J Mater Sci: Mater Med (2008) 19:1275–1281
DOI 10.1007/s10856-007-3237-9

Synthesis and characterization of arginine–glycine–aspartic peptides conjugated poly(lactic acid-co-L-lysine) diblock copolymer

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Received: 25 April 2007 / Accepted: 5 July 2007 / Published online: 20 August 2007

Abstract A biodegradable Copolymer of poly(lactic acid-co-lysine)(PLA–PLL) was synthesized by a modified method and novel Arginine–Glycine–Aspartic (RGD) peptides were chemical conjugated to the primary ε-amine groups of lysine components in four steps: I to prepare the monomer of 3-(N^{ε}-benzoxycarbonyl-L-lysine)-6-L-methyl-2,5-morpholinedione; II to prepare diblock copolymer poly(lactic acid-co-(Z)-L-lysine) (PLA–PLL(Z)) by ring-opening polymerization of monomer and L,L-lactide with stannous octoate as initiator; III to prepare diblock copolymer PLA–PLL by deprotected the copolymer PLA–PLL(Z) in HBr/HoAc solution; IV the reaction between RGD and the primary ε-amine groups of the PLA–PLL. The structure of PLA–PLL–RGD and its precursors were conformed by FTIR-Raman and ^{1}H NMR. Low weight average molecular weight (9,200 g/mol) of the PLA–PLL was obtained and its PDI is 1.33 determined by GPC. The PLA–PLL contained 2.1 mol% lysine groups as determined by ^{1}H NMR using the lysine protecting group's phenyl protons. Therefore, the novel RGD-grafted diblock copolymer is expected to find application in drug carriers for tumor therapy or non-viral DNA carriers for gene therapy.

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1 Introduction

Angiogenesis is a requirement for tumor growth and metastasis as it provides nutrients and oxygen for growing tumor cell [1–4]. The dormant tumors begin to grow rapidly and usually have large gaps in their endothelium when vascularized. Therefore, the extravasation of nanoparticles allowed to the extravascular space surrounding the tumor cells [5]. In generally, it is believed that the permeated nanocarriers of <200 nm with prolonged blood circulation properties are accumulated in solid tumors due to the enhanced permeation and retention (EPR) effect [6].

Polymeric micelles are core/shell structures formed through self-assembly of amphiphilic block copolymers [7]. Many nanoparticles size of polymeric micelles (such as pluronic105, chitosan) [8, 9] are prepared in cancer therapy as they make the carriers unrecognized by the phagocytic cells of the reticuloendothelial system (RES) and prolong their blood circulation. The small size of polymeric micelles are also expected to facilitate carriers's extravasation from tumor vasculature and to ease the penetration of the extravasated carriers within a solid tumor tissue [7].

During the past decades, Poly(lactic acid) (PLA) has been approved by the FDA in clinic and investigated for many applications, such as tissue engineering and drug delivery [10, 11]. The cationic polymer, poly(L-lysine) (PLL) has been widely applied in gene delivery vector [12, 13]. Based on the electrostatic binding with the tumor cell, PLL-based cationic and biodegradable polymeric micelles are expected to permeate tumor cell more feasible [14]. To reduce its cytotoxicity, PLL has been modified by conjugating with polyethylene glycol (PEG), histidine, and targeting ligands including polysaccharides, transferrin due to the conjugated polymers disperse the cytotoxicity of

PLL [15–18]. We propose PLA–PLL cationic copolymer would show such reduced PLL cytotoxic effect.

In the 1980s, Pierschbacher and Ruoslahti found that the tripeptide arginine–glycine–aspatic (Arg–Gly–Asp or RGD) were the minimal cell-recognizable sequence in many extracellular matrix proteins and blood proteins [19]. Integrin $\alpha_v\beta_3$ is a receptor for the extracellular matrix proteins with exposed RGD tripeptide sequence and normally expressed at low levels on epithelial cells and mature endothelial cells but highly expressed on the activated endothelial cells in the neovasculature of tumors, including osteosarcomas, glioblastomas, melanomas, lung carcinomas, and breast cancer [20–25]. Therefore, The overexpression of integrin $\alpha_v\beta_3$ during tumor growth, invasion, and metastasis presents an interesting drug-targeted method [26].

The objective of this study is to synthesize RGD conjugated diblock copolymer PLA–PLL (PLA–PLL–RGD) by a modified method of Barrera's [27, 28] and characterize it by FTIR-Raman, ^{1}H NMR and GPC. The potential of RGD–PLA–PLL for tumor targeted drug release carrier will be explored in our group.

2 Experimental

2.1 Materials and method

N^{ε}-(Carbonylbenzoxy)-L-lysine, D-alanine and RGD peptides were purchased from GL biochem (China), L,L-lactide was purchased from GLACO (China) and recrystallized from dry toluene and dry ethyl acetate and stored at −20 °C before use. N,N-Diisopropylethylamine was purchased from J & K chemica. N-hydroxylsuccinimide (NHS), dicyclohexylcarbodiimide (DCC) and Dimethylamino pyridine (DMAP) were purchased from Acros and used without further purification. Stannous octoate was purchased from Zhixing chemical Ltd. (China) and distilled under reduced pressure, and dry toluene was distilled in to and out of it three times under vacuum to remove any trace of water. Chloroform was distilled from anhydrous $MgSO_4$ and stored in 4A sieve, $SOCl_2$ was purified by distillation, triethylamine was distilled from sodium and stored in KOH.

^{1}H NMR spectras were recorded on a Bruker Avance 400 NMR spectrometer. FTIR-Raman spectras were measured using a Nicolet NEXUS-670 Fourier transform infrared spectrometer. GPC measurements were carried out on a Polymer PL-GPC 50 gel permeation chromatograph system equipped with an RI detector, THF was used as an eluent at a flow rate of 1.0 mL/min. Narrowly dispersed polystyrene standards from Polymer PL-GPC 50 were employed for preparing a calibration curve.

2.2 Preparation of monomer

The method of the synthesis of monomer was modified with Barrera's [27, 28]. A solution of D-alanine (10 g, 0.11 mol) and 116 mL of >40% aqueous HBr in 160 mL distilled water, and cracked ice was added into 680 mL. $NaNO_2$ (20.8 g, 0.30 mol) and Na_2SO_4 (140 g, 0.11 mol) were added into the mix solution in turns with intensive stirring. When the temperature of stirred mix solution warmed up to the constant point and keeping stirring for 30 min, filtered off the solid three times and was extracted four times from 100 mL anhydrous Et_2O. Dried the Et_2O over Na_2SO_4 and $CaCl_2$ overnight, and evaporated the Et_2O solution. The forerun of the residue was distilled at 20–60 °C under vacuum using rotoevaporation to give a colorless crude lipid (3.52 g). Next, 2.3 mL (3.76 g, 0.032 mol) of $SOCl_2$ was added into the crude lipid, the reaction was heated to 60 °C for 8 h under nitrogen environment. Distilled the reaction solution under vacuum to give colorless lipid, D-α-Bromopropionyl Chloride (2.32 g, the total yield of two reactions: 13%, mp: 38–39 °C, 0.095 kpa). ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.92–1.93 (J = 6.9 Hz, –CH_3, 3H), 4.64–4.65 (J = 6.9 Hz, –CH, 1H) and IR (neat, cm^{-1}) 1,775 (CO).

N^{ε}-(Carbonylbenzoxy)-L-lysine (8.23 g, 0.029 mol) was added in 200 mL anhydrous chloroform and a mix solution of D-α-Bromopropionyl Chloride (2.32 g, 0.014 mol) in 50 mL anhydrous chloroform was slowly dropwise added over a period of 30 min with stirring. The reaction was bubbled with nitrogen over 1 h and sealed to stir for 24 h under room temperature. The reaction was followed by TLC, when the reaction was completed, filtered off the solids of the reaction mix and removing the solvent of chloroform with rotoevaporation, then purified the crude production by silica gel chromatography using the eluents chloroform/methanol 98/2(v) to give the colorless lipid, (R)-N^2-(2-Bromo-l-oxopropyl)- N^{ε}-(Carbonylbenzoxy)-L-lysine (4.92 g, yield: 85%). ^{1}H NMR (DMSO, 400 MHz) δ = 1.27–1.29(–CH_2, 2H), 1.37–1.39 (–CH_2, 2H), 1.64–1.76 (–CH_2, –CH_3, 5H), 2.96–2.97 (–CH_2, 2H), 4.16 ($BrCHCH_3$, 1H), 4.58–4.60 ($HNCHCH_2$, 1H), 5.0 (CH_2Ph, 2H), 7.21 (Carbamate NH, 1H), 7.30–7.38 (aromatic protons, 5H), 8.45–8.47 (amide NH, 1H), 12.64 (–COOH, 1H).

The previous lipid was redissolved in 20 mL anhydrous chloroform and a mix solution of N, N-Diisopropylethylamine (2.02 mL, 0.012 mol) in 10 mL anhydrous chloroform was dropwise added slowly with stirring over 20 min. The reaction solution was heat to reflux for 8 h under nitrogen protected and followed by TLC. End the reaction, evaporated off chloroform to give yellow waxy colloid and purified the crude production by silica gel chromatography using the eluents chloroform, chloroform/

methanol 95/5(v) to give white powder, 3-[N^{ε}-(Carbonyl-benzoxy)-L-lysine]-6-L-methyl-2,5-morpholinedione. The sample was recrystallized from EtOAc and stored in vacuum drying container (2.96 g, yield: 74%). ^{1}H NMR (DMSO, 400 MHz) δ = 1.38–1.40 (–CH_3, 3H), 1.87 (–CH_2, 6H), 3.14 (–CH_2 next to carbamate, 2H), 4.31–4.34 (HNCHCH_2, 1H), 5.0 (CH_2Ph, 2H), 5.04–5.07 (OCHCH_3, 1H), 7.25 (Carbamate NH, 1H), 7.30–7.36 (aromatic protons, 5H), 8.31, 8.46 (amide NH, 1H) and IR (KBr, cm^{-1}) 3,330, 3,220 (amide and carbamate NH), 3,140–3,035 (aromatic), 1,755 (CO carboxylic acid), 1,695 (CO carbamate and amide I), 1,535 (amide II type from carbamate).

2.3 Preparation of PLA–PLL

With free ε-amine groups PLA–PLL was synthesized through the ring-opening polymerization of 3-[N^{ε}-(Carbonylbenzoxy)-L-lysine]-6-L-methyl-2,5-morpholinedione and L,L-lactide catalyzed by Stannous octoate, and deprotected in HBr/HoAc. The polymerization tube was heated to 140 °C for 6 h and cooled under nitrogen, then washed with anhydrous acetone three times. Recrystallized the monomer from EtOAc before use. The desired amounts of monomer, L,L-lactide, Stannous octoate in 0.1 mL anhydrous chloroform were added into the tube. After the chloroform was eliminated by high vacuum for 1 h and the oxygen was eliminated by displace of nitrogen six times, the tube was sealed under high vacuum, then the tube was shaken at 100 °C for 24 h. The residues were cooled to stop the reaction in freezer and precipitated by pouring into excessive methanol before redissolved in chloroform. The precipitate was collected and dried in vacuum.

The poly(lactic acid-co-(Z)-L-lysine) was deprotected by stirring the slurry of the copolymer in HBr/HoAc solution under nitrogen for 30–60 min. the copolymer was then washed with Et_2O and MeOH and collected via vacuum filtration. It was then redissolved in anhydrous chloroform, neutralized with excessive N, N-diisopropylethylamine, precipitated from methanol, and dried to obtain PLA–PLL with free ε-amine groups.

2.4 Preparation of PLA–PLL–RGD

The tripeptide RGD were grafted onto the PLA–PLL by first activating the side chain carboxyl groups of the PLA–PLL with NHS and then coupling with the RGD. For the carboxyl group activation, 0.9 g of PLA–PLL (0.1 mmol) and 0.35 g of NHS (3 mmol) were dissolved in a mix solution of 20 mL anhydrous CH_2Cl_2 and 20 mL anhydrous DMF in a dried flask and treated with 0.63 g DCC (3 mmol) and 0.003 g DMAP (0.025 mmol) for 24 h at room temperature, then dicyclohexylurea formed was removed by filtration. The copolymer solution was precipitated into an excessive methanol. The production (PLA–PLL–NHS) was obtained via vacuum filtration and purified by washing with anhydrous methanol, then dried under vacuum for 24 h and stored at −20 °C.

For the coupling of RGD, 0.9 g (0.1 mmol) of PLA–PLL–NHS and 20 mg of RGD (0.06 mmol) were dissolved in a mix solution of 5 mL anhydrous CH_2Cl_2 and 5 mL anhydrous DMF. After adding 0.02 mL of triethylamine (0.15 mmol), the reaction mixture was stirred at room temperature for 4 days. The product mixture was precipitated in excessive methanol and then washed with anhydrous methanol and dried in vacuum.

3 Results and discussion

In the present study, Cyclic dimer lactone of L-lactic acid and protected L-lysine was synthesized in according with the outline of synthesis route which illustrates in Scheme 1. D-alanine was first bromination with HBr and then reacted with $SOCl_2$ to give D-α-Bromopropionyl Chloride, N^{ε}-(Carbonylbenzoxy)-L-lysine was subsequently added and esterification was occurred, the last final ring-closing step was carried out with N, N-Diisopropylethylamine added. The obtained monomer was confirmed by IR and ^{1}H NMR in Fig. 1. The esterification was confirmed by the signal of the peak at 7.30–7.36 ppm which indicated the existence of the aromatic group of the N^{ε}-(Carbonylbenzoxy)-L-lysine, and the proton peak at 12.64 ppm (–COOH) of (R)-N^2-(2-Bromo-l-oxopropyl)-N^{ε}-(Carbonylbenzoxy)-L-lysine disappeared in the final step powerfully verificated the ring-closing reaction. From the reaction results, the whole yield of monomer mainly determined in the first two reactions as the trace quantity water existed in reagents.

Sn $(OCt)_2$-catalyzed ring-opening polymerization of L-lactide has been intensively studied for some decades. Polymerization was initiated through a nucleophilic attack at the carbon and catalyzed transesterification reaction between the activated lactone and hydroxyl groups through Lewis acid, such as $Sn(OCt)_2$ [29]. In the present study, the diblock copolymer PLA–PLL(Z) was synthesized in according with the ring-opening mechanism, the outline route of copolymerization between the monomer and L,L-lactide is shown in Scheme 2. The first copolymerization mechanism is similar with $Sn(OCt)_2$-catalyzed ring-opening polymerization of L-lactide and glycolide was carried out at 100 °C for 24 h, the obtained (R)-N^2-(2-Bromo-l-oxopropyl)-N^{ε}-(Carbonylbenzoxy)-L-lysine was confirmed by IR and H^1 NMR in Fig. 2. The peak at 7.36 ppm (g) which presents the aromatic group of the N^{ε}-

Scheme 1 Synthesis of monomer, 3-[N$^{\varepsilon}$-(Carbonylbenzoxy)-L-lysine]-6-L-methyl-2,5-morpholinedione

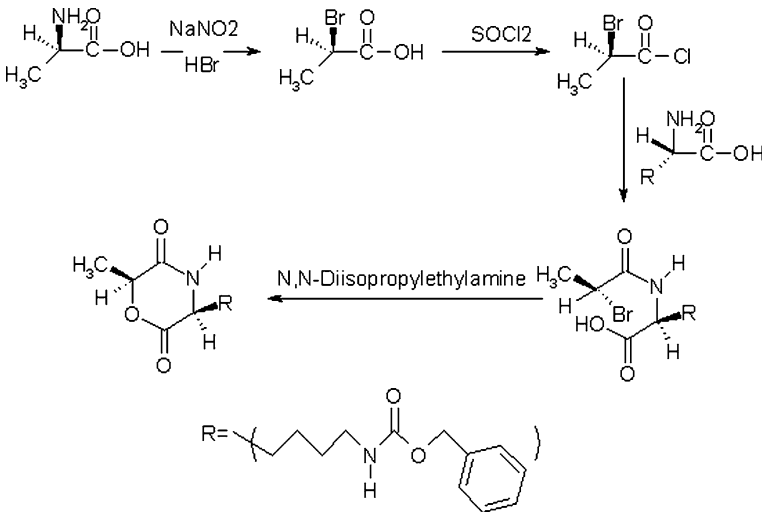
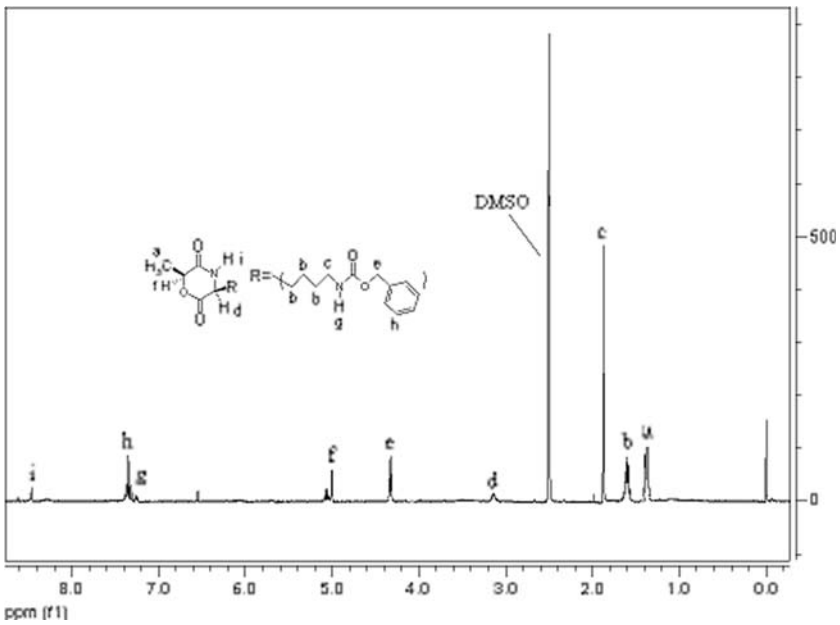
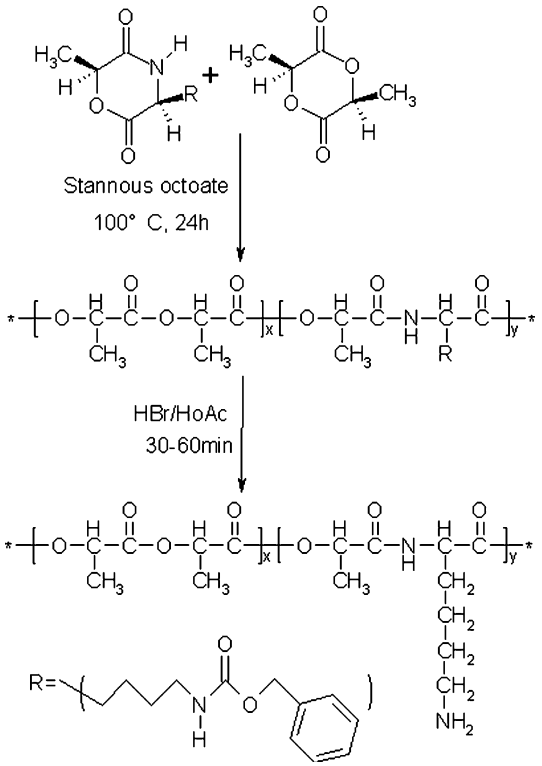


Fig. 1 H^{1} NMR spectrum of 3-[N$^{\varepsilon}$-(Carbonylbenzoxy)-L-lysine]-6-L-methyl-2,5-morpholinedione in DMSO



(Carbonylbenzoxy)-L-lysine indicates the cyclic dimer lactone successfully polymerized with L,L-lactide. 2.1 mol% lysine residues of PLA–PLL(Z) is calculated by ^{1}H NMR using the lysine protecting group's phenyl protons (7.36 ppm, g) and lactide's methyl protons (1.52 ppm, a), and the Mn value of PLA–PLL(Z) is 6,960 also obtained from ^{1}H NMR. In Fig. 3, the absorption for the CO ester at 1,755 cm^{-1}, symmetrical CH_3 at 1,455 cm^{-1} and CH at 2,950, 2,933 cm^{-1} were found in all spectra of these copolymer samples, amide I bands at 1,685 cm^{-1} and amide II bands at 1,530 cm^{-1} also observed. The copolymerization further confirmed by IR from the absorption at 3,035 cm^{-1}, 3,089 cm^{-1} and 3,140 cm^{-1} which represents the aromatic group of N$^{\varepsilon}$-(Carbonylbenzoxy)-L-lysine. After deprotected the PLA–PLL(Z) in mix acid solution (HBr/HoAc), the PLA–PLL was obtained. Figure 4 illustrates the GPC chart of the PLA–PLL, the Mn value of PLA–PLL from GPC is 6,560 that well consistents with the Mn value of PLA–PLL(Z) from ^{1}H NMR, the Mw value of PLA–PLL is 9,200 g/mol, PDI is 1.33 obtained from GPC. Therefore, the molecular weight data of the PLA–PLL samples did not change much after deprotect the terminal Carbonylbenzoxy group. However, compared with the general ring-opening process [27, 28], the low weight value of PLA–PLL in this present possible due to the trace water in the copolymerization which may initiate the hydrolysis of PLA–PLL(Z) in copolymerization process [28]. A respective IR spectrum of the PLA–PLL is illustrated in Fig. 3b, the deprotected occurred that verified by the absorption peak of 3,035 cm^{-1}, 3,089 cm^{-1} and 3,140 cm^{-1} (aromatic group of N$^{\varepsilon}$-(Carbonylbenzoxy)-L-lysine) disappeared in PLA–PLL.



Scheme 2 Synthesis of PLA–PLL

The tripeptide RGD were grafted onto the PLA–PLL by first activating the side chain carboxyl groups of the PLA–PLL with NHS and then coupling with the RGD. The outline route is illustrated in Scheme 3. The RGD groups successfully grafted to the diblock copolymer PLA–PLL confirmed by IR in Fig. 3c as strengthen of absorption peak

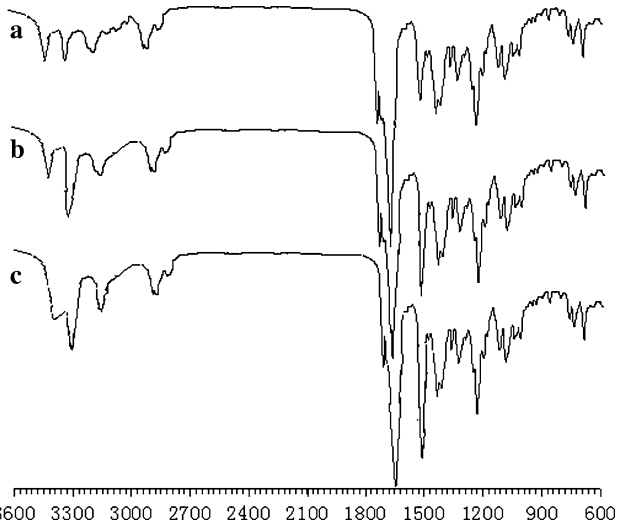


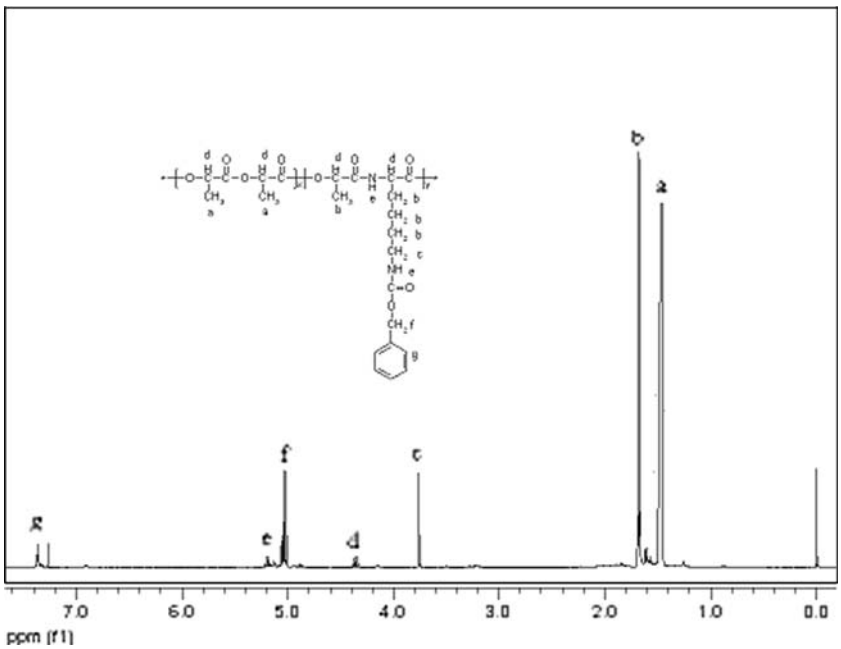
Fig. 3 IR spectra of PLA–PLL(Z) (trace a), PLA–PLL (trace b) and PLA–PLL–RGD (trace c)

of 3,400 cm^{-1} (NH), 1,685 cm^{-1} and 1,530 cm^{-1} (amide I and amide II). The potential of using this PLA–PLL–RGD for tumor targeted drug release carriers will be explored in our group.

4 Conclusions

A new biodegradable biomaterial of PLA–PLL–RGD was successfully synthesized in four-step process. I. The cyclic dimer lactone of L-lactic acid and protected L-lysine was synthesized; II. The protected PLA–PLL was synthesized through ring-opening polymerization; III. The PLA–

Fig. 2 H^1 NMR spectrum of poly(lactic acid-co-(Z)-L-lysine) in $CDCl_3$



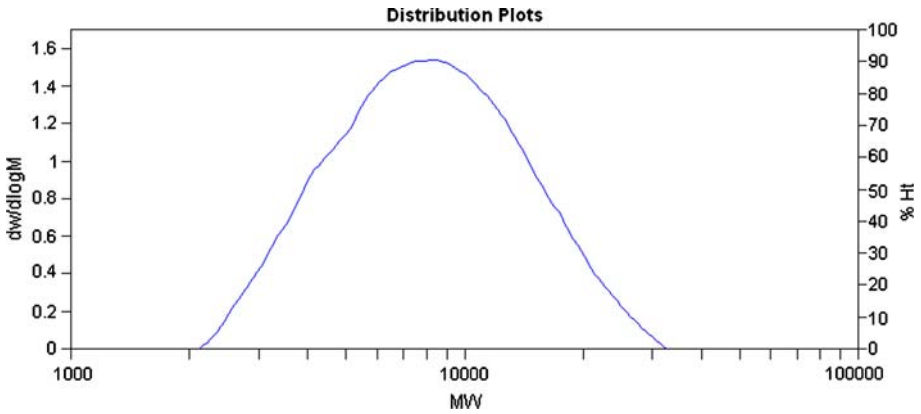
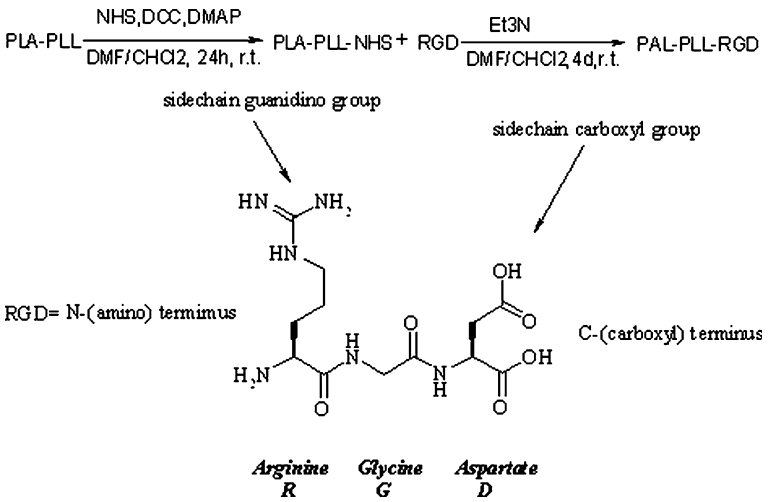


Fig. 4 GPC spectrum of the PLA–PLL



Scheme 3 Synthesis of PLA–PLL–RGD

PLL(Z) was deprotected in the HBr/HoAc mix solution; IV. The tripeptide RGD were grafted onto the PLA–PLL by first activating with NHS and then coupling with the RGD. The structure of the block copolymer and its precursors were confirmed by ^{1}H NMR, IR and GPC. The percent of lysine residues groups of the PLA–PLL is 2.1 mol% determined by ^{1}H NMR using the lysine protecting group's phenyl protons and the Mw value of the PLA–PLL is 9,200 g/mol by GPC. The novel copolymer combines cationic poly (L-lysine), hydrophobic poly (L-lactide) and functional ligands RGD is expected as the carrier for tumor targeted drug release system and its potential application under researching in our group.

Acknowledgements This research was performed with support from Natural Science Foundation of China via Grant 30572271 and 50673058, Key Foundation Project of Shanghai via Grant 05JC14072. Science and Technology Develop Foundation of Shanghai via Grant 054119539. The author is grateful to Lan Li and Dr Aojia Zhou for discussion to this research.

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