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Preparation and Properties of Poly(benzyl glutamate)-Poloxamer-Poly(benzyl glutamate) and Poly(glutamic acid)-Poloxamer-Poly(glutamic acid) Triblock Polymers

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ABSTRACT: The novel block copolymer poly(benzyl glutamate) (PBLG)–polomamer–PBLG were synthesized from glutamic acid and poloxamer in six steps with three different molecular weights, and another new block copolymer, poly(glutamic acid) (PGA)–poloxamer–PGA, was obtained by the benzyl deprotection of PBLG–poloxamer–PBLG. The obtained compounds were characterized by IR spectroscopy, gel permeation chromatography, and ^{1}H-NMR. The *in vitro* biological degradation and water absorption of PBLG showed that a greater proportion of PBLG in the copolymer led to a slower degradation and weaker water absorption, so the speed of degradation and water absorption could be adjusted through adjustment of the ratio of poloxamer. Both PBLG–poloxamer–PBLG and PGA–poloxamer–PGA exhibited lower cytotoxicity and good biocompatibility in the methyl thiazolyl tetrazolium (MTT) assay. The results show that both block polymers are promising as drug-carrier materials. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 129: 1187–1192, 2013

KEYWORDS: adsorption; biodegradable; polyamides; properties and characterization; synthesis and processing

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INTRODUCTION

Poly(amino acid) materials are widely researched as biomedical materials because of their advantageous biodegradation, low cytotoxicity, excellent biocompatibility, and so on.[1,2] These degradable high-polymer materials can be used for controlled drug-delivery systems to carry bioactive agents and drugs for the purposes of controlled release and target delivery. Poly(benzyl glutamate) (PBLG) and poly(glutamic acid) (PGA) are new biodegradable materials that have attracted considerable attention as drug carriers and gene vectors.[3–7] PBLG has been used as carrier of sustained-release preparations, and the carboxyls in the side chains of PGA can be combined with drugs by covalent conjugation to obtain stable compounds.[8] However, the hydrophobicity of PBLG and the strong water absorption caused by too many carboxyls in the side chain of PGA restrict their application as drug carriers. Furthermore both PBLG and PGA have the disadvantage of difficult control in their degradation cycle and speed. The hydrophilicity, hydrophobicity, and crystallinity of a polymer material can be changed by its copolymerization with different monomers. The speed of degradation can be controlled by the molecular weight and the ratio of different comonomers. The properties of PBLG and PGA can be improved by the introduction of another component, such as poly(ethylene glycol),[9] Polyether imide (PEI),[10–13] poly(ethylene oxide),[14,15] polyester,[16] another poly(α-amino acid),[17] or poly[2-(dimethylamino)ethyl methacrylate],[18] to get block, graft, or hyperbranched copolymers.

Poloxamer is a kind of triblock polymer composed of poly(ethylene oxide) and polyoxypropylene, which has been widely used in the pharmaceutical industry as a very good auxiliary material because of its advantages, which include stability, good solubility, noncytotoxicity, and nonantigencity.[19] It is usually used as a solubilizer, emulsifier, drug carrier, stroma, stabilizer, or sorbefacient.[20] In addition, poloxamer 188 is the only synthesized macromolecule approved for intravenous injection. However, there are no active groups, such as —COOH, in the structure of poloxamer, so it cannot be combined with some special drugs by covalent conjugation.

In this paper, the hydrophilic amine-terminated poloxamer188 was used to induce polymerization of hydrophobic BLG and a new type block copolymers called PBLG-Poloxamer-PBLG were prepared, then another new block copolymers PGA-poloxamer-PGA was obtained by the benzyl deprotection of PBLG-Poloxamer-PBLG.

EXPERIMENTAL

Material

We used poloxamer 188 (≥98%, BASF, Germany) and l-glutamate (≥98%, Kelong Chemistry, Chengdu, China). Anhydrous

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tetrahydrofuran (THF) (Chengdu, China) was obtained by refluxing with sodium for 8 h and distilled. Dichloromethane was dried with calcium chloride and then distilled. Pyridine was anhydrated by refluxing with KOH for 24 h and distilled. All of other reagents were analytical grade and were used without further purification.

Preparation of L-Glutamate–γ-Benzyl Ester–*N*-Carboxylic Acid Anhydride (BLG–NCA or Monomer 1)

$PhCH_2OH$ (6.7 mL) and H_2SO_4 (60% in water, 8.1 g) were added dropwise to a flask containing THF (10 mL) and L-glutamate (7.3 g). The mixture was heated to 70°C, stirred until the reaction solution became clear, and reacted for a further 30 min. Then, THF and water were removed. After it was cooled to room temperature, the resulting mixture was poured into a saturated $NaHCO_3$ solution (50 mL). The precipitate (compound **1**) was filtered, washed with ethanol three times, and dried *in vacuo* at 30°C. Then, a white powder obtained (yield = 49.18%).

A solution of bistrichloromethyl carbonate (BTC; 1.4 g) in THF was added dropwise to a solution of compound **1** (2 g) in THF (20 mL) under stirring. The mixture was heated to 50°C, stirred until the reaction solution became clear, and reacted for further 30 min. The white precipitates were separated out when petroleum ether was added to the resulting solution and were then filtered, washed with petroleum ether three times, and dried *in vacuo* below 30°C to obtain monomer **1** with a 63.64% yield.

^{1}H-NMR (400 MHz, CDC_{l3}, δ): 6.08 (—NH, 1H); 2.61 (—CH_2, 2H); 4.36 (—CH, 1H); 1.55 (—CH_2, 2H); 2.12, 2.30 (—CH_2, 2H); 5.15 (—CH_2, 2H); 7.37 (—Ph, 5H). IR characteristic absorption peak (V_{max}) (cm^{-1}, film): 3334.71, 741.75, 1655.65, 2933.96, 2854.59, 1781.63, 1255.00, 1112.

Preparation of the Double-Amino-Terminated Poloxamer (Poloxamer–NH_2 or Monomer 2)

Phthalimide (3 g) was added to a three-necked flask containing absolute ethyl alcohol (120 mL) at 80°C. A solution of KOH (1 g) in methanol (16 mL) was added dropwise to the flask after phthalimide was dissolved. The reaction mixture was heated to 90°C and stirred for 3 h. After cooling, the light green precipitate was filtered and washed with absolute ethyl alcohol to obtain the phthalimide potassium (yield = 60.21%). A solution of paratoluene sulfonyl chloride (2.5 g) in pyridine (8 mL) was added dropwise to the solution of poloxamer 188 (22 g) dissolved in dichloromethane (90 mL) and stirred at 30°C for 12 h. The mixture solution was precipitated in anhydrous ether, and the white solids (compound **2**) were filtered, washed with anhydrous ether, and dried (yield = 71.92%). Compound **2** (15 g) and phthalimide potassium (1 g) were added to a three-necked flask containing *N*,*N*-dimethyl formamide (74 mL), heated to 120°C, and stirred for 4 h. After cooling, anhydrous ether was poured into the resulting mixture. The white solids (compound **3**) were obtained after filtering, washed with anhydrous ether, and dried at an 72.14% yield. Compound **3** (9 g) was added to a three-necked flask containing anhydrous ethanol with stirring. Hydrazine hydrate (3 mL) was added to the solution, heated to 80°C, and stirred for 12 h. The resulting solution was dropped into anhydrous ether after it was concentrated, and the precipitate was filtered, washed with anhydrous ether, and dried to obtain the white precipitates (monomer **2**; yield = 83.91%).

Preparation of the PBLG–Poloxamer–PBLG Triblock Polymers (Product 1)

Monomer **2** and monomer **1** (molar ratio = 1 : 20) were added to a three-necked flask containing moderate dichloromethane. The solution was stirred at 35°C for 72 h and was then poured into anhydrous ether to precipitate. The precipitate was filtered, washed, and dried to obtain the powderlike yellow solid (product **1**). The procedure used for different proportions of monomer **2** to monomer **1,** 1 : 40 and 1 : 80, was the same that used for 1 : 20.

^{1}H-NMR (400 MHz, CDC_{l3}, δ): 1.15 (—CH_3), 3.51 (—CH_2), 3.41 (—CH—O), 3.58 (—CH_2CH_2—O), 3.92 (—CH—N), 8.36 (—NH), 2.24 (—CH_2), 2.49 (—CH_2), 5.12 (—O—CH_2), 7.25 (—Ph). IR V_{max} (cm^{-1}, film): 2872, 1453, 1350, 1112, 1654, 1732, 1545, 3291, 1654, 3060, 747.

Preparation of the PGA–Plolxamer–PGA Triblock Polymers (Product 2)

PBLG–poloxamer–PBLG (1 : 20, 3.6 g), dichloroacetic acid (40 mL), and 33% HBr/CH_3COOH (9 mL) were added to a three-necked flask and stirred at the room temperature for 3 h. The resulting solution was precipitated in acetone, and the solids (product **2**) were filtered and dried (yield = 46.43%). The procedures used for the reaction of PBLG–poloxamer–PBLG (1 : 40) and PBLG–poloxamer–PBLG (1 : 80) were the same as those used for PBLG–poloxamer–PBLG (1 : 20).

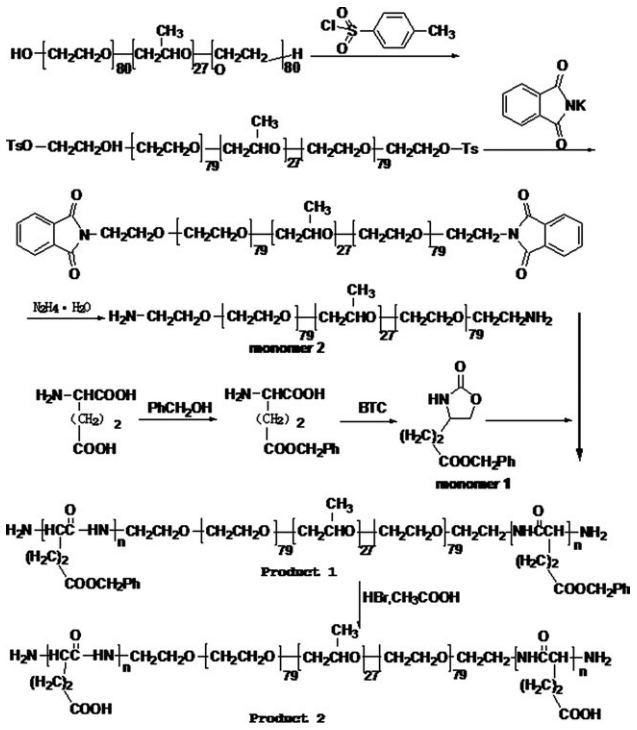
^{1}H-NMR (400 MHz, D_2O, δ): 1.05 (—CH_3), 3.45 (—CH_2), 3.47 (—CH—O), 3.66 (—CH_2CH_2—O), 3.84 (—CH—N), 5.92 (—NH), 2.22 (—CH_2), 2.54 (—CH_2), 8.34 (—COOH).

In Vitro Biological Degradation of the PBLG–Poloxamer–PBLG

Buffer solution was prepared by the dissolution of trometamol (9.68 g), cysteine (2.42 g), and ethylene diamine tetraacetic acid (24.32 g) in distilled water (2000 mL); this resulted in a buffer solution of pH 8.01. PBLG–poloxamer–PBLG (1 : 20) was dissolved in a mixed solvent of THF and dioxane (volume ratio = 3 : 1) to obtain a 50 g/L solution. The solution was poured into watch glasses, and the solvent was evaporated naturally at room temperature to obtain the polymer films, which were separated from the watch glasses by immersion in distilled water and dried at 60°C. The *in vitro* enzymolysis procedure was based a procedure from the literature.[8] Papain (100 mg) was added to a conical flask containing buffer solution (100 mL) to result in an enzyme solution of 1 mg/mL. The polymer films (ca. 100 mg) whose weight was exactly record (W_0) was added to the solution at 37°C and was retrieved from the solution every 4 h to be washed, dried, and weighed (W_t). The viscosity of the sample was tested by an Ubbelohde viscometer after dissolution in DMSO (0.01 g/mL) (Kelong chemistry, Chengdu, China). The weight loss ratio was calculated as follows:

$$\text{Weight loss ratio} = (W_0 - W_t)/W_0 \times 100\%$$

The procedure was repeated six times to get the average results, and the measurements of PBLG–poloxamer–PBLG (1 : 40 and 1 : 80) were the same.



Scheme 1. Schematic diagram of synthetic route.

Water Absorption Ability of the PBLG–Poloxamer–PBLG

The polymer film (ca. 100 mg) whose weight was exactly record (M_0) was added to a conical flask with distilled water in a thermostat at 37°C for a week; we then wiped the surface water off of the polymer film with filter paper, recorded the weight (M). The procedure was repeated three times to get the average results, and the measurements of PBLG–poloxamer–PBLG (1 : 40 and 1 : 80) were the same. The water absorption ratio was calculated as follows:

$$\text{Water absorption ratio} = (M - M_0)/M_0 \times 100\%$$

Methyl Thiazolyl Tetrazolium Cell Toxicity Assay

The pancreatic enzyme digestion logarithm period cells were seeded in a 96-well plate at a density of 1000–10,000 cells per well and were incubated for 24 h at 37°C (CO_2, 5%). The supernatant was removed, and the cells were incubated for 48 h

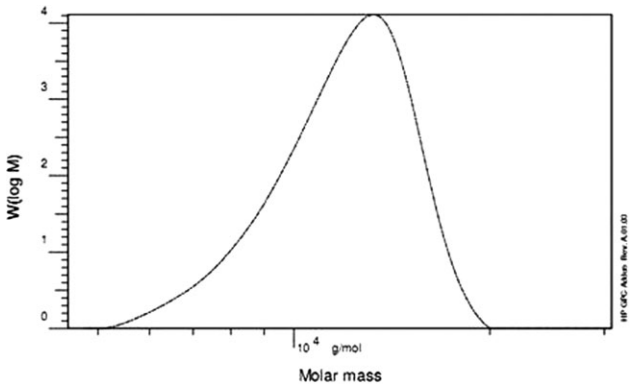


Figure 1. GPC spectra of product 1 (1 : 20); W = log M.

Table I. Analysis of ^{1}H-NMR

Proton belonging	Chemical shift (ppm)		
	Poloxamer	Product **1**	Product **2**
a: CH_3	1.11	1.15	1.05
b: CH_2	3.52	3.51	3.45
c: CH—O	3.50	3.41	3.47
d: CH_2CH_2—O	3.66	3.58	3.66
e: CH—N		3.92	3.84
f: NH		8.36	5.92
g: CH_2		2.24	2.22
h: CH_2		2.49	2.54
i: O—CH_2		5.12	
j: Ph		7.25	

with 200 μL of PBLG–poloxamer–PBLG (1 : 20) solution in DMSO of different concentrations (20, 10, 5, 0.25, 0.625, 0.3125, and 0 μL/mL) at 37°C (CO_2, 5%). MTT solution (5 mg/mL) was added to every plate, and the cells were incubated for another 1–4 h at 37°C. Subsequently, the wells were emptied, 150 μL of DMSO was used to dissolve the formed crystals, and the absorbance was read at 570 nm. The absorbance value was recorded as A_n (n is the concentration of PBLG–poloxamer–PBLG), and the relative cell inhibition rate of PBLG–poloxamer–PBLA was calculated as follows:

$$\text{Relative cell inhibition rate}(\%) = (A_0 - A_n)/A_0 \times 100\%$$

The procedure was repeated three times to get the average results, and the measurements of PBLG–poloxamer–PBLG (1 : 40, 1 : 80) and PGA–poloxamer–PGA were the same.

RESULTS AND DISCUSSION

Synthesis and Characterization

The preparation of products **1** and **2** is illustrated in Scheme 1. First, L-glutamic acid was reacted with benzyl alcohol to afford γ-glutamate-L-benzyl (BLG) for the purpose of protecting the side chain carboxyl of glutamate. Then, monomer **1** was prepared by the reaction of benzyl glutamate and triphosgene in THF. Next, both ends of the tosyl poloxamer were prepared by the reaction of poloxamer and tosyl chloride. Then, double-sided phthalimide poloxamer was obtained by the reaction of both ends of the tosyl poloxamer and potassium phthalimide. Double-amino-terminated poloxamer was prepared by the reaction of double-sided phthalimide poloxamer and hydrazine hydrate. Finally, triblock polymer PBLG–poloxamer–PBLG was prepared by the reaction of double-amino-terminated poloxamer and BLG–NCA, and double-amino-terminated poloxamer was as an initiator. In this procedure, poloxamer–NH_2 was a kind of neutral nucleophilic reagent, and there was a lone pair in the molecule so that it caused charge separation during the period of initiation and increase.

The structures of the synthesized compounds were confirmed by gel permeation chromatography (GPC), IR spectroscopy, and ^{1}H-NMR. The single peak in Figure 1 meant that there was

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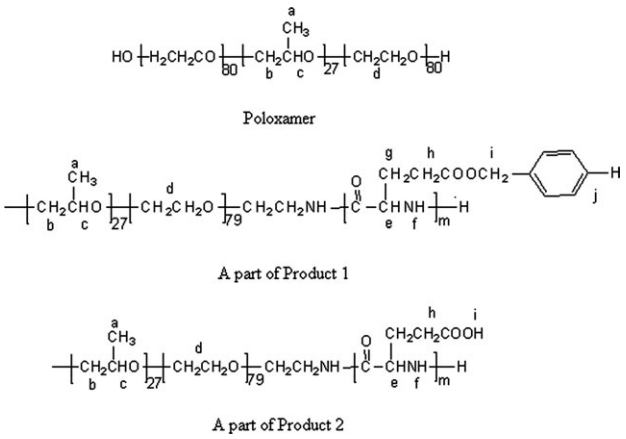

Figure 2. Structure of the poloxamer: parts of products **1** and **2**.

only one substance in the outcome, and the molecular weight was about 12,100, which was greater than that of poloxamer 188, so it could be inferred that there were about seven PBLGs on each side of the poloxamer. The peaks at 1654 and 1545 cm^{-1} on the IR spectra for the product implied the structures of C=O and N—H and C—H, respectively. Compared to the IR spectra for *N*-carboxylic acid anhydride (NCA), the disappearance of the peaks of the carbonyl groups in NCA at 1855 and 1785 cm^{-1} also confirmed that product **1** was produced. Table I summarizes the comparison of the ^{1}H-NMR spectra for poloxamer and the products, and Figure 2 shows the structure of the three compounds, the appearance of the benzene ring protons at 7.25 ppm, and other peaks showed that PBLG–poloxamer–PBLG was successfully prepared.

According to the different ratios between the initiator and monomer, three triblock copolymers were obtained with different molecular weights. With the benzyl removed under the action of 33% HBr/CH_3COOH, PGA–poloxamer–PGA (product

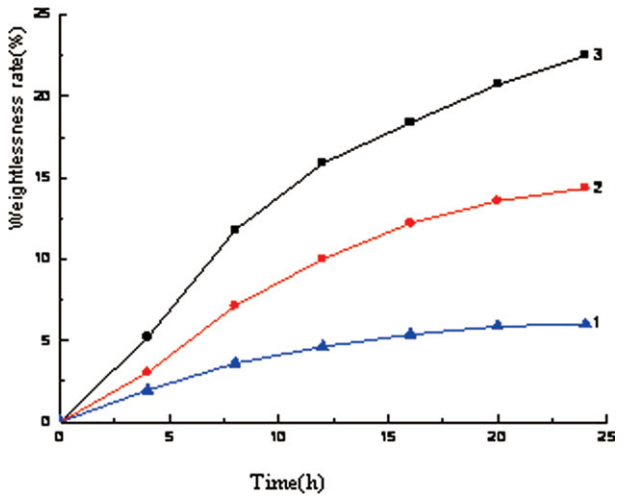

Figure 3. Relationship between the weight loss and time in three kinds of polymer: (1) product **1** (1 : 80), (2) product **1** (1 : 40), and (3) product **1** (1 : 20). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

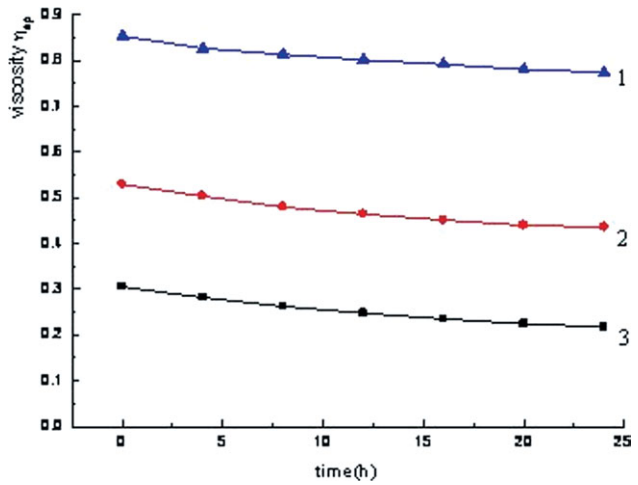

Figure 4. Relationship between the specific viscosity (η_{ap}) and hydrolysis in the enzyme solution: (1) 1 : 80, (2) 1 : 4), and (3) 1 : 20 of product **1**. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

2) was prepared. The ^{1}H-NMR spectra for products **1** and **2** were similar, except for the disappearance of the benzene ring protons at 7.25 ppm, which showed that benzyl was successfully removed.

In Vitro Biological Degradation of Product 1

According to many works in the literature, the degradation of poly(amino acid) *in vivo* is usually carried out by enzymolysis with lipoidase, cathepsin B, collagenase, and so on. Papain, which is a kind of endopeptidase as an analogue of collagenase, was used in this research as a catalyst in the process of degradation. The relationship between the weight loss of three kinds of polymer and the time is shown in Figure 3. The results indicate that the weight loss ratio increased with time. The higher the proportion of PBLG was, the smaller the weight loss ratio was,

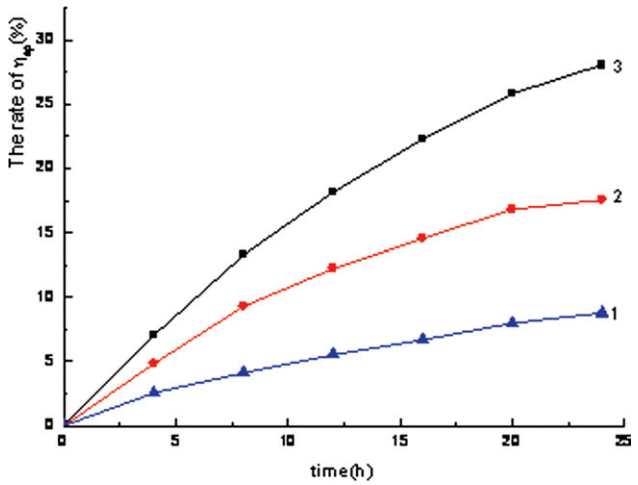

Figure 5. Relationship between the rate of specific viscosity (η_{ap}) and hydrolysis in the enzyme solution: (1) 1 : 80, (2) 1 : 40, and (3) 1 : 20 of product **1**. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Table II. Relative Cell Inhibition Rate of Product **1**

	Name of the polymer					
	PBLG–poloxamer–PBLG (1 : 20)		PBLG–poloxamer–PBLG (1 : 40)		PBLG–poloxamer–PBLG (1 : 80)	
Solution concentration (μg/mL)	Average absorbance value	Cell inhibition rate	Average absorbance value	Cell inhibition rate	Average bsorbance value	Cell inhibition rate
40	0.310	2.52	0.302	5.03	0.302	5.03
20	0.321	−0.94	0.322	−1.26	0.328	−3.14
10	0.330	−3.77	0.324	−1.89	0.357	−12.26
5	0.320	−0.63	0.346	−8.81	0.334	−5.03
2.5	0.334	−5.03	0.352	−10.69	0.346	−8.81
1.25	0.332	−4.40	0.310	2.52	0.333	−4.72
0	0.318	0	0.318	0	0.318	0

which meant that the speed of degradation was slower. The relationship between the specific viscosity and the hydrolysis time in the solution of enzyme is shown in Figure 4. The viscosity is a token of the molecular weight values. Along with the time-prolonged enzymolysis process, the viscosity of the polymer solution decreased constantly; this indicated that the molecular weight decreased constantly. The relationship between the rate of specific viscosity and the hydrolysis time in the enzyme solution is shown in Figure 5. The higher the proportion of PBLG was, the smaller the viscosity change rate was.

Water Absorption Ability of the PBLG–Poloxamer–PBLG

The rates of water absorption of 1 : 20, 1 : 40, and 1 : 80 product **1** after a week were 287.58, 200.43, and 152.49%; this indicated that the water absorption ability of the polymer decreased with increasing proportion of PBLG because of the steric hindrance caused by hydrophobic benzyl, so the higher the proportion of poloxamer was, the stronger the water absorption ability was, and the faster the degradation speed was.

MTT Cell Toxicity Assay of Product 1

Poly(amino acid) is a biodegradable macromolecule with the advantages of low toxicity and good biocompatibility, and it can be easily absorbed and metabolized in the body. As shown in Table II, the polymers, which had no cell toxicity and could evenly promote the growth of cells in low concentrations, were very biocompatible.

MTT Cell Toxicity Assay of Product 2

The results of MTT cell toxicity assay is shown in Table III. When the concentration gradients of the three materials were 1.25–40 μL/mL, the relative cell inhibition rates were all less than 20%, some even below 0. It led to the conclusion that the polymers had no cell toxicity and could evenly promote the growth of cells in low concentrations. They were very biocompatible.

CONCLUSIONS

The novel block copolymer PBLG–polomamer–PBLG was synthesized by the hydrophobic BLG–NCA and hydrophilic double-amino-terminated poloxamer, and another new block copolymer PGA–poloxamer–PGA was obtained by the benzyl deprotection of PBLG–poloxamer–PBLG. According to the different ratios between poloxamer–NH_2 (as the initiator) and BLG–NCA (as the monomer), three triblock copolymers were obtained with different molecular weights. The structures of the

Table III. Relative Cell Inhibition Rate of Product **2**

	Name of the polymer					
	PGA–poloxamer–PGA (1 : 20)		PGA–poloxamer–PGA (1 : 40)		PGA–poloxamer–PGA (1 : 80)	
Solution concentration (μg/mL)	Average absorbance value	Cell inhibition rate	Average absorbance value	Cell inhibition rate	Average absorbance value	Cell inhibition rate
40	0.330	13.16	0.330	13.16	0.349	8.16
20	0.340	10.53	0.320	0.360	0.328	13.68
10	0.400	−2.5	0.360	5.26	0.356	6.32
5	0.390	−2.63	0.350	7.89	0.341	10.26
2.5	0.400	−2.5	0.340	10.53	0.336	11.58
1.25	0.380	−15.79	0.400	−5.26	0.383	−0.79
0	0.380	0	0.380	0	0.384	0

obtained compounds were confirmed by IR spectroscopy, GPC, and ^{1}H–NMR. The results show that PBLG–poloxamer–PBLG was successfully prepared. The *in vitro* biological degradation, water absorption, and cytotoxicity of PBLG–poloxamer–PBLG were investigated, and also, the impact of different PBLG and PGA proportions on the copolymer's properties were studied. The results showed that the greater proportion of PBLG in the copolymer led to a slower degradation and weaker water absorption, so the speed of degradation and water absorption could be adjusted by the ratio of PBLG. PGA–poloxamer–PGA was prepared by the removal of the benzyl with 33% HBr/CH_3COOH. The results of ^{1}H-NMR showed that the benzyl was successfully removed. When the benzyl side chains were put off PBLG, many carboxyl side chains appeared so that other drugs and monoclonal antibodies could be bonded with PGA. The cytotoxicity of both PBLG–poloxamer–PBLG and PGA–poloxamer–PGA was investigated by MTT assay. The results show that both exhibited a lower toxicity and both had good biocompatibility. The results show that the two block polymers are promising as drug-carrier materials.

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