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The synthesis, deprotection and properties of poly(γ-benzyl-L-glutamate)

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Diethylamine, di-*n*-hexylamine, dicyclohexylamine and triethylamine have been used as initiators for the ring-opening polymerization of γ-benzyl-L-glutamate *N*-carboxyanhydride (BLG NCA) to synthesize poly(γ-benzyl-L-glutamate) (PBLG). The relationship between the molecular weight of PBLG and the molar ratio of monomer and initiator was studied. With dicyclohexylamine as initiator, the influence of monomer concentration, and reaction temperature and time on the polymerization of BLG NCA was examined. Three reagents were used for the deprotection of benzyl groups in PBLG, including hydrobromic acid/acetic acid (33 wt.%), NaOH aqueous solution and trimethylsilyl iodide (TMSI). Through examining the molecular weight of PLGA obtained using different deprotection methods, it was revealed that TMSI could minimize chain cleavage in the process of deprotection and retain the degree of polymerization. The biocompatibilities of PBLG obtained using different initiators were evaluated by a live/dead assay against L929 fibroblast cells. The *in vitro* cytotoxicities of PLGA obtained using different deprotecting agents were evaluated by a methyl thiazolyl tetrazolium assay. The results revealed that both PBLG and PLGA exhibited good biocompatibilities.

biocompatibility, biomaterials, deprotection, poly(γ-benzyl-L-glutamate), polymerization

1 Introduction

Synthetic poly(amino acid)s are commonly used polymers composed of α-amino acids by the connection of peptide bonds (–CO–NH–), which can be degraded into α-amino acids and metabolized in the human body. Their excellent biocompatibilities and good biodegradabilities *in vivo* make poly(amino acid)s ideal biomedical materials [1]. At present, poly(amino acid)s have been widely used in many fields, such as drug carriers [2–5], tissue engineering materials [6, 7], and gene vectors [8, 9].

Poly(γ-benzyl-L-glutamate) (PBLG) and its derivatives are one of the most widely investigated synthetic poly(amino acid)s, and the degradation product *in vivo* is L-glutamic acid, which is an essential amino acid for the human body. PBLG can be conveniently synthesized through the ring-opening polymerization (ROP) of γ-benzyl-L-glutamate *N*-carboxyanhydrides (BLG NCA) [10]. The mechanism of NCA polymerization has been intensively studied for more than 40 years in order to realize control over the chain growth [11]. Traditional ROP of NCA mainly involves two polymerization mechanisms: i) normal amine mechanism (NAM) and ii) activated monomer mechanism (AMM) (Scheme 1) [10–13]. NAM is mostly applicable to primary amines and strong nucleophilic reagents, and the initiation rate of NAM is always slow and

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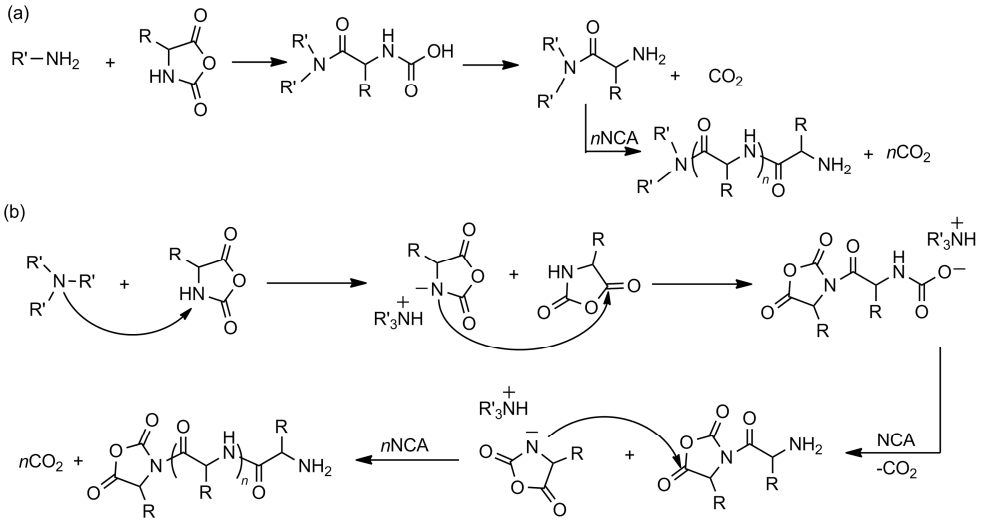

Scheme 1 The normal amine mechanism (NAM, (a)) and activated monomer mechanism (AMM, (b)) of NCA polymerization.

does not afford poly(amino acid)s with high molecular weight [14–16]. Relatively strong alkalis including some secondary amines [16] and tertiary amines primarily follow AMM [17]. The initiation rate is relatively fast, and the AMM can afford poly(amino acid)s with high molecular weight. However, pre-control of the molecular weight is difficult and the molecular weight dispersion is wide [12].

In recent years, many researchers have synthesized poly(amino acid)s with controlled molecular weight and low polydispersities. A number of influencing factors have been studied, including initiator species, reaction pressure, and reaction temperature. Initiators based on zerovalent nickel organic compounds (such as bipyNi(COD), bipy = 2,2′-bipyridyl and COD = 1,5-cyclooctadiene) have been developed, and the controlled polymerization of NCA with low molecular weight dispersity was achieved [18, 19]. However, the residual nickel in the poly(amino acid)s severely limited its application in biological fields. Cheng *et al.* [20, 21] adopted 1,1,1,3,3,3-hexamethyldisilazane (HMDS) as the initiator of BLG NCA, and found that HMDS could effectively control the polymerization of BLG NCA as well as giving a low polydispersity (polydispersity index, *PDI* = 1.19–1.26) . High vacuum techniques (HVT) were also employed for the ROP of NCA by Hadjichristidis's group, resulting in both high molecular weight and low polydispersities [22, 23]. The effect of decreased reaction temperature on the NCA polymerization was studied recently. When the polymerization temperature was decreased from 20 to 0 °C, the extent of side reactions was significantly reduced and the number of active chains increased, resulting in well-defined poly(amino acid)s with low polydispersities [24]. As the temperature was increased from 20 to 60 °C, it was found that almost all the end-groups were pyroglutamates, and chain end termination occurred throughout the polymerization, which led to an increasing number of dead chain-ends as the polymerization proceeded [25].

Poly(L-glutamic acid) (PLGA) is prepared from poly(γ-benzyl-L-glutamate) (PBLG) by removing the protecting benzyl groups. The commonly used deprotecting agent is hydrobromic acid/acetic acid (HBr/HOAc, 33 wt.%) [2, 26, 27]. Although this can remove the benzyl groups efficiently, at the same time it causes serious molecular strand breaking [28, 29]. This is a fatal disadvantage as far as controlling the molecular weight is concerned. Strong alkalis, such as KOH and NaOH [30, 31], have also been used for the deprotection of benzyl groups in PBLG. However, in strong alkaline conditions, the hydrolysis of amide bonds is severe, and this process is irreversible. Palladium/carbon catalytic hydrogenation, as a moderate method, has been shown to be effective by many researchers, but it only applies for PBLG with molecular weight lower than 10.0 kDa, because the stable α-helical secondary structure of PBLG with higher molecular weights prevents access of the hydrogenation catalyst to the amide bond [32, 33]. Trimethylsilyl iodide (TMSI) has also been used for the deprotection of PBLG [34], but relatively few studies have been reported.

Since HMDS, a secondary amine, shows good control over the polymerization of BLG NCA [21], it is of interest whether this is also the case for other secondary amines. In this paper, diethylamine, di-*n*-hexylamine, dicyclohexylamine and triethylamine have been used as initiators for the ROP of BLG NCA. The polymerization characteristics and mechanism have been investigated through controlling the molar ratio of monomer and initiator (*M*/*I*). The molecular weight of PLGA obtained from HBr/HOAc (33 wt.%), NaOH aqueous solution and TMSI were contrasted, and the results showed that TMSI could avoid chain cleavage to the greatest extent. The thermal stabilities, and degradability and biocompatibility properties of PBLG or PLGA were

also investigated.

2 Experimental section

2.1 Materials

γ-Benzyl-L-glutamate (BLG) was purchased from Sigma-Aldrich and used without any further purification. BLG NCA was synthesized as our previous work with slight modification [35–38]. Triphosgene, diethylamine, di-*n*-hexylamine, dicyclohexylamine, triethylamine and TMSI were purchased from Aladdin Reagents (Shanghai, China). Triphosgene was recrystallized twice before use. Dicyclohexylamine, triethylamine, hexylamine and diethylamine were purified by distillation before use. HBr/HOAc (33 wt.%) was supplied by J&K Scientific Ltd. 1,4-Dioxane, tetrahydrofuran, *n*-hexane and diethyl ether were purchased from Sinopharm Chemical Reagent Co., Ltd., and 1,4-dioxane, tetrahydrofuran (THF) and *N*,*N*-dimethylformamide (DMF) were further distilled before use. Other chemicals were analytical grade and used without any further purification.

2.2 Synthesis of PBLG

PBLG was synthesized through ROP of BLG NCA in 1,4-dioxane using dicyclohexylamine, triethylamine, di-*n*-hexylamine or diethylamine as initiators. Typically, BLG-NCA (2.0 g, 6.6 mmol) was dissolved in 60.0 mL of dry 1,4-dioxane in a flame-dried flask, and then 1.52 mL of 0.10 M dicyclohexylamine, triethylamine, di-*n*-hexylamine or diethylamine in 1,4-dioxane solution was added under vigorously stirring (*M*/*I* = 50/1). After stirring for 3 days at 15 °C, the mixture was precipitated into excess diethyl ether/ethanol (2/1, *v*/*v*) mixture. The obtained product was further washed twice with diethyl ether and dried under vacuum at room temperature for 24 h, affording 80–89% yield.

2.3 Deprotection of benzyl groups in PBLG

In this work, the deprotection of benzyl groups in PBLG was carried out by three different methods, namely HBr/HOAc (33 wt.%), NaOH aqueous solution and TMSI. The PBLG was all initiated by dicyclohexylamine with a number-average molecular weight (M_n) of 6.07×10^5 g mol^{-1}, as measured by gel permeation chromatography (GPC). The detailed methods were as follows:

a) HBr/HOAc (33 wt.%). 1.0 g of PBLG was dissolved in 10.0 mL of dichloroacetic acid at 25 °C in a flask. After adding 3.0 mL of HBr/HOAc (33 wt.%), the solution was slowly stirred at 30 °C for additional 1 h and the product was precipitated into excess diethyl ether. The precipitate was dissolved in 20.0 mL of DMF, dialyzed with a membrane (MWCO 3500) against deionized water for 3 days and then lyophilized (Yield: 79–83%).

b) NaOH aqueous solution. 1.0 g of PBLG was dissolved in 60.0 mL of 1,4-dioxane at room temperature in a flask. Then 6.8 mL of 1.0 M NaOH aqueous solution (1.5 equiv. of NaOH per benzyl group) was slowly added under vigorously stirring [39, 40]. The reaction mixture was stirred at room temperature for 12 h. The product was precipitated into excess diethyl ether. The precipitate was dissolved in 10.0 mL of DMF, dialyzed with a membrane (MWCO 3500) in deionized water for 3 days and then lyophilized (Yield: 80–83%).

c) TMSI. Deprotection was accomplished by dissolving the PBLG (1.0 g) in 50.0 mL of dry dichloromethane followed by addition of excess TMSI (0.5 mL), and then the mixture was stirred under nitrogen at 40 °C for 24 h. After the reaction, the product was dialyzed with a membrane (MWCO 3500) in deionized water (after addition of a small amount of 1.0 M HCl) for 3 days to remove all the impurities and lyophilized until dry. The yield was 81–85%. Deprotection kinetics of PBLG by TMSI were measured as follows: 1.0 g of PBLG was dissolved in 50.0 mL of dry dichloromethane at room temperature in a dry flask. Then 0.5 mL of TMSI was added by syringe and the mixture was stirred under nitrogen. At time points of 0, 4, 8, 12, 16, 20, 24 and 26 h, 5.0 mL aliquots of the reaction mixture were taken out by syringe and dialyzed with a membrane (MWCO 3500) in deionized water (after addition of a small amount of 1.0 M HCl) for 3 days. The final product was characterized by ^{1}H NMR spectroscopy.

2.4 Characterizations of polymers

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on Bruker AV 400 M NMR spectrometer in trifluoroacetic acid-*d* (TFA-*d*) or deuterium oxide (D_2O). M_n, weight-average molecular weights (M_w) and PDI were determined by GPC using a series of linear Tskgel Super columns (AW3000 and AW5000) and Waters 515 HPLC pump, with OPTILAB DSP Interferometric Refractometer (Wyatt Technology) as the detector. The eluent was DMF containing 0.01 M lithium bromide (LiBr) at a flow rate of 1.0 mL min^{-1} at 50 °C. Monodisperse polystyrene standards purchased from Waters Co. with a molecular weight range of 1790–200,000 g mol^{-1} were used to generate the calibration curve. The viscosity-average molecular weight (M_η) was determined using an HH-W600 Ubbelohde viscometer (Ningbo Tianheng, China): the capillary diameter of the viscometer was 0.38 mm, and the experiment was performed in 0.4 M NaCl/0.01 M NaH_2PO_4 buffer solution (pH 7.05) at 25.5 °C. M_η was obtained from the Mark–Houwink equation: intrinsic viscosity $[\eta] = K_m M_\eta^{\alpha}$, where $K_m = 2.93 \times 10^5$, and $\alpha = 0.923$ [26, 41]. Thermal properties of the PBLG and PLGA were examined by differential scanning calorimetry (DSC Q100, TA, USA) under N_2 atmosphere. The sample was heated from −30 to 200 °C at a rate of 10 °C min^{-1}, kept at 200 °C for 5 min, and cooled to −30 °C at a rate of 10 °C min^{-1}, and the data collection was carried

out on the second heating run.

2.5 Live/dead assays

To evaluate the cytotoxicities of PBLG synthesized with different initiators, a live/dead assay against L929 cells (a mouse fibroblast cell line) was employed. First, PBLG (theoretical *PD* = 50) was dissolved in THF at a concentration of 5.0 g L^{-1}, and the solution was spin coated on a clean circular slide (diameter: 14 mm, thickness: 1 mm) by a desktop spin coating machine (KW-4A, Institute of Microelectronics, Chinese Academy of Sciences). The slide was dried for 48 h in vacuo at room temperature to eliminate the remaining THF, yielding a PBLG film. The PBLG film was washed three times with phosphate buffered saline (PBS) at pH 7.4 and placed in a UV disinfection cabinet for 3 h before cell culture. L929 cells were planted in 24-well plants at 2.0×10^4 cells per well in 1.0 mL of complete Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS, Gibco BRL, Gaithersburg, MD) and cultured at 37 °C for 72 h. A control group, just a clear circular slide, not exposed to any chemicals or inserts, was maintained in parallel. After 72 h incubation, DMEM was removed, and each well was rinsed three times with PBS. Then, 20.0 μL of PBS containing calcein acetoxymethylester (Calcein-AM, 2.0 μg mL^{-1}) and propidium iodine (PI, 3.0 μg mL^{-1}) was added into each well, incubated for 30 min at room temperature, and examined using fluorescence microscopy.

2.6 Methyl thiazolyl tetrazolium (MTT) assays

The cytotoxicities of PLGA obtained with different deprotecting agents were investigated with a MTT assay toward L929 cells. The cells were seeded in 96-well plates at 10,000 cells per well in 180 μL of complete DMEM for 24 h, followed by removing culture medium and adding polymer solutions (200 μL in complete DMEM) at different concentrations (0–200 mg L^{-1}). PEI25k was used as positive control. The cells were subjected to MTT assay after being incubated for another 72 h. The absorbance of the solution was measured on a Bio-Rad 680 microplate reader at 490 nm. Cell viability (%) was calculated based on the following equation:

$$\text{Cell viability (\%)} = \frac{A_{\text{sample}}}{A_{\text{control}}} \times 100$$

wherein, A_{sample} and A_{control} represent the absorbances of the sample and control wells, respectively.

3 Results and discussion

3.1 Synthesis and characterization of PBLG

PBLG was synthesized by ROP of BLG NCA using diethylamine, di-*n*-hexylamine, dicyclohexylamine and triethylamine as initiators. All polymerizations were conducted with different feed molar ratios (*M*/*I* = 10, 20, 50, 100 and 200) at 15 °C in 1,4-dioxane. Figure 1 shows the ^{1}H NMR spectra of PBLG obtained using different initiators for *M*/*I* of 50 in TFA-*d*. The signals at 1.92, 4.65, 4.95 and 7.20 ppm are the characteristic resonances of PBLG. The small peak at 1.05 ppm in Figure 1(A) can be assigned to the methyl protons of diethylamine ($CH_3CH_2)_2N$–, 3H). The signals at 0.75 and 1.23 ppm in Figure 1(B) can be attributed to the methyl ($CH_3CH_2CH_2CH_2CH_2CH_2)_2N$–, 3H) and methylene protons ($CH_3CH_2CH_2CH_2CH_2CH_2)_2N$–, 8H) of di-*n*-hexylamine, respectively. The resonances at 1.18 and 3.55 ppm in Figure 1(C) are the signals of dicyclohexylamine. However, in Figure 1(D), the signal of triethylamine was not observed. This suggests that in the AMM of NCA polymerization initiated with triethylamine, the initiator did not enter the poly(amino acid) backbone.

The initiation characterizations of the four amines were investigated by varying the *M*/*I*. Figure 2 shows the change of M_n as a function of *M*/*I*. As shown in Figures 2(a) and 2(b), the M_n of PBLG increased with increasing *M*/*I*, while it was still no more than 8×10^4 g mol^{-1} with *M*/*I* at 200. In Figure 2(c), the M_n was about 1.2×10^5 g mol^{-1} even for *M*/*I* of 10, and a M_n of about 7.0×10^5 g mol^{-1} was achieved with *M*/*I* of 200. When the initiator was triethylamine, the M_n was not related to the *M*/*I* (Figure 2(d)). At a *M*/*I* of 10, the value of M_n was already more than 5.0×10^5 g mol^{-1}, and it reached about 7.2×10^5 g mol^{-1} with a *M*/*I* of 200. According to the characterization of the two polymerization mechanisms (NAM and AMM) and combining the results in Figures 1 and 2, it can be concluded that diethylamine and di-*n*-hexylamine followed the NAM pathway. As for dicyclohexylamine, the variation in M_n with *M*/*I* showed some similarities with triethylamine (Figure 2). However, in Fig-

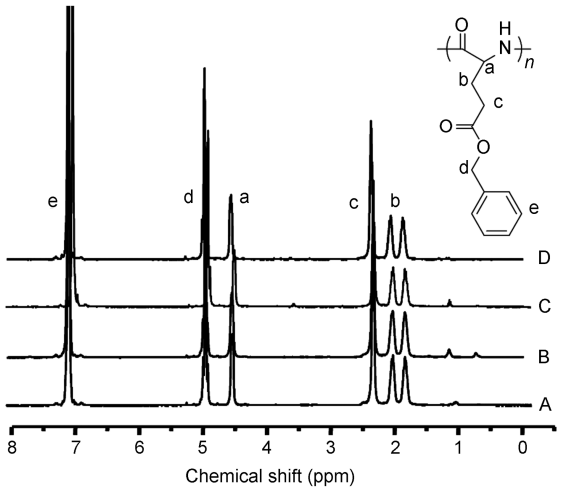

Figure 1 ^{1}H NMR spectra of PBLG obtained using different initiators: diethylamine (A), di-*n*-hexylamine (B), dicyclohexylamine (C) and triethylamine (D) (in TFA-*d*).

ure 1(C), peaks of dicyclohexylamine were visible whilst no signals of triethylamine were observed (Figure 1(D)), showing that dicyclohexylamine but not triethylamine entered the polymer chains. These observations suggest that the initiation of dicyclohexylamine occurred by both NAM and AMM mechanisms [13], whereas triethylamine initiated the ROP of NCA through the AMM.

3.2 Effect of monomer concentration, temperature and reaction time on the polymerization of BLG NCA

In order to further study the initiation characterization of dicyclohexylamine, the effects of monomer concentration ([M]), temperature and reaction time were investigated. The effect of [M] on the polymerization was studied as follows: the reaction was conducted in 1,4-dioxane at 12 °C for 72 h, and [M] ranged from 0.095 to 0.38 mol L^{-1} with *M*/*I* at 100. The conversions of BLG NCA at different concentrations are shown in Figure 3(a). When [M] changed from 0.095 to 0.127 mol L^{-1}, the conversion increased from 60 to 72% in the test duration. When [M] was increased above 0.253 mol L^{-1}, the conversion remained constant at ~85%. At low [M], the conversion was relatively low. This is because low [M] leads to low effective collision probability. When [M] was higher, the viscosity of the reaction system increased very rapidly, and this is not conducive to improving the conversion. The relationship between M_n and *PDI* with [M] is shown in Figure 3(b); M_n did not show much change with

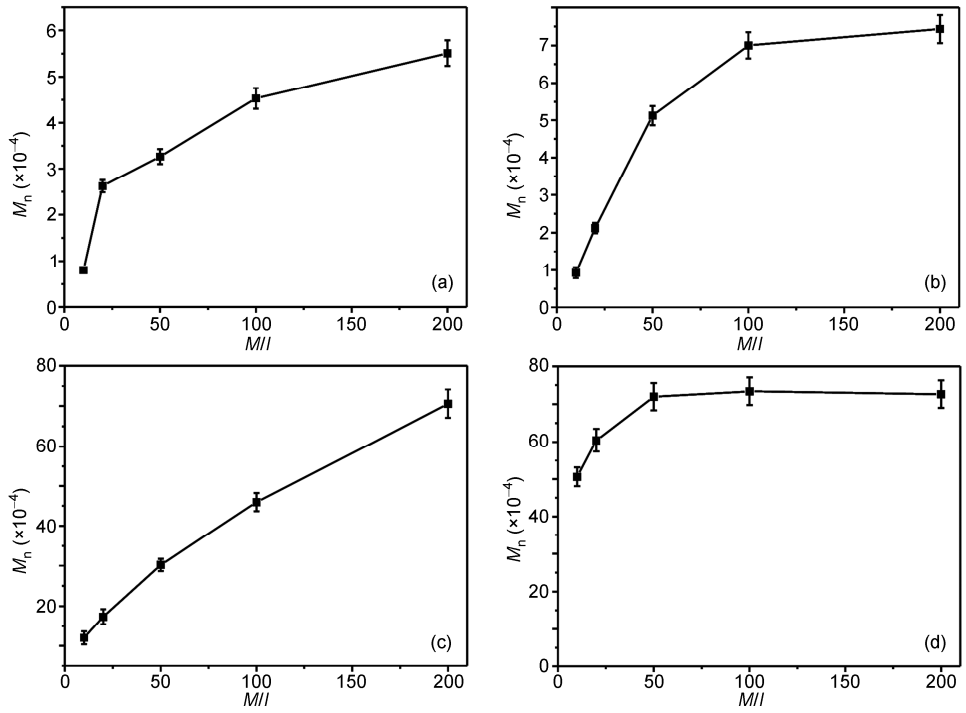

Figure 2 The relationship between M_n of PBLG and *M*/*I*. The initiators were diethylamine (a), di-*n*-hexylamine (b), dicyclohexylamine (c) and triethylamine (d), respectively. Data are represented as mean ± standard deviation (n = 3).

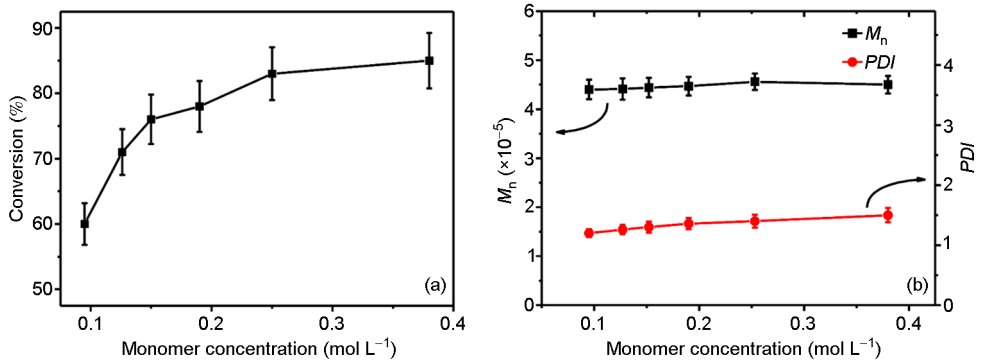

Figure 3 (a) The conversions of BLG NCA at different monomer concentrations at 12 °C for 72 h; (b) the M_n and *PDI* of PBLG synthesized at different concentrations of BLG NCA. The conversion was determined by ^{1}H NMR spectroscopy in TFA-*d*. Data are represented as mean ± standard deviation (n = 3).

increasing [M], but the polydispersities showed a slight increase. The viscosity of the reaction system increased at high [M], and the collisions between monomer and active chains was relatively difficult, resulting in a wide *PDI*.

Reaction temperature is an important influence on the ROP of NCA monomers in most polymerization processes. The relationship between monomer conversion and reaction temperature is shown in Figure 4(a). The conversion decreased continuously with increasing temperature. When the reaction temperature was increased from 12 to 40 °C, the conversion decreased from about 90% to 80%. From Figure 4(b), the M_n showed a slight decrease and the *PDI* showed an increase with increasing reaction temperature, indicating that lower reaction temperature led to higher monomer conversion, higher M_n and narrower polydispersities. At high temperature, the extent of side reactions—such as the formation of succinimide units in the main chain—increased, and such an end-capping reaction terminates the chain growth [25, 42, 43].

Reaction time also had an important effect on the polymerization of BLG NCA. The relationship between reaction time and monomer conversion and molecular weight were investigated under the same conditions. The reaction was conducted in 1,4-dioxane at 12 °C with [M] of 0.253 mol L^{-1} and *M*/*I* at 100. At the time points 2, 5, 12, 24, 48 and 72 h, the monomer conversion and M_n were measured. The conversion of BLG NCA increased with extended reaction time (Figure 5(a)). It increased rapidly at first, and then the rate of increase decreased for longer reaction times. When the reaction time was over 48 h, the conversion did not change significantly. The variation of M_n with the reaction time (Figure 5(b)) showed almost the same tendency as that of monomer conversion. At the beginning of the reaction, the monomer conversion and M_n increased rapidly. As the polymerization progressed, the system viscosity increased, [M] decreased and movement of the active chain became more difficult. All of these phenomena are not conducive to the chain growth, so the monomer conversion and M_n remained essentially constant.

3.3 Deprotection of benzyl groups in PBLG

In this work, three deprotecting agents (*i.e.* HBr/HOAc (33 wt.%), NaOH aqueous solution and TMSI) were used to remove the benzyl groups in PBLG, and the results are shown in Figure 6. The disappearance of the characteristic peaks at about 5.0 (d) and 7.05 ppm (e) in Figures 6(B), 6(C) and 6(D) indicated that the benzyl groups in PBLG had been successfully removed with all the three reagents. The M_η of PLGA obtained using different deprotecting agents

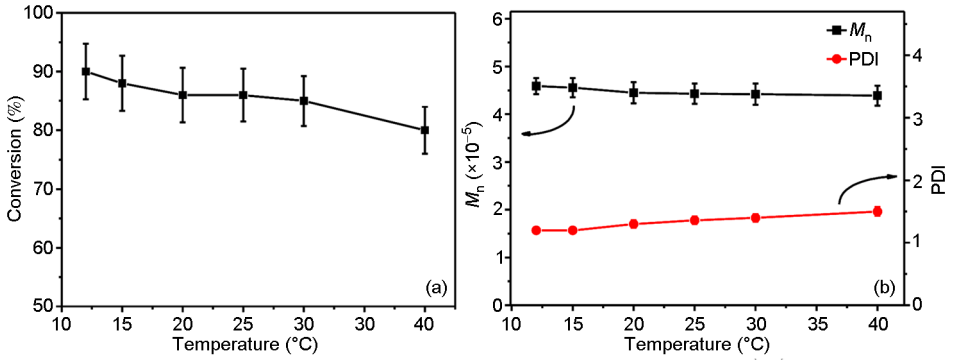

Figure 4 The conversions of BLG NCA at different temperatures (a); the M_n and PDI of PBLG at different temperatures (b). The monomer conversion was determined by ^{1}H NMR spectroscopy in TFA-*d*. Data were represent as mean ± standard deviation (n = 3).

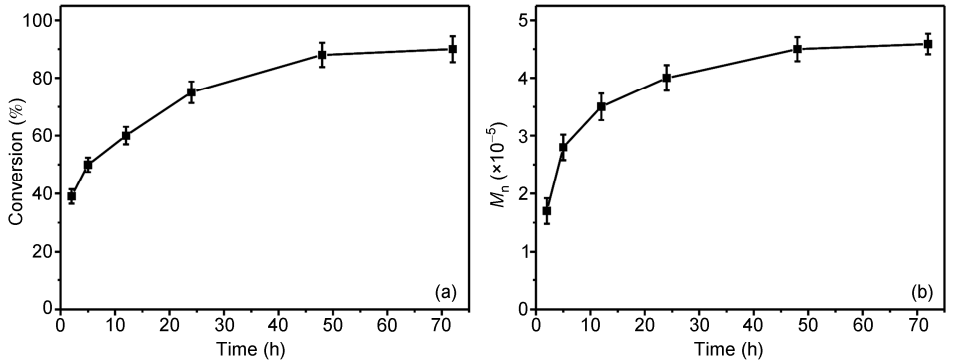

Figure 5 The conversions of BLG NCA (a) and M_n of PBLG (b) at different reaction times. The monomer conversion was determined by ^{1}H NMR spectroscopy in TFA-*d*. Data are represented as mean ± standard deviation (n = 3).

was measured to evaluate the degree of chain cleavage. The M_η showed remarkable differences as follows, NaOH aqueous solution: 7.2×10^4 g mol^{-1}, HBr/HOAc (33 wt.%): 9.2×10^4 g mol^{-1} and TMSI: 2.9×10^5 g mol^{-1} (Figure 7). The M_η of PLGA obtained from TMSI was the highest, indicating that TMSI could avoid chain cleavage to the greatest extent, and retain the molecular weight of PLGA after deprotection.

In order to understand the deprotection process of TMSI in detail, the deprotection kinetics was studied. As shown in Figure 8, spectra were recorded for the products after 0, 4, 8, 12, 16, 20, 24 and 26 h. As the time increased, the peaks at 5.1 and 7.2 ppm became smaller and smaller, and disappeared at 26 h, indicating that the benzyl groups in PBLG had been removed completely (Figure 8(a)). It should be noted that PBLG was converted to PLGA when all the benzyl groups in PBLG were removed, and this is insoluble in TFA-*d*, so D_2O with a small amount of NaOD was used as the solvent for ^{1}H NMR measurements. The quantitative statistical results are shown in Figure 8(b). They reveal that the deprotection of benzyl groups in PBLG was fast at first, and then the deprotection rate gradually decreased.

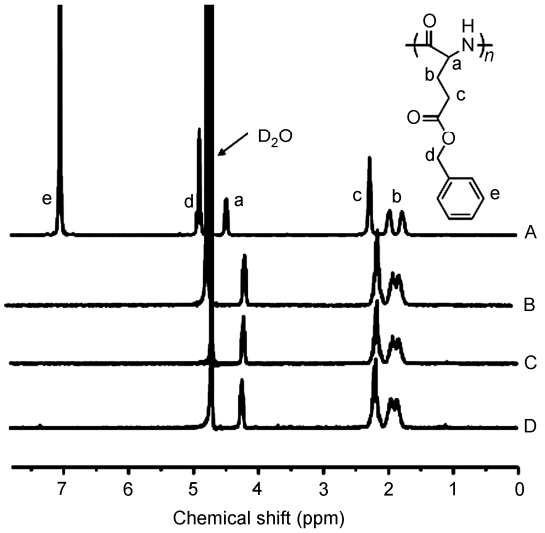

Figure 6 ^{1}H NMR spectra of PBLG (A) and PLGA obtained by deprotection with HBr/HOAc (33 wt.%) (B), NaOH aqueous solution (C) and TMSI (D) (in TFA-*d* (A) and D_2O (B, C and D)).

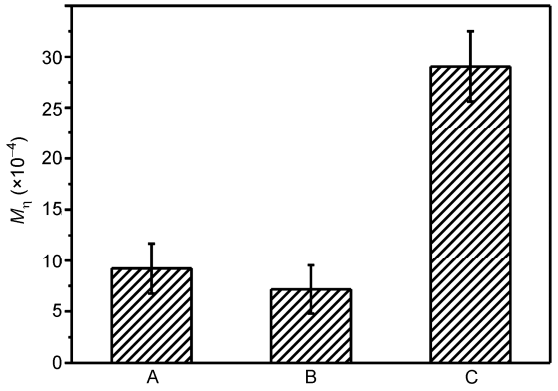

Figure 7 The M_η of PLGA prepared with HBr/HOAc (33 wt.%) (A), NaOH aqueous solution (B) and TMSI (C) as deprotecting agents. Data are represented as mean ± standard deviation (n = 3).

3.4 Thermal properties of PBLG and PLGA

The TGA data indicated that there was no significant difference in thermal stability for PBLG with different molecular weights (Figures 9(Aa), 9(Ab), 9(Ac) and 9(Ad)). The thermal degradation began at about 260 °C, and was mainly caused by the fracture of peptide bonds in the main chains and the ester bonds of the side groups. In the DSC traces (Figure 9(B)), at about 20.3 °C, the unfreezing of the side chain and backbone motions of PBLG were observed and the activity became weaker with increasing molecular weight. As for PLGA, the decomposition temperature (T_d = 207 °C) was significantly lower than that for PBLG (T_d = 260 °C) (Figure 9(Ae)), indicating that the removal of the benzyl groups in PBLG reduced the thermal stability. At the same time, the glass and melting transitions were not observed in the DSC trace of PLGA (Figure 9(Be)). The carboxylic acid functional pendant groups in PLGA chains are strongly polar groups, which will result in strong intermolecular and intramolecular interactions, such as hydrogen bonding, in PLGA thus preventing the movement and rotation of the molecular chains [44].

3.5 Degradation of PLGA

Figure 10 shows the degradation of PLGA in PBS at pH 7.4, 37 °C at a concentration of 1.0 g L^{-1}. The M_η of PLGA decreased severely in the first week. Five weeks later, it was only about 2000 g mol^{-1}, which indicated the good degradability of PLGA. This suggests that the amide bond in PLGA is readily hydrolyzed in PBS. If an extended degradation time is required, cross-linking and copolymerization with aliphatic polyesters are appropriate methods [45].

3.6 Cytotoxicities of PBLG synthesized with different initiators

The cytotoxicities of PBLG synthesized with different initiators were investigated through a live/dead assay, where viable cells fluoresced green through the reaction of Calcein-AM with intracellular esterases and non-viable cells fluoresced red due to the diffusion of PI into cells with damaged membranes. The viability of cells cultured in the presence of PBLG is shown in Figure 11, and there was no significant difference in cell viability after culturing for 72 h with PBLG with different initiators as compared with the control. Furthermore, the almost complete absence of dead cells suggested that PBLG samples synthesized with all four initiators exhibited good cytocompatibilities.

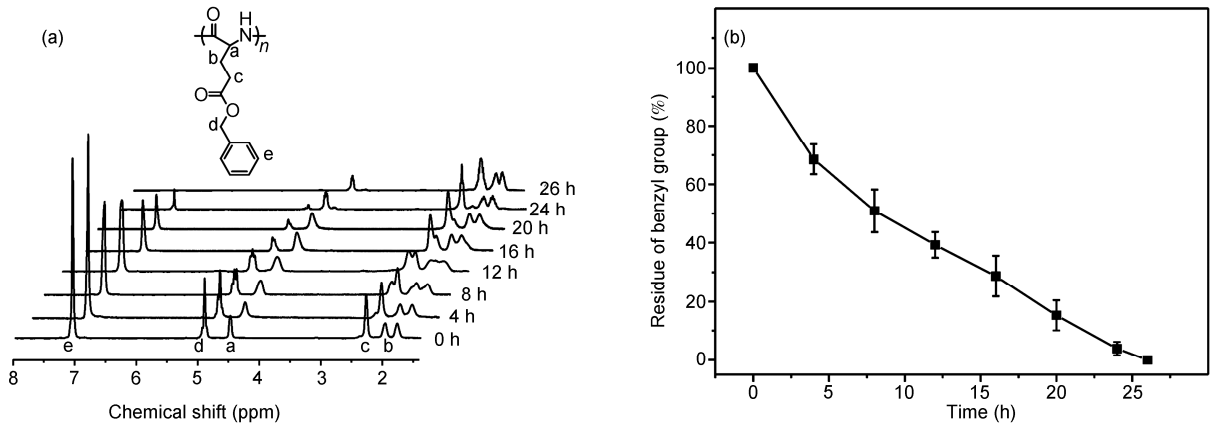

Figure 8 (a) Deprotection kinetics of PBLG by TMSI. The solvent for the products at 0, 4, 8, 12, 16, 20 and 24 h was TFA-*d* and for that at 26 h was D_2O. The resonance of H_2O was automatically eliminated by the software of the Bruker AV 400 M NMR spectrometer; (b) the quantitative statistical results of the deprotection kinetics of PBLG by TMSI, which were determined by the spectrometer. Data are represented as mean ± standard deviation (n = 3).

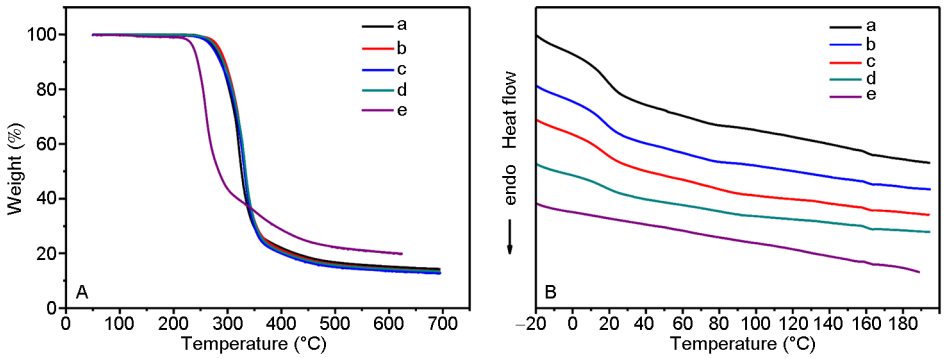

Figure 9 TGA (A) and DSC (B) curves of PBLG (a, b, c and d) initiated by dicyclohexylamine with different M_n and PLGA (e). The DSC curves were the second heating scan in nitrogen atmosphere. The M_n of (a), (b), (c) and (d) were 8.2×10^3, 7.0×10^4, 4.3×10^5 and 7.1×10^5 g mol^{-1}, respectively. (e) PLGA formed by deprotection of PBLG (a) with TMSI as deprotecting agent.

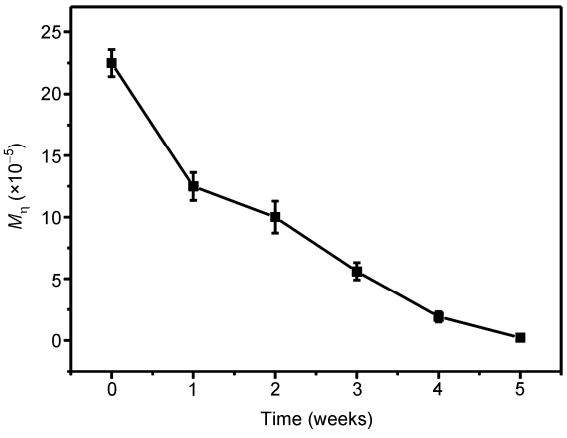

Figure 10 The degradation of PLGA with an initial M_η of PLGA at 2.3×10^5 g mol^{-1} in PBS at pH 7.4, 37 °C at a concentration of 1.0 g L^{-1} (with dicyclohexylamine as initiator and TMSI as deprotecting agent). Data are represented as mean ± standard deviation (n = 3).

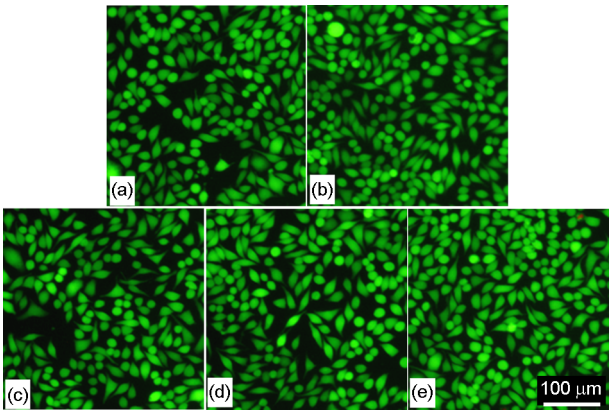

Figure 11 Fluorescence micrographs of L929 cells in monolayer culture exposed to PBLG obtained using different initiators (a: diethylamine, b: di-*n*-hexylamine, c: dicyclohexylamine, d: triethylamine) and the control (e: no addition of poly(amino acid)) for 72 h. Cells were stained by Calcein-AM and PI, and green and red colors indicate viable and dead cells, respectively.

3.7 *In vitro* cytotoxicities of PLGA

The *in vitro* cytotoxicities of PLGA obtained using different deprotecting agents to L929 cells were evaluated by a MTT assay. The cells were treated with PLGA in PBS at pH 7.4 at different concentrations for 72 h. PEI25k was used as positive control. As shown in Figure 12, it was observed that the viabilities of L929 cells were around 90 to 110% at all test concentrations up to 200 mg L^{-1}, indicating the low toxicities of the PLGA obtained using the different deprotecting agents.

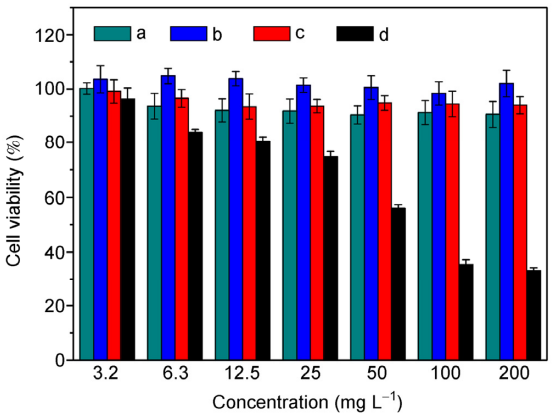

Figure 12 *In vitro* cytotoxicities of PLGA obtained using different deprotecting agents to L929 cells with PEI25k as control. (a), (b) and (c) represent PLGA obtained using HBr/HOAc (33 wt.%), NaOH aqueous solution and TMSI, respectively, and (d) corresponds to PEI25k as a positive control. Data are represented as mean ± standard deviation (n = 3).

4 Conclusion

Diethylamine, di-*n*-hexylamine, dicyclohexylamine and triethylamine have been used as initiators for the polymerization of BLG NCA. The NAM initiation mechanisms was observed for diethylamine and di-*n*-hexylamine, triethylamine followed the AMM mechanism, while initiation by dicyclohexylamine followed both NAM and AMM pathways. The initiation characteristics of dicyclohexylamine were further studied, and the results revealed that low monomer concentration and reaction temperature were favored the formation of products with low polydispersities. HBr/HOAc (33 wt.%), NaOH aqueous solution and TMSI were used for the removal of pendent benzyl groups in PBLG, and comparison of the results showed that TMSI minimized the chain cleavage and controlled the molecular weight of PLGA when compared with the other two. All the samples of PBLG initiated by various agents and PLGA deprotected with different reagents showed good cytocompatibilities, indicating that PBLG and PLGA are promising biomedical materials.

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