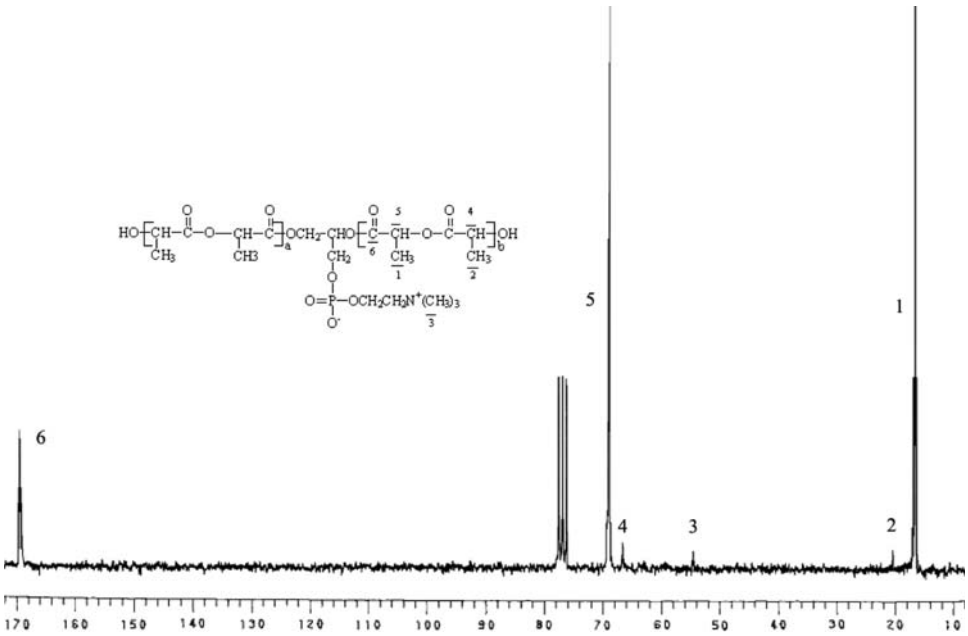

Table 3 N and P contents at PLLA–PC 30/1 surface under dry and wet conditions obtained from angle-dependent XPS spectral

Angle	P_{2p} atomic percentage (%)		N_{1s} atomic percentage (%)	
	Wet	Dry	Wet	Dry
30°	0	0	0.39	0
45°	0.42	0.34	0.39	0.36
75°	0.66	0.47	0.44	0.24
90°	0.61	0.49	0.33	0.16

surface due to strong interaction with H_2O, whereas the –OPO– is not located at the outmost surface and hardly detectable. The peak for P_{2p} is detected at 135 eV when the angle is above 45°. Intensity of both nitrogen and phosphorus is larger for wet surfaces than for dry ones. For example, N content increases by 110% and P content by 23% when releasing angle is 90°. Therefore, all the data obtained at different angles indicate a surface rearrangement of PLLA–PC copolymer under aqueous condition. The results are consistent with the surface rearrangement occurred on PLA–BMA–MPC copolymer surface at room temperature or at 40 °C reported by Watanabe et al. [17, 18]. This phenomenon is explained on the basis of the dynamic molecular motion at the polymer surface.

Water absorption was used to evaluate the hydrophilicity of the copolymers, using PLLA 50 as control. As shown in Fig. 6, water absorption of PLLA 50 is only 4% after 2 days, and slightly increased in the following 6 days. In contrast, water absorption of the copolymers is much higher and increases over time. After 2 days, water absorption of PLLA–PC 46/1 is 51%, and that of

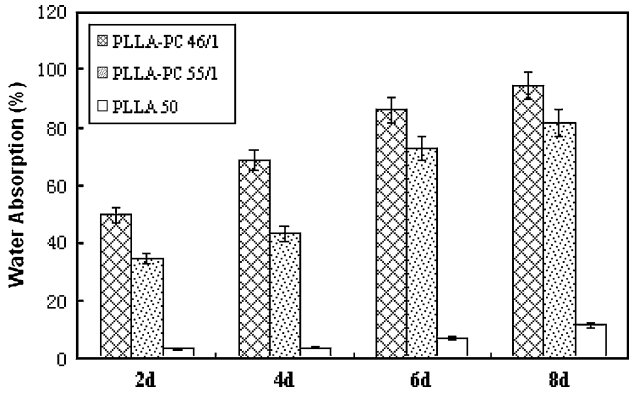

Fig. 6 Water absorption of the synthesized PLLA–PC copolymers in comparison with PLLA

PLLA–PC 55/1 is 35%. After 8 days, water absorption increases to 95 and 85% for PLLA–PC 46/1 and PLLA–PC 55/1, respectively. These results indicate a significant improvement in the hydrophilicity of PLLA by PC. The higher the PC content, the higher the hydrophilicity of PLLA–PC copolymers.

Protein adsorption was firstly carried out with fibrinogen (FN), the most commonly used protein for the identification of hemocompatible biomaterial surface, and the data are summarized in Fig. 7. PLLA 50 presented a FN adsorption 0.937 μg/cm^2, while FN adsorption 0.335 μg/cm^2 on PLLA–PC 55/1 surface, i.e., it was about 1/3 of that on PLLA. The lowest FN adsorption was found on PLLA–PC 46/1 (0.106 μg/cm^2), thus indicating that introduction of PC strongly decreases the FN adsorption. The higher the PC content, the lower the FN adsorption.

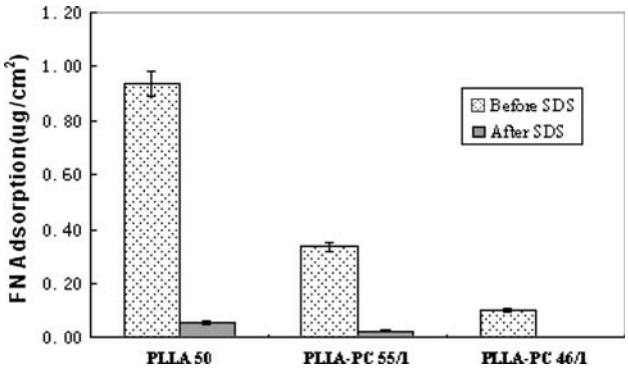

Fig. 7 The fibrinogen adsorption on PLLA–PC copolymer surfaces in comparison with PLLA (Fibrinogen labeled with 125I was mixed with unlabeled fibrinogen with 1:19 at a total concentration of 1 mg/mL and serially diluted with Tris–HCl buffer solution, TBS, 50 mM, pH = 7.4)

The material surface was rinsed with SDS and the remaining FN was determined. In all cases, FN adsorption greatly decreased after SDS rinsing. On the other hand, the remaining FN is lower in the cases of copolymers, indicating a diminished interaction between FN and the surface. Therefore, one can assume that the addition of PC enhances the protein resistance of PLLA. This might be explained by the fact that the hydrophilic surface of the copolymers reduces the FN adsorption [19–21].

Figure 8 shows the adherent platelets (white dots) on the polymer surface after contacting with human PRP for 60 min. A large number of platelets are observed on the PLLA 50 surface. In the case of copolymers, however, the surface seems prone to prevent platelets from adhering, since much less platelets are observed, especially in the case of PLLA–PC 46/1.

Moreover, the cell morphology on the copolymer surface appears totally different from that on PLLA 50 (Fig. 9). Almost all platelets on PLLA 50 were found sticking out many flake pseudopodias, which made them much larger than the static platelets. In contrast, platelets on copolymer remained their inactivated round shape. Therefore, it could be deduced that PC moieties could diminish adhesion and activation of platelets on PLLA surface.

In general, cell adhesion on the material surface was mediated via protein adsorption. Proteins, especially adhesive proteins, such as fibrinogen and collagen play an important role in the process of cell attachment and spreading. It has been suggested that receptors on the cell membrane, e.g., integrin, recognize the adhesive proteins via RGD (arginine–glycine–aspartic acid) tripeptide, and the cells attach on the surface via protein adsorption layer. Therefore, the number of cell adhesion is dependent on the amount of protein adsorption. This has been proved by a number of research groups. In fact, platelets were found to be correlated to fibrinogen adsorption and distribution on mixed hydroxyl-/methyl-terminated self-assembled monolayer [19] or on fluorinated surface-modified poly(ether-urethane) [22]. In our study, a similar trend was observed, i.e., platelet adhesion decreased with FN adsorption decrease. It is thus suggested that the presence of PC in PLLA backbone, which results in a more hydrophilic surface, might contribute to the decreased platelet adhesion and activation.

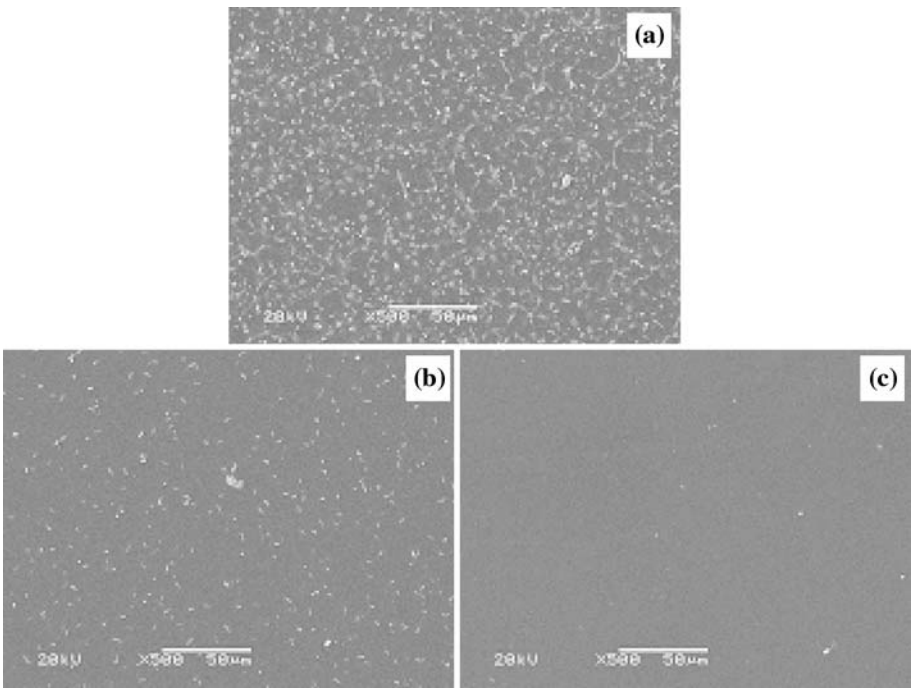

Fig. 8 SEM pictures of polymer surfaces after contact with human platelets for 60 min: **a** PLLA 50; **b** PLLA–PC 55/1; **c** PLLA–PC 46/1

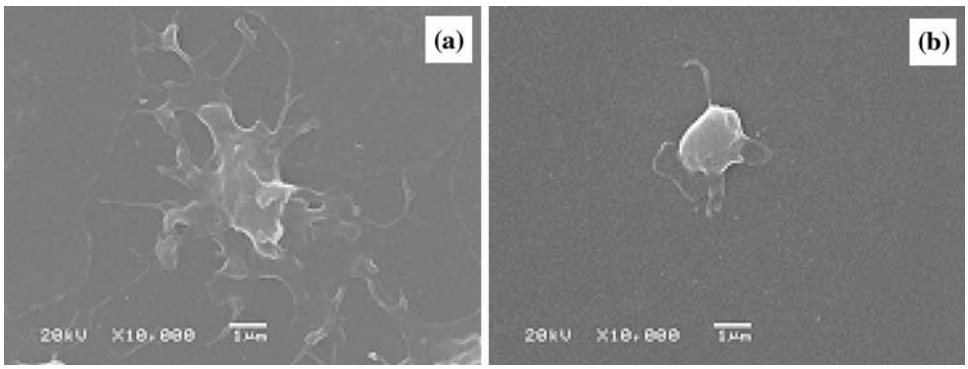

Fig. 9 Morphology of platelets adhered on polymer surfaces: **a** PLLA 50; **b** PLLA–PC 46/1

Conclusions

GPC was firstly prepared from lecithin in eggs and the structure was confirmed by FT-IR and ^{1}H-NMR analysis. PLLA–PC copolymers were then synthesized by ring-opening polymerization of LLA in the presence of GPC, and characterized by using FT-IR, ^{1}H-NMR, and ^{13}C-NMR. In the synthesis process, the reaction time was found to be a very important factor influencing on the yield. Copolymers with the largest molecular weights were obtained at 122 °C for 48 h.

In addition, the properties and biocompatibility of the copolymers were characterized in this study. A surface rearrangement was observed due the dynamic molecular motion. Increase in hydrophilicity induced to decrease in fibrinogen adsorption and platelet adhesion, due to the hydrophilic PC moieties in the copolymers. The higher the PC content, the higher the hydrophilicity, and the lower the fibrinogen adsorption and platelet adhesion.

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