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Synthesis and Characterization of Hydroxyl-Terminated Poly(γ-benzyl-L-glutamate)

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Synthesis and Characterization of Hydroxyl-Terminated Poly(γ-benzyl-L-glutamate)

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In order to provide an active end group of hydroxyl group and improve the hydrophility of poly(γ-benzyl-L-glutamate) (PBLG), ethanolamine (EA) was utilized as the initiator to initiate N-carboxy-γ-benzyl-L-glutamate anhydride (Bz-L-Glu-NCA) polymerization. The prepared hydroxyl-terminated PBLG (HO-PBLG) was fully characterized by FTIR, ^{1}H-NMR, XPS, XRD, DSC, and GPC. The results of FTIR and XRD indicated that the chain conformation of HO-PBLG predominantly presented α-helix. The water contact angle was measured to confirm that the hydrophilicity was improved by the introduction of hydroxyl group. Chondrocytes studies showed that the cells attachment efficiency on the HO-PBLG film was good and the cells grew well.

Keywords: hydroxyl-terminated poly(γ-benzyl-L-glutamate); ethanolamine; synthesis; biocompatibility

1 Introduction

Amino acids used as the important constituent unit of organism, synthetic poly(amino acid)s have attracted much attention in recent years (1–4). As a type of the important synthetic poly(amino acid)s, poly(γ-benzyl-L-glutamate) (PBLG) has been widely used in the biomedical field such as drug carrier, biosensor, due to its biodegradability, non-toxicity of the degradation products (5–11). For these applications, incorporation of functional end group onto the chains is essential for targeting the drug-delivery complexes, as well as anchoring of the substrate materials.

The best technique for synthesis of high molecular weight PBLG is ring-opening polymerization of Bz-L-Glu-NCA (12). The conventional Bz-L-Glu-NCA polymerization was initiated using primary amines and tertiary amines (13–15). Recently, Deming et al. reported primary amine-transition metal initiators for the preparation of PBLG (12, 16, 17). However, these products have no functional end group for the further reaction. Hydroxyl groups of chain ends were allowed to further join drugs or incorporate cell signaling peptides, including the sequence RGD (18). In order to provide active end group of hydroxyl group to combine other materials and improve the biocompatibility of PBLG by increasing the hydrophility of PBLG to some extent, ethanolamine (EA) was utilized as the initiator.

In this study, HO-PBLG was synthesized through anionic ring-opening polymerization of Bz-L-Glu-NCA initiated by EA and the chain conformation was studied. Moreover, chondrocytes were cultured on the HO-PBLG film, aiming to primarily test the biocompatibility of the HO-PBLG film.

2 Experimental

2.1 Materials

L-glutamic acid was purchased from Sigma (USA) and dried for 48 h under vacuum. Tetrahydrofuran (THF) was distilled over sodium. EA, dichloromethane, ethyl acetate and n-hexane were distilled before used. Other reagents were commercially available and used as received.

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS) and trypsin were purchased from Gibco (USA).

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2.2 Preparation of HO-PBLG Film

The monomer of Bz-L-Glu-NCA was synthesized according to the method introduced by Blout (13) as following: Bz-L-Glu-NCA (5 g, 19 mmol) was weighed and introduced in a flame-dried flask. The desired amount of EA was dissolved in 100 mL dried dichloromethane and added via cannula. The solution was stirred for 72 h under nitrogen at room temperature. The product was precipitated from dichloromethane using ether as a precipitator and dried under high vacuum. To prepare the HO-PBLG film, the product was firstly dissolved in chloroform (5% wt/vol), and then the solution was cast onto the glass plate. When the solvent evaporated spontaneously, the HO-PBLG film was obtained.

2.3 Characterization

FTIR spectrum was recorded on the FTIR spectrometer (Nexus, Nicolet, USA) using KBr pellets method and ^{1}H-NMR spectrum was recorded on the NMR instrument (DRX-400NMR, Bruker, Germany-Switzerland) at 400 MHz using $CDCl_3$ as solvent.

X-ray photoelectron spectroscopy (XPS) analysis was acquired on an X-ray photoelectron spectrometer (Ultra DCD, Kratos Axis, Britain) using a monochromated Al-K_α source at a pressure of 2×10^{-9} Torr and a scan area of 0.7×0.3 mm^2. Element analysis and quantification spectra from the individual peaks were obtained with 40 eV pass energy. A value of 285.0 eV for the binding energy of the C_{1s} component was used to correct the charge of specimens under irradiation. A Gaussian function was assumed for the curve-fitting process.

X-ray diffraction (XRD) analysis was performed on an X-ray diffractometer (X Pert' PRO, PANalytical, Netherlands) using a flat camera and 40 keV Cu-k_α ($\lambda = 0.154$ nm) radiation. Measurement was made in the range from 2 to 40° in a 0.02° step size.

Differential scanning calorimetry analysis (DSC) was recorded on a DSC apparatus (204 F1, NETZSCH, Germany) at a rate of 10°C/min under nitrogen. DSC calibration was performed with indium, and the transition temperature was determined at the midpoint between upper and lower intersection of the baseline with the tangent to the transition step. The used sample was prepared from monomer/initiator (M/I) = 100.

The molecular weight and molecular weight distribution of HO-PBLG were determined with gel permeation chromatography (GPC) (515, Waters, USA) using standard polystyrene as the reference and THF as the eluent at 35°C.

The water contact angle was measured on contact angle system (OCA15, dataphysics, Germany) at 25°C and 65% relative humidity on five different regions of each sample surface and the average values were taken.

2.4 Cell Culture on the HO-PBLG Film

In order to primarily evaluate the biocompatibility of HO-PBLG, the sample of M_n = 18,388 was selected. The film was sterilized by 75% (v/v) ethanol, washed with phosphate buffer solution (PBS, pH 7.2), then placed in 96-well plates. The chondrocytes (passage 1) were seeded evenly onto the HO-PBLG at a density of about 5×10^3 cells per well. The cells were cultured in DMEM supplemented with 20% FBS and were maintained in an incubator (Thermo Forma, USA) with 5% CO_2 at 37°C. The cell attachment efficiencies at different time were determined using a hemacytometer to count the number of cells.

After the cells were cultured for 3 days, the films were fixed by immersing in 2.5% (v/v) glutaraldehyde for 1 h at 4°C and dehydrated by ethanol, and then dried at ambient temperature for SEM observation (30XLFEG, Philips, Netherlands) to visualize the morphology of the cells.

3 Results and Discussion

3.1 Synthesis of HO-PBLG

The FTIR spectrum of HO-PBLG is shown in Figure 1. The peak at 3291 cm^{-1} was assigned to the N-H stretching motion. The peak at 1651 cm^{-1} was attributed to the C═O stretching motion of the amide group (amide I) and the peak at 1548 cm^{-1} was attributed to the associated absorbance of C-N stretching motion and N-H deformation motion of amide group (amide II), which indicated that the linkage of amide was formed. The peak at 1732 cm^{-1} corresponded to C═O stretching vibration of the ester group in the side chain. The absorptions at 697 cm^{-1} and 750 cm^{-1} were characteristic peaks of the phenyl group. In addition, characteristic peaks of monomer Bz-L-Glu-NCA at 1850 and 1780 cm^{-1} disappeared. Based on the above results, PBLG was successfully synthesized. The amide I and II bands were observed respectively at 1651 and 1548 cm^{-1}, which revealed that the chain conformation of HO-PBLG predominantly exhibited the α-helical form (19, 20).

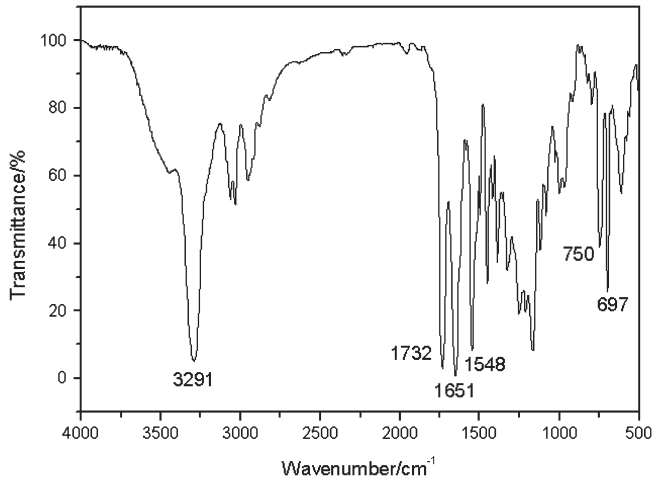

Fig. 1. FTIR spectrum of HO-PBLG.

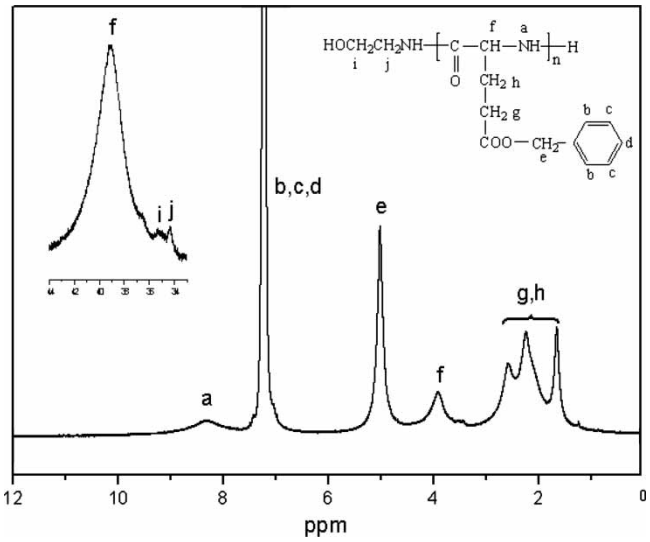

Fig. 2. ^{1}H-NMR spectrum of HO-PBLG.

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The structure of HO-PBLG is further confirmed by ^{1}H-NMR spectrum. As shown in Figure 2, the peak at 8.18 ppm was attributed to the proton signal of amide (a) in the backbone, which showed the formation of the amide. The proton signals in the benzene ring (b, c, d) were overlapped at 7.24 ppm and the proton signals of methylene (e) adjacent to the benzene ring were detected at 5.02 ppm. The peaks at 3.91 and 2.58∼1.67 were assigned to the proton signals of methenyl (f) in the backbone and the methylene (g, h) in the side chain, respectively. The proton signals of methylene (i) in the terminal group were detected at 3.53, which showed that the methylene was adjacent to the hydroxyl end group (21, 22) and the proton signals of methylene (j) in the end group adjacent to the amide were detected at 3.44. The results confirmed that HO-PBLG was successfully synthesized.

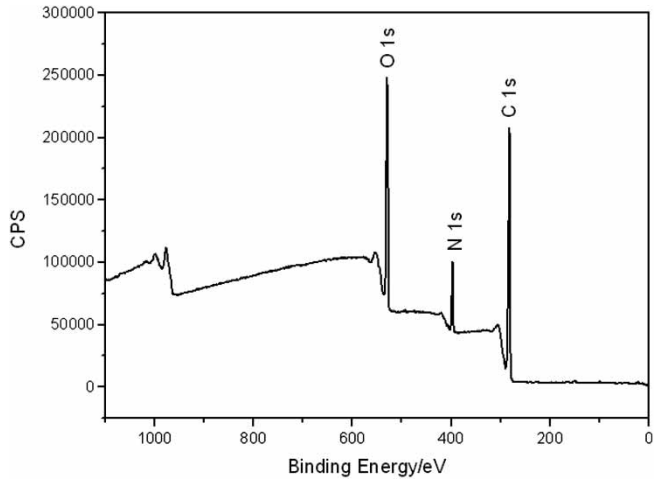


Fig. 3. XPS survey spectrum of HO-PBLG.

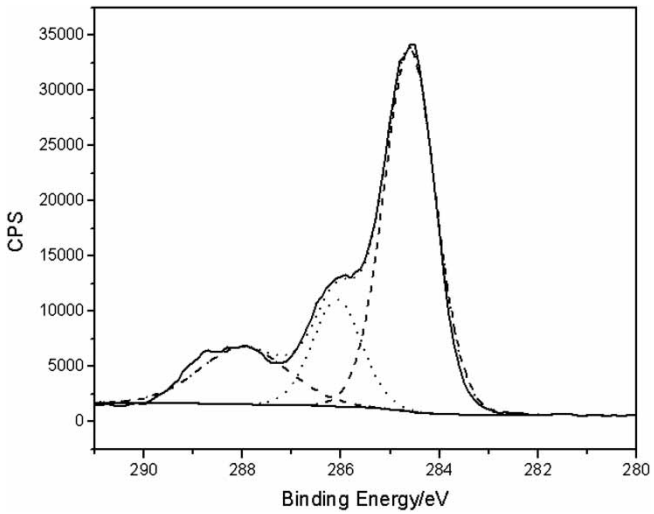


Fig. 4. C_{1s} core level scan spectrum of HO-PBLG.

XPS analysis has been employed to get information on the structure and chemical states of the surface of HO-PBLG film and the survey spectrum of HO-PBLG is presented in Figure 3. As expected, C_{1s}, N_{1s}, and O_{1s} peaks appeared on the surface of HO-PBLG film and their binding energies were 285.2, 399.2, and 532.2 eV, respectively. The found percentages of C, N, and O atoms in HO-PBLG based on the survey spectrum were 69.92%, 6.79% and, 23.29%, respectively. The calculated percentages of C, N, and O atoms in HO-PBLG based on the structure HO-PBLG were 69.90%, 6.80%, and 23.30%, respectively. The result showed that the found atomic percentage was in agreement with the calculated atomic percentage.

The spectrum is fitted with Gaussian peaks to elucidate the state of chemical bonds. The C_{1s} spectra of HO-PBLG could be resolved into three components, as shown in Figure 4. The peaks near 284.6, 286.1, and 288.6 eV were assigned to the functional groups of C-C (or C-H), C-O, and C═O (COOR or CONH) on the surface of HO-PBLG film, respectively. The relative intensity of the fitted C_{1s} peaks is presented in Table 1. From Table 1, the found relative intensity of the fitted C_{1s} peaks agreed with the calculated relative intensity of the fitted C_{1s} peaks based on the structure HO-PBLG and further demonstrated that HO-PBLG was successfully obtained.

XRD pattern of HO-PBLG is shown in Figure 5. As shown, the diffraction peaks appeared at $2\theta = 7.24^\circ$, 12.52°, 14.50°, and 21.76°, which were respectively assigned to $(1\bar{1}0)$, (110), (200), and (300) lattice planes of the α-crystal of PBLG

Table 1. Relative intensity of the fitted C_{1s} peak of HO-PBLG

Position (eV)	Relative intensity (at %)	Possible chemical state
284.6	66.3	C-C, C-H
286.1	17.0	C-N, C-O
288.0	16.7	COOR, CONH

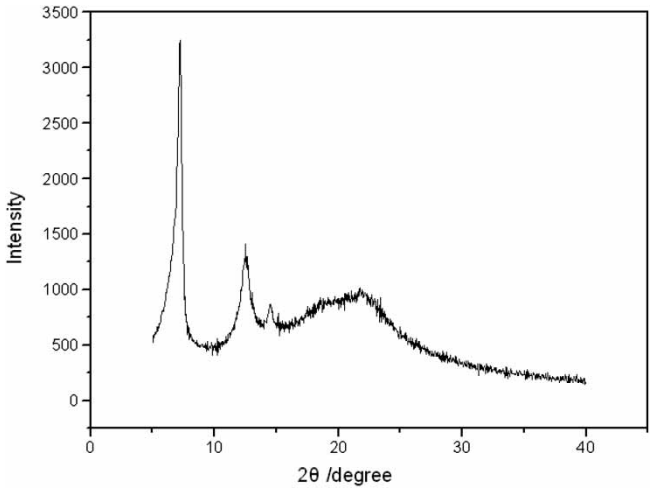


Fig. 5. XRD pattern of HO-PBLG.

(triclinic cell, a = 1.54 nm, b = 1.54 nm, c = 2.70 nm, $\alpha = 58.0^\circ$, $\beta = 58.0^\circ$, $\gamma = 122^\circ$). The α-helical conformation was stabilized by intra-molecular hydrogen bonds between the amide oxygen and the amide proton at the next fourth residue. The results also indicated that the chain conformation of HO-PBLG presented α-helix.

The DSC result, shown in Figure 6, is enlightening with respect to the imposed thermodynamic changes. From the DSC curve, there existed a glass transition for HO-PBLG at 18°C with an associated change in specific heat of $\Delta c_p = 0.41 J/(g \cdot K)$, which was similar to reported data (23). However, the absence of the low melting temperature was at about 373 K in the DSC curve, which reflected the absence of the first-order transformation between two helical conformations: from a 7/2 to an 18/5 α-helical conformation. The α-helix, stabilized by intra-molecular hydrogen bonds, has its residues on a spiral pitch of

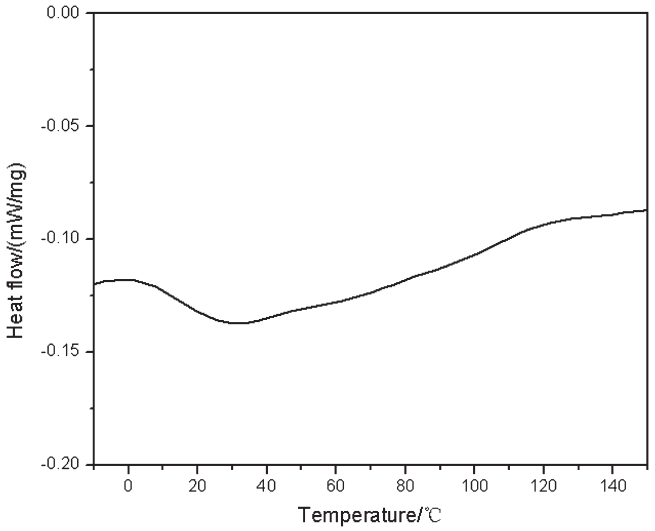


Fig. 6. DSC curve of HO-PBLG.

Table 2. Molecular weight, polydispersity and contact angle of HO-PBLG

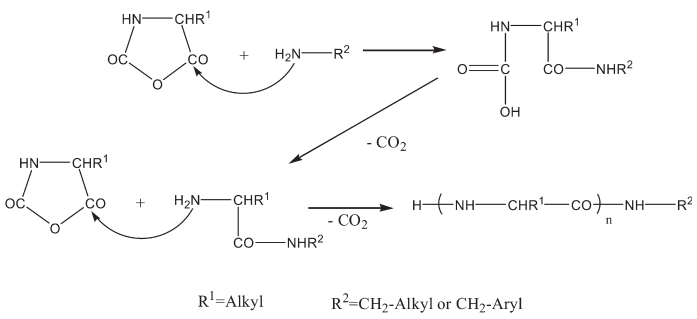
No.	M/I molar ratio[a]	M_n	M_w	M_w/M_n	Contact angle (°)
1	100	18388	32725	1.78	73.7 ± 0.6
2	200	35115	68470	1.95	74.4 ± 0.7
3	400	62832	113732	1.81	77.2 ± 0.6
4	500	76872	142210	1.85	78.6 ± 0.4

[a]M/I: the monomer to initiator molar ratio.

approximately 0.54 nm, which is right handed for L-α-amino acids and has about 3.6 residues per turn, giving 18 residues in five turns and the α-helical secondary conformation is the norm for peptides. In addition, a second helical conformation has been reported with seven residues in two turns (7/2). From the above experimental result, the chain conformation of the prepared HO-PBLG was 18/5 α-helix.

The molecular weight and polydispersity of HO-PBLG are characterized by GPC and the results are summarized in Table 2. From Table 2, it could be seen that the molecular weight increased from 18,388 g/mol to 76,872 g/mol with the increase in M/I from 100 to 500 and the molecular weight distribution index was in the range of 1.78∼1.95. When primary amines were used as initiators, the initiation step (see Scheme 1) was somewhat faster than the propagation steps because the initiators were slightly more nucleophilic than the amino groups of the active chain ends. Therefore, it is possible to control the molecular weight via M/I in principle. From the above experimental result, it can be concluded that HO-PBLG with the different molecular weight can been prepared by changing M/I.

The water contact angle of HO-PBLG is used to characterize the hydrophilicity of HO-PBLG and the results are also listed in Table 2. According to the literature (24), the water contact angle of PBLG initiated by triethylamine was 82°. From Table 2, the water contact angle of HO-PBLG ranged from 78.6° to 73.7° with the decrease in M/I from 500 to 100, suggesting that the hydrophilicity of HO-PBLG was improved with the increase of the relative percent of



Sch. 1. Normal route of primary amine-initiated NCA polymerization.

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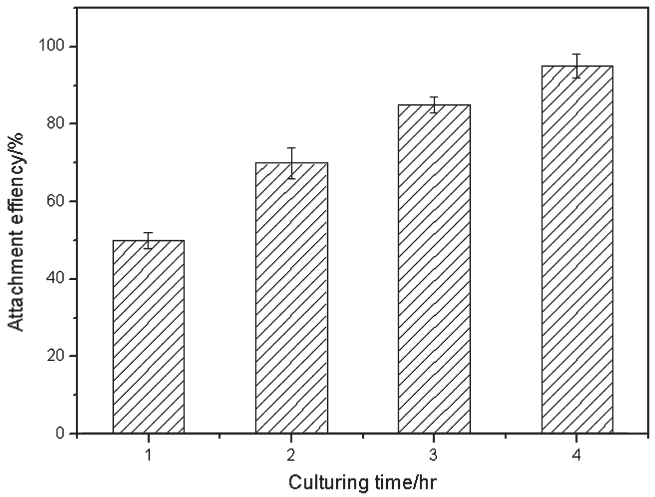

Fig. 7. Cell attachment efficiencies on the HO-PBLG film at different times.

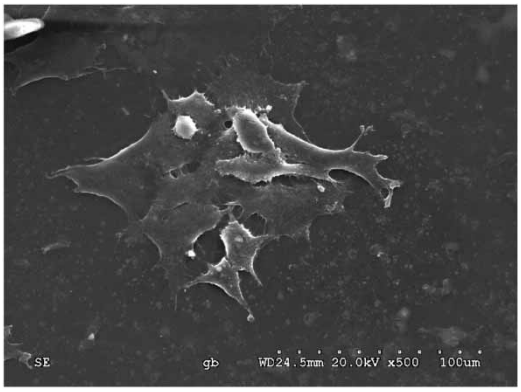

Fig. 8. SEM photograph of the cells on HO-PBLG at 3 days.

hydroxyl group in HO-PBLG. The above results confirmed that the hydrophilicity of HO-PBLG could be improved to a certain extent by incorporation of hydroxyl group.

3.2 Cell Studies

Cell attachment experiments with chondrocytes are utilized to illustrate the cell affinity of the HO-PBLG and the attachment efficiencies of the cells on the HO-PBLG film after 1, 2, 3, and 4 h are shown in Figure 7. As shown, the cell attachment efficiency on HO-PBLG film got to 50 $\pm$ 2% after the cells were cultured for 1 h. At 4 h, the cell attachment efficiency arrived at 95 $\pm$ 2%. It indicated that the cell attachment efficiency went up with the increase of the time and the cells attachment capacity of HO-PBLG was excellent.

After the cells were cultured for 3 days, SEM was used to visualize cells on the film and SEM image was presented in Figure 8. As shown, the cells tightly integrated with film and the cells exhibited typical polygonal morphology, a characteristic of chondrocytes' morphology, which showed that the cells on the HO-PBLG film grew favorably.

4 Conclusions

HO-PBLG with different molecular weight was successfully synthesized *via* anionic ring-opening polymerization of Bz-L-Glu-NCA initiated by EA. DSC study showed that the glass transition for HO-PBLG at 18°C and the chain conformation of the prepared HO-PBLG is 18/5 α-helix and not contain 7/2 α-helix. The number average molecular weight was affected by M/I and the polydispersity index was found in the range of 1.78$\sim$1.95. The contact angle test showed that the hydrophilicity of HO-PBLG was improved to some extent by the incorporation of the hydroxyl group. Preliminary cell studies indicated the potential of HO-PBLG in medical applications.

5 Acknowledgments

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