Synthesis and Characterization of RGD Peptide Grafted Poly(ethylene glycol)-*b*-Poly(L-lactide)-*b*-Poly(L-glutamic acid) Triblock Copolymer

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Advances in tissue engineering require biofunctional scaffolds that can provide not only physical support for cells but also chemical and biological cues needed in forming functional tissues. To achieve this goal, a novel RGD peptide grafted poly(ethylene glycol)-*b*-poly(L-lactide)-*b*-poly(L-glutamic acid) (PEG−PLA−PGL/RGD) was synthesized in four steps (1) to prepare diblock copolymer PEG−PLA-OH and to convert its −OH end group into $-NH_2$ (to obtain PEG−PLA-NH_2), (2) to prepare triblock copolymer PEG−PLA−PBGL by ring-opening polymerization of NCA (*N*-carboxyanhydride) derived from benzyl glutamate with diblock copolymer PEG−PLA-NH_2 as macroinitiator, (3) to remove the protective benzyl groups by catalytic hydrogenation of PEG−PLA−PBGL to obtain PEG−PLA−PGL, and (4) to react RGD (arginine-glycine-(aspartic amide)) with the carboxyl groups of the PEG−PLA−PGL. The structures of PEG−PLA−PGL/RGD and its precursors were confirmed by ^{1}H NMR, FT-IR, amino acid analysis, and XPS analysis. Addition of 5 wt % PEG−PLA−PGL/RGD into a PLGA matrix significantly improved the surface wettability of the blend films and the adhesion and proliferation behavior of human chondrocytes and 3T3 cells on the blend films. Therefore, the novel RGD-grafted triblock copolymer is expected to find application in cell or tissue engineering.

Introduction

Hydrophobic aliphatic polyesters based on hydroxyalkanoic acids, such as polyglycolide (PGA), polylactide (PLA), poly(ϵ-caprolactone) (PCL), and their copolymers, are well-known as very important synthetic biodegradable materials. Due to their low immunogenicity, good biocompatibility, and excellent mechanical properties, they may be widely used in pharmaceutical and other medical applications,[1,2] such as sutures, implants for bone fixation, carriers in drug delivery, and temporary matrixes or scaffolds in tissue engineering.[3−5] An important problem is inadequate interaction between the polymers and cells, leading to in vivo foreign body reactions, such as inflammation, infections, local tissue necrosis, and implant encapsulation, as well as thrombosis.[6,7] Furthermore, due to the lack of functional groups, they cannot be modified easily with biologically active moieties.

Many methods have been developed to improve hydrophobic aliphatic polyesters, including polymer design and modification, promoting endothelialization, and coupling with bioactive reagents to obtain enhanced interaction with cells.[8] Chemical modifications of aliphatic polyesters were realized by preparing co-polyesters, such as PEG−polyester, polyester−poly(amino acid).[9−11] Surface-grafting biological moieties was often used to improve the polymer surface, but bulk modification was seldom described.[12,13]

In the 1980s, Pierschbacher and Ruoslahti found that the tripeptide arginine-glycine-aspatic acid (Arg-Gly-Asp or RGD) was the minimal cell-recognizable sequence in many extracellular matrix protein and blood protein.[14] Since then, RGD-containing peptides have been incorporated into various polymers such as acrylic acid-containing copolymer, poly(ethylene glycol)−poly(propylene fumarate) and poly(vinyl alcohol).[15−17] These polymers showed improved cell adhesion. Although biodegradable polyester−RGD conjugates are expected to be the best scaffold materials for guiding tissue regeneration (GTR) in vivo and ex vivo,[18,19] because of their complete absorbability, controlled degradability, low immunogenicity, and excellent mechanical properties, very few polyester−peptide conjugates have been synthesized and studied,[12] because these biodegradable polyesters (e.g. PLA) lack functional groups that can react with peptides.

In this paper, therefore, a novel RGD-grafted triblock copolymer PEG−PLA−PGL/RGD was prepared. Incorporation of PEG into PLA could minimize protein adsorption[20,21] and decrease nonspecific cell adhesion.[22,23] The mechanical properties and degradation properties of PEG−PLA−PGL could be easily controlled by varying the relative lengths of the three blocks. The number of grafted RGD peptides could be adjusted either by changing the length of the PGL block or by changing the grafting rate of RGD. Therefore, this polymer is expected to have enhanced cell adhesion and can serve as a biodegradable scaffold for cell and tissue engineering.

Experimental Section

Materials. Monomethoxy-poly(ethylene glycol) with a molecular weight of 750 (PEG750) was obtained from Aldrich. Prior to use, it was dried by an azeotropic distillation in toluene. L-Lactide (LA) was purchased from Purac and recrystallized in ethyl acetate three times. *N-tert*-Butoxycarbonyl-L-phenylalanine (Phe-BOC), N-hydroxylsuccinimide (NHS), and dicyclohexylcarbodiimide (DCC) from GL Biochem (Shanghai, China) Ltd. were used as received. (Dimethyl-

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amino)pyridine (DMAP, 99%) obtained from Acros, Pd/C (10%) supplied by Suzhou Xukou Corp., and RGD purchased from CL Bioscientific Inc. (Xi'an, China) were used without further purification. The *N*-carboxy anhydride of γ-benzyl-L-glutamate (BGL-NCA) was prepared according to Daly's method.[24] Hexane, methylene chloride, and chloroform were refluxed over CaH_2 and distilled under nitrogen. Tetrahydrofuran (THF) was dried and distilled in the presence of sodium immediately before use. DMF was dried over CaH_2 and distilled before use.

Measurements. FT-IR spectra were recorded on a Bio-Rad Win-IR instrument. ^{1}H NMR spectra were measured by an AV-400 NMR spectrometer at room temperature. Gel permeation chromatography (GPC) measurements were conducted on a Waters 410 GPC with THF as eluent (flow rate 1 mL/min, at 35 °C). The molecular weights were calibrated against polystyrene (PS) standards. The water contact angle was measured by drop shape analysis (DSA 10, KRüSS GmbH) to investigate the wettability of the film samples. Atomic force microscopy (AFM) experiments were performed by using SPI 3800/SPA300HV (Seiko Instrument Inc.) at room temperature in air, and the AFM images were obtained in tapping mode. Commercially available SiN_4-cantilevers were used with a spring constant of 42 N/m and resonant frequency of 300 kHz, and the tip was of OMCL-ACTS-W type. The surface morphology was also examined by a field emission scanning electron microscopy (XL30 ESEM FEG, Philips), which allows low-voltage operation and high magnification.

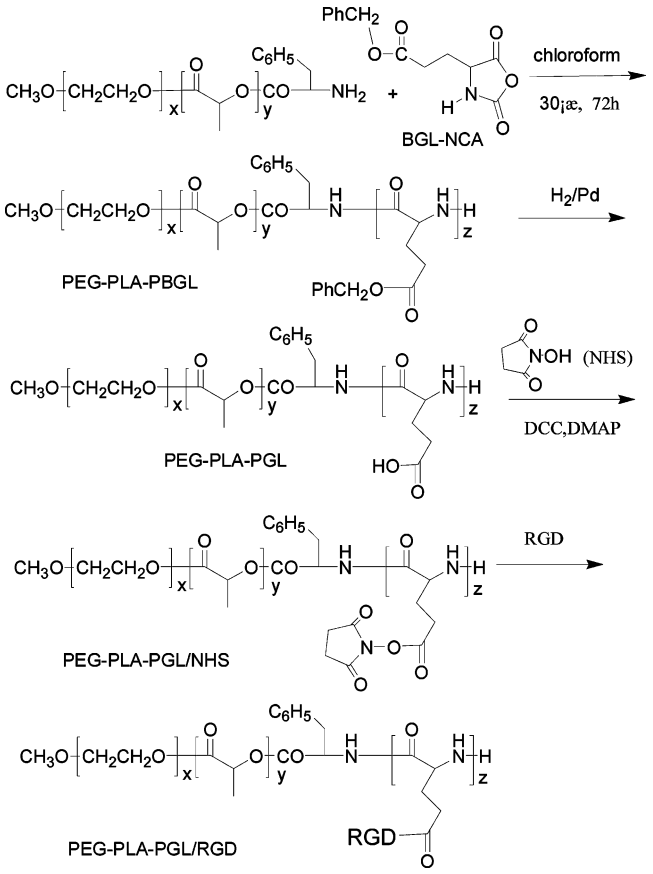
Amino Acid Analysis. The sample was first hydrolyzed in 6 N HCl at 110 °C for 24 h. Then it was reacted with FDNB (1-fluoro-2,4-dinitrobenzene) and was analyzed by a high-pressure liquid chromatograph (HPLC) equipped with a C_{18} column and detected at 360 nm at room temperature to determine the amounts of glycine and aspartic acid. On the basis of these two contents, the amount of RGD and the coupling efficiency of RGD were calculated.

Surface elemental compositions of the peptide-containing samples were analyzed on an Escalab MKII photoelectron spectrometer (VG Scientific). The XPS experiments were performed in the spectroscopy chamber using a standard Mg anode X-ray source (Mg Kα X-rays at 1253.6 eV) and a 150 mm hemispherical electron energy analyzer. The spectra were obtained for each sample using a 90° takeoff angle.

Syntheses of Triblock Copolymers PEG−PLA−PBGL, PEG−PLA−PGL, and PEG−PLA−PGL/RGD. The synthetic approach to these three polymers is shown in Scheme 1. The PEG−PLA−PBGL was synthesized in three steps: (1) block copolymer PEG−PLA-OH was prepared by ring-opening polymerization (ROP) of L-lactide in the presence of methoxy-poly(ethylene glycol) (M_n = 750) with stannous octanoate as catalyst; (2) its end group −OH was converted into $-NH_2$, i.e., to prepare PEG−PLA-NH_2; and (3) ROP of the *N*-carboxy anhydride of γ-benzyl-L-glutamate (BGL-NCA) was carried out in the presence of PEG−PLA-NH_2 as macroinitiator. The experimental details were described in ref 25. The following is a typical procedure for the third step. In a dried flask, 4.15 g of PEG−PLA-NH_2 (1.0 mmol) and 2.63 g of BGL-NCA (10 mmol) were dissolved in dried chloroform (65 mL), and the solution was stirred for 72 h at 30 °C. The product mixture was precipitated with an excess of a mixture of acetic acid and methanol (1:3, v/v) under vigorous stirring to give a white solid, while the unreacted PEG−PLA remained dissolved in the mixture. After removal of the PEG−PLA-NH_2 solution, purified PEG−PLA−PBGL was obtained under vacuum at 40 °C for 24 h.

The protective benzyl groups in the PEG−PLA−PBGL were removed to obtain PEG−PLA−PGL by catalytic hydrogenation. Then 0.5 g of PEG−PLA−PBGL (0.072 mmol) was dissolved in 15 mL of THF. A small amount of Pd/C (10%) was suspended in 5 mL of methanol. These two liquids were added into a hydrogenator. After purging with nitrogen three times, the reaction mixture was stirred under 1.0 MPa hydrogen pressure at 50 °C for 3 days. After removal of the Pd/C powders, the solution was dropped into an excess of petroleum ether. The precipitate (PEG−PLA−PGL) was dried in vacuo at 40 °C for 24 h.

Scheme 1. Synthesis of Copolymer PEG−PLA−PGL/RGD



As shown in Scheme 1, the RGD was grafted onto the PEG−PLA−PGL by first activating the side chain carboxyl groups of the PEG−PLA−PGL with NHS and then coupling with the RGD. For the carboxyl group activation, 2.2 g of PEG−PLA−PGL (0.40 mmol) and 1.4 g of NHS (12 mmol) were dissolved in 40 mL of DMF in a dried flask and treated with 2.5 g of DCC (12 mmol) and 0.01 g of DMAP (0.082 mmol) for 24 h at room temperature. Then, the solid dicyclohexylurea formed was removed by filtration. The copolymer solution was precipitated into an excess of ether. The product (PEG−PLA−PGL/NHS) was purified by washing with methanol under sonication, dried under vacuum at 40 °C for 24 h, and then kept in a refrigerator.

For the coupling of RGD, 0.1 g (0.015 mmol) of PEG−PLA−PGL/NHS and 60 mg of RGD (0.18 mmol) were dissolved in 5 mL anhydrous DMF. After adding 0.06 mL of triethylamine (0.44 mmol), the reaction mixture was stirred at room temperature for 4 days. The product mixture was precipitated in excessive ether and then dialyzed with a cellulose membrane (cutoff M_n = 3500) for 2 days. After dialysis, the solution was immediately lyophilized for 2 days.

Cell Experiments. Test Cells. Human chondrocytes from articular cartilage and 3T3 cells were chosen as the test cells. The former were isolated and cultured according to the method reported by Hong Zhongkui et al.[26] The chondrocytes were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum (FBS, GIBCO), 50 mg/L l-ascorbic acid (Sigma), 10 mmol/L HEPES (Sigma), 1.0×10^5 U/L penicillin (Sigma), and 100 mg/L streptomycin (Sigma). The 3T3 cells were purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences, and cultured with DMEM supplemented with 10% FBS, 1.0×10^5U/L penicillin, and 100 mg/L streptomycin.

Test Films. The RGD-grafted copolymer was tested in the form of films. The PEG−PLA−PGL/RGD was blended with poly(lactic acid-*co*-glycolic acid) (PLGA) by mixing the dried polymers together (5 wt % PEG−PLA−PGL/RGD in PLGA), dissolving in DMF, and casting on round cover slides (15.5 mm in diameter). Pure PLGA films were

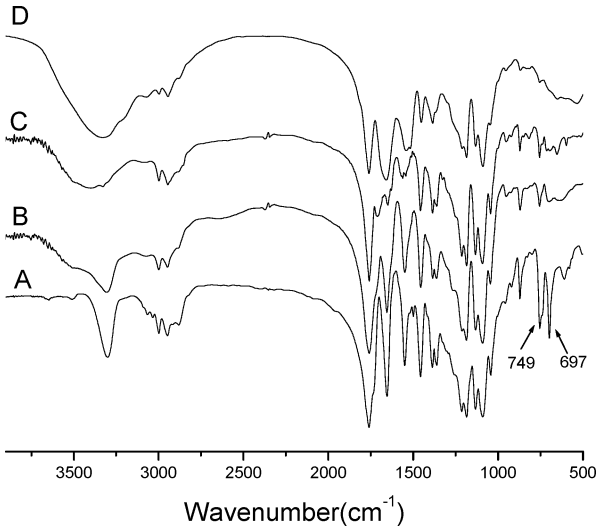


Figure 1. The FT-IR spectra of (A) PEG-PLA-PBGL, (B) PEG−PLA−PGL, (C) PEG−PLA−PGL/NHS, and (D) PEG−PLA−PGL/RGD.

prepared in the similar way and were used as the control. The slides were kept under vacuum for 48 h or more to remove the last traces of DMF and exposed to UV light for 30 min for sterilization.

Cell Adhesion and Spreading. The cover slides coated with the polymer films were placed in each well of 24-well tissue culture plates (NUNC). The chondrocytes were seeded on the cover slides at a density of 2.5×10^4 cells/well, incubated in a humidified incubator at 37 °C and 5% CO_2, and examined after 2, 6, and 12 h. Following incubation, the cover slides were washed three times with PBS and fixed with 3% glutaraldehyde in PBS at room temperature for 30 min. After thorough washing with PBS, the cells were dyed with one drop of Giemsa stain (Sigma) for 30 min, washed with deionized water, dried in air, and then enveloped with neutral balsam. The stained cells were observed under an inverted microscope (TE2000-U, Nikon). Nine pictures of each cover slide were taken with a digital camera (DXM1200F, Nikon) and analyzed with NIH Image J.

Cell Viability. Cell viability and proliferation were examined by the MTT assay.[27] The round slides coated with the polymer films were placed in each well of 24-well tissue culture plates (NUNC), completely covering the bottom surface of the well. Four cover slides were used for each sample. 3T3 cells (2.5×10^4) were placed on each well and cultured for 6 days. After incubation, 80 μL of MTT solution (5 mg/mL in PBS) was added to each well and incubated at 37 °C and 5% CO_2 for an additional 4 h. The medium was removed and replaced with 0.5 mL of 0.04 mol/L HCl/2-propanol to solubilize the converted dye. The solution (200 μL) from each well was transferred to another 96-well plate, and the absorbance values of the converted dye were measured at a wavelength of 540 nm by using a Thermo Electron MK3 micrometer.

Statistical Analysis. All data reported are means ± standard deviations. The degree of statistical significance between two groups was estimated by *t*-test; *p* values less than 0.05 were considered to be significant.

Results and Discussion

Synthesis of the Triblock Copolymers. PEG−PLA−PBGL was prepared by reacting PEG−PLA-NH_2 with BGL-NCA as reported previously.[25] Its IR spectrum is shown in Figure 1A. The absorption peak at 3292 cm^{-1} is assigned to ν_{NH} stretching, and the peaks at 1653 cm^{-1} (amide I) and 1548 cm^{-1} (amide II) are attributed to the amide group, indicating the formation of the polypeptide block. The peak at 1734 cm^{-1} (ν_{CO}) is characteristic of the PLA block. The absorptions at 697 and 749 cm^{-1} from the phenyl group are characteristic of the PBGL

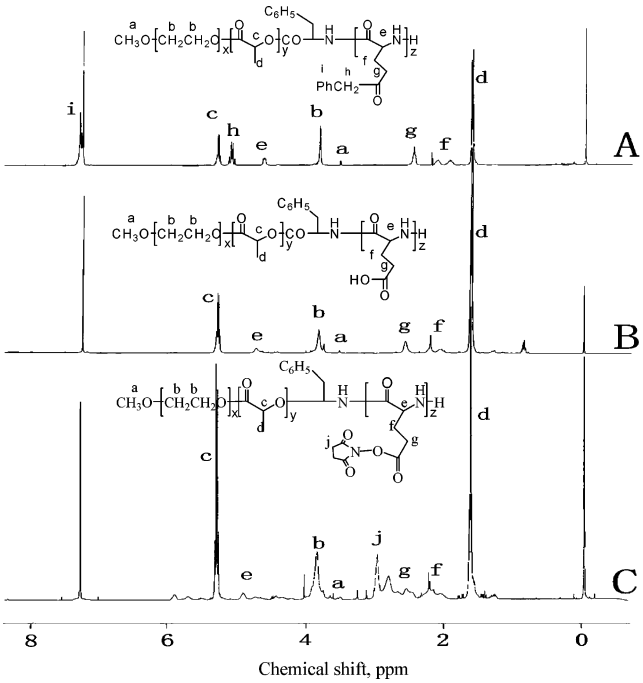


Figure 2. The ^{1}H NMR spectra and their assignments: (A) PEG−PLA−PBGL, (B) PEG−PLA−PGL, and (C) PEG−PLA−PGL/NHS in a $CDCl_3$/TFA-*d* mixture (1:1, v/v).

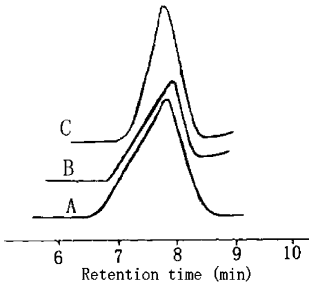


Figure 3. The GPC traces of PEG−PLA−PBGL (A), PEG−PLA−PGL (B), and PEG−PLA−PGL/NHS (C).

block carrying the protective groups. The peak at 1127 cm^{-1} (ν_{C-O-C}) belongs to the PEG block. The peak at 1087 cm^{-1} (ν_{C-O-C}) corresponds to the PLA block.

The structure of the triblock copolymer PEG−PLA−PBGL is also confirmed by the ^{1}H NMR spectra (Figure 2A). The peak *i* at 7.35 ppm is attributed to the benzene ring of the protecting group. The peaks at 5.13, 4.67, 2.50, 2.18, and 1.98 ppm are assigned to protons of the PBGL block. The peaks at 3.86 and 3.58 ppm are assigned to protons of the PEG block. The peaks at 5.32 and 1.65 ppm are assigned to protons of the PLA block. The degree of polymerization of the PBGL block (DP_{PBGL}) in the triblock copolymer can be obtained from the integral ratio of CH_3O- (3.38 ppm, *a*) to $C_6H_5CH_2OCOCH_2-$ (5.13 ppm, *h*) or $C_6H_5CH_2OCOCH_2-$ (2.50 ppm, *g*), as shown in the formula $DP_{PBGL} = 3h/2a$ or $3g/2a$. The value thus determined is 13.

The GPC (Figure 3A) trace of the triblock copolymer shows a unimodal shape with $M_w/M_n = 1.17$ and $M_n = 7300$, as shown in Table 1. This further indicates that the copolymerization was completed successfully and there were no homopolymers in the copolymer obtained.

PEG−PLA−PGL. It is well-known that the benzyl protective group of the polymer can be removed by catalytic hydrogenation with the use of Pd/C as catalyst. The deprotection of PEG−PLA−PBGL is confirmed with FT-IR and ^{1}H NMR

Table 1. Molecular Weight Data of the Block Copolymers

polymer	$M_n/10^3$ ^{1}H NMR	$M_n/10^3$ GPC	$M_w/10^3$: GPC	M_w/M_n
$PEG_{750}-PLA_{3400}-PBGL_{2800}$	7.0	7.3	8.4	1.17
$PEG_{750}-PLA_{3400}-PGL_{1400}$	5.6	6.3	7.5	1.19
$PEG_{750}-PLA_{3400}-PGL/NHS_{2500}$	6.7	8.6	10.6	1.22

as shown in Figures 1B and 2B, respectively. The OH stretching in the carboxyl group at 3440 cm^{-1} is strengthened greatly and the δ_{CH} vibration of the benzyl at 749 and 697 cm^{-1} disappeared after hydrogenation, while the resonance peaks of the protective benzyl groups at 5.13 and 7.35 ppm completely disappear. These indicate complete removal of the protective groups in the polymer. The GPC peak of the deprotected polymer (Table 1, Figure 3B) shifts a little to the lower molecular weight side, but its M_w/M_n remains unchanged compared to that of the protected one, suggesting that main-chain cleavage in the polymer did not occur during the deprotection reaction.

PEG−PLA−PGL/NHS. PEG−PLA−PGL was activated with NHS in the presence of DCC at room temperature. The ^{1}H NMR spectrum of PEG−PLA−PGL/NHS is shown in Figure 2C. A proton peak of succinimide distinctly appeared at 2.95 ppm, revealing the presence of the *N*-hydroxysuccinimidyl ester of PGL. The formation of PEG−PLA−PGL/NHS was also confirmed by the IR spectrum (Figure 1C): there appeared two new absorption peaks at 1710 and 1780 cm^{-1} attributable to the succinimide ring. Its GPC chromatograph shows a unimodal shape and an obviously increased molecular weight (Figure 3C), which further supports the incorporation of NHS.

PEG−PLA−PGL/RGD. It was synthesized by reacting RGD with the activated PEG−PLA−PGL, as shown in Scheme 1. It is well-known that the amino acid sequences containing RGD in a protein often display cell attachment activity, but for a short peptide, this activity can be retained only when the C-terminal of the RGD tripeptide is blocked (e.g., RGD-NH_2 in which the C-terminal COOH is converted to $CONH_2$) or connected with a flanking amino acid (e.g., RGDS and GRGDSP).[14,28] So we chose RGD-NH_2 in the present study (but it is still abbreviated as RGD in the following text for the sake of simplicity). Without any protection, it was directly reacted with the NHS-activated PEG−PLA−PGL to obtain the final product PEG−PLA−PGL/RGD. Because the solubility of RGD and PEG−PLA−PGL/RGD was limited in $CDCl_3$ or $CDCl_3$/TFA-*d*, TFA-*d* was employed as their common solvent for ^{1}H NMR measurements. Correspondingly, the resonance peaks of PLA block (5.10, 1.40 ppm in Figure 4), PEG block (3.64, 3.30 ppm), and PGL block (4.84, 1.79−2.61 ppm) displayed a certain shifts compared with those measured in $CDCl_3$/TFA-*d* (Figure 2). Nevertheless, ^{1}H NMR spectrum of the reaction product (Figure 4) showed the characteristic proton peaks of RGD, such as a broad multiplet from 1.6 to 2.0 ppm ($-CH_2CH_2CH_2NHC(=NH)NH_2$ of arginine, p), a multiplet at 2.9 ppm ($-CH_2CONH_2$ of aspartic acid, s), and a multiplet at 4.1 ppm ($-NHCH_2CO-$ of glycine, r). The FT-IR spectrum (Figure 1D) showed a stronger NH stretching band from 3100 to 3500 cm^{-1} and two enhanced peaks at 1653 cm^{-1} (amide I) and 1548 cm^{-1} (amide II) due to the formation of the amide bonds between the side carboxylic acid and the amine group of RGD compared to PEG−PLA−PGL. Therefore, the RGD had been attached to the copolymer PEG−PLA−PGL.

Table 2. Amino Acid Analysis of the RGD-Grafted Triblock Coplymers

sample	feed ratio ($n_{RGD}/n_{polymer}$)	glutamic acid (μmol/mL)	glycine (μmol/mL)	aspartic acid (μmol/mL)	coupling ratio[a] (mol %)
1	2:1	413	21	23	5.3
2	4:1	445	67	73	15.7
3	11:1	410	122	121	39.4
4	20:1	400	200	189	48.6

[a] Coupling efficiency = (Gly/Glu + Asp/Glu)/2.

Amino Acid Analysis. The amino acid analysis was conducted to determine the contents of glycine and arginine and the coupling ratio of RGD in the PEG−PLA−PGL/RGDs synthesized. As shown in Table 2, the coupling ratio was improved with increasing RGD ratio in the reaction feed. When the molar ratio of RGD to polymer increased from 2:1 to 20:1, the coupling ratio increased from ca. 5% to ca. 49%. This implied that the coupling ratio could be adjusted by simply changing the feed ratio.[29]

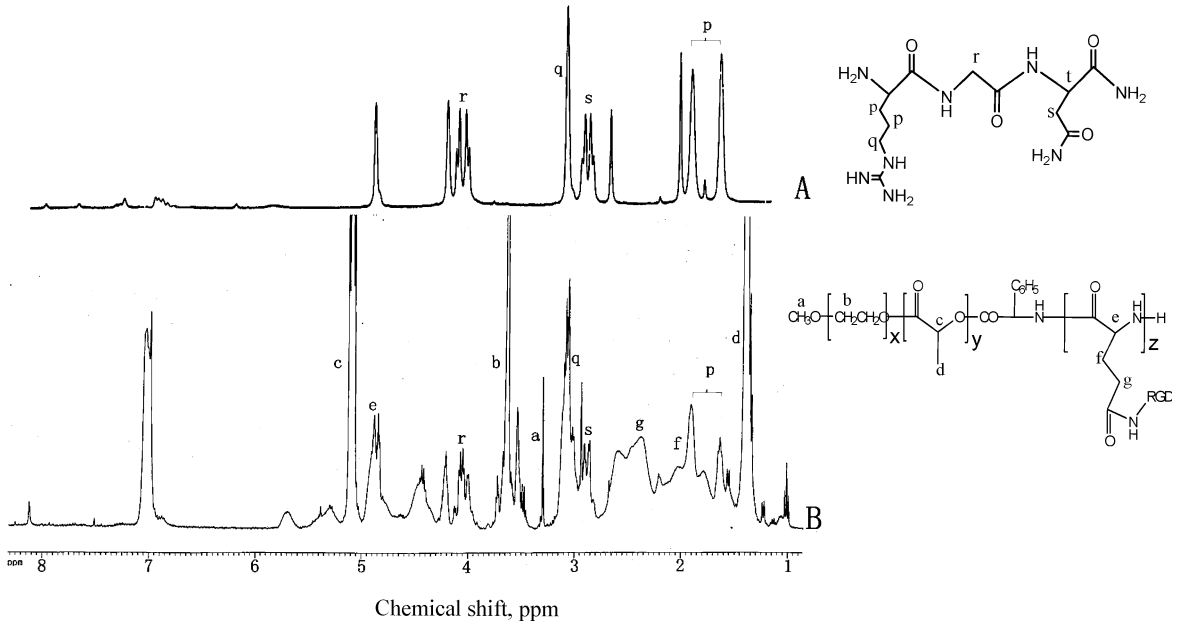


Figure 4. ^{1}H NMR spectra and their assignments: (A) RGD-NH_2 and (B) PEG−PLA−PGL/RGD in TFA-*d*.

Table 3. Surface Elemental Analysis

sample	C (mol %)	O (mol %)	N (mol %)	N/C
PEG−PLA−PGL	68.63	29.66	1.71	0.025
PEG−PLA−PGL-NHS	68.65	27.04	4.01	0.058
PEG−PLA−PGL/RGD (5.3%)[a]	70.53	25.56	3.91	0.056
PEG−PLA−PGL/RGD (15.7%)[a]	66.09	29.52	4.39	0.066
PEG−PLA−PGL/RGD (39.4%)[a]	65.89	28.04	6.08	0.113
PEG−PLA−PGL/RGD (48.6%)[a]	66.85	25.08	8.06	0.121

[a] The percent in brackets was the coupling ratio of RGD.

XPS Analysis. The XPS was used to determine the elemental composition of the polymers synthesized. According to the molecular formulas shown in Scheme 1, nitrogen is only present in the PBGL segment and the grafted peptide; therefore, the atom ratio of nitrogen to carbon can provide a qualitative measure of the amount of grafted peptide at the material surface. As indicated in Table 3, the N/C ratio increases from 0.0249 for PEG−PLA−PGL to 0.056−0.121 for PEG−PLA−PGL/RGDs, depending on the coupling ratio of RGD, indicating the successful coupling of RGD.

Blend Films. Because the molecular weight of the final PEG−PLA−PGL/RGD was not high enough, its film could not survive the cell tests. To investigate its cell-adhesion enhancement, it was blended into high molecular weight PLGA (200 000 g/mol) to examine the differences between the blends and the PLGA matrix. The blends containing the copolymer (coupling ratio 48.6%) of 1, 5, and 10 wt % and the pure PLGA were solution-cast onto round cover slides or Si wafers for the subsequent experiments.

The surface structure of these films was examined by AFM and SEM. As shown in Figure 5, the PLGA film presented a smooth surface (Figure 5A), the blend film of PEG−PLA−PGL/RGD in PLGA exhibited a little rough but even surface when the content of PEG−PLA−PGL/RGD was 5% (Figure 5B), and obvious phase separation and a discontinuous surface were observed (Figure 5C) when the minor component was 10%. When the 5% sample was observed with SEM, phase separation was also detected (Figure 6B). The minor phases were evenly dispersed in the PLGA matrix. The experimental fact that the electron radiation always caused damage to the minor phases because of their low molecular weight and low T_g provided a support to the two-phase structure of the blend film.

The surface wettability of these films was determined by water contact angle measurement. It was found that the wettability of the blend films of PEG−PLA−PGL/RGD in PLGA is better than the blend films of PEG−PLA−PGL in PLGA and both of them have better wettability than pure PLGA film, as shown in Table 4. The results suggest that the grafted RGD peptide can alter the hydrophobic nature of the matrix polymer and improve the surface wettability, although they were not continuously distributed in the PLGA matrix.

Cell Adhesion and Spreading. Cell adhesion of various films was evaluated by culturing the human chondrocytes in a culture medium of DMEM containing 10% of FBS (fetal bovine serum). The test sample was a 5 wt % blend of PEG−PLA−PGL/RGD (coupling ratio 48.6%) in PLGA and the control sample was pure PLGA. Figure 7 shows the cell morphology during the incubation. The cells become adhered to the substrate, spread on the surface first, and begin to grow and to proliferate sometime later. This process is obviously dependent on the film materials. The cells adhere and spread better and proliferate faster on the RGD-containing film than on the control film. The

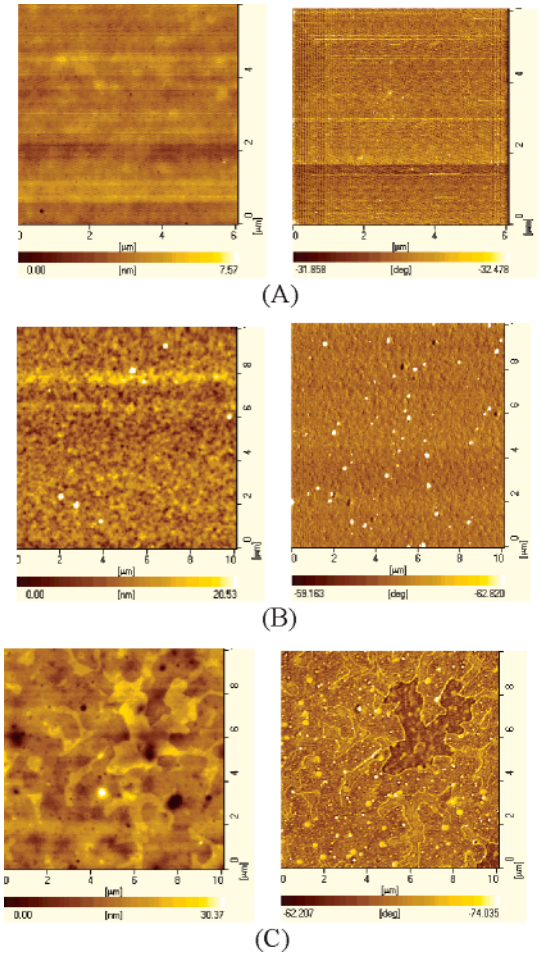


Figure 5. AFM height images (left) and phase images (right) of blend films of PEG−PLA−PGL/RGD in PLGA. PEG−PLA−PGL/RGD contents: (A) 0 wt %, (B) 5 wt %, and (C) 10 wt %.

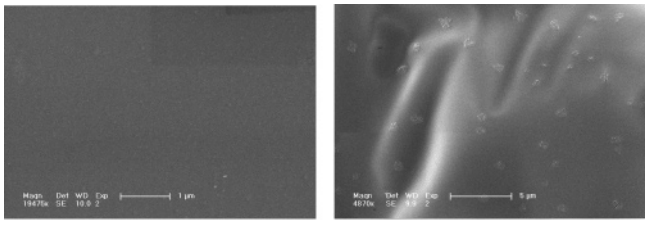


Figure 6. SEM pictures of (A) PLGA film, and (B) 5% blend film of PEG−PLA−PGL/RGD in PLGA.

Table 4. Water Contact Angle Data of the Copolymer/PLGA Blends

	water contact angle (deg)	
% in blend	PEG−PLA−PGL	PEG−PLA−PGL/RGD
0	102.9	
1	78.9	76.8
5	74.4	70.0
10	74.7	57.0

cell counting data are collected in Figure 8. The cell number over 0.4 mm^2 on the RGD-containing film is significantly ($p < 0.01$) higher than that on PLGA film (31 vs 17 at 2 h, 34 vs 23 at 6 h, and 77 vs 44 at 12 h after seeding). In addition to cell counting, cell area on the film surface was also analyzed. As shown in Figure 9, the difference between the two samples becomes greater and greater along with the increase of incuba-

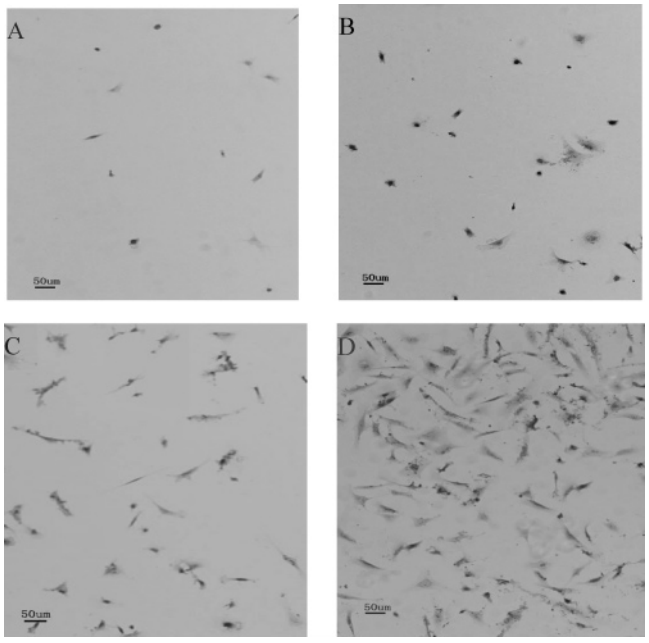


Figure 7. Microscopic images of adhered and spread human chondrocytes. Film sample: (A and C) PLGA and (B and D) a 5% blend of PEG−PLA−PGL/RGD in PLGA. Incubation time: A and B, 6 h; C and D, 12 h.

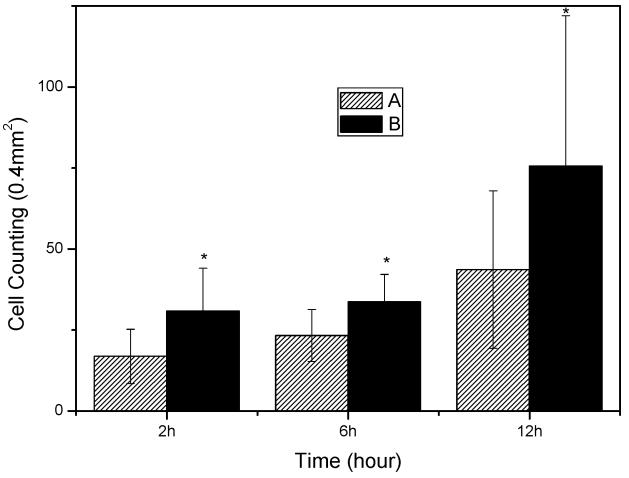


Figure 8. Attached human chondrocytes on the following substrates: (A) PLGA film and (B) 5% blend film of PEG−PLA−PGL/RGD in PLGA. Columns and error bars represented the means ± SD of nine samples. Statistical significance was indicated by a * ($p < 0.01$).

tion time (151.4 vs 149.1 at 2 h, 251.6 vs 201.9 at 6 h, 361.5 vs 268.0 at 12 h after seeding).

To summarize, incorporation of RGD peptide into the triblock copolymer really improves the cell adhesion and spreading ability of the substrate and promotes the cell proliferation of the human chondrocytes, indicating the successful coupling of RGD and the efficacy of the RGD peptide in improving the adhesion property of the matrix.

Cell Viability. The MTT assay was used to measure relative cell viability. Figure 10 shows the viability of the 3T3 cells seeded on the PLGA films and the 5% blend films of PEG−PLA−PGL in PLGA. On the sixth day after seeding, the OD value on 5% blend films of PEG−PLA−PGL in PLGA was 1.06, while it was 0.91 on the control PLGA films, which could be attributed to the improved adhesion and spreading ability of RGD. This difference was not statistically significant compared to the cell counting tests for human chondrocytes. There were

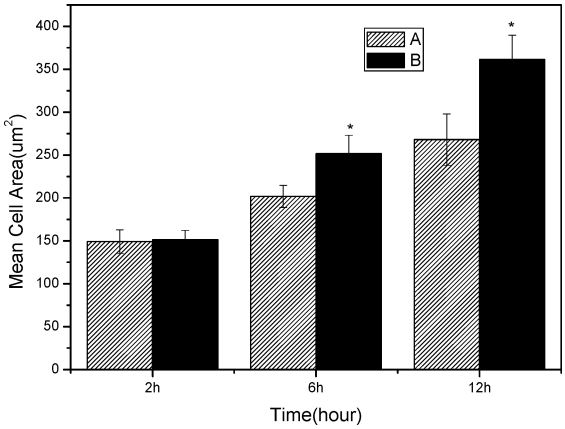


Figure 9. Cell area of human chondrocytes on the following substrates: (A) PLGA film and (B) 5% blend film of PEG−PLA−PGL/RGD in PLGA. Columns and error bars represented the means ± SD of nine samples. Statistical significance was indicated by a * ($p < 0.01$).

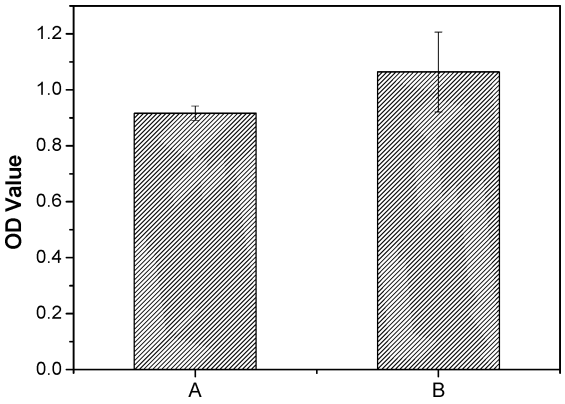


Figure 10. Viability values of 3T3 cells evaluated by MTT assay after 6 days incubation at 37 °C for (A) PLGA film and (B) 5% blend film of PEG−PLA−PGL/RGD in PLGA. Columns and error bars represented the means ± SD of four samples.

two possible reasons: (1) Two different kinds of cells were used in cell counting test and the MTT assay. They might show different affinities for RGD peptide. (2) The test duration of the MTT assay was 6 days while that of cell counting was only 12 h. Probably, the influence of RGD peptide became less significant with culture time.

Conclusion

Cell-adhering RGD tripeptide was successfully grafted on to PEG−PLA−PGL in two steps: (1) activation of the side-chain carboxylic acid with NHS in the presence of DCC and (2) coupling RGD and PEG−PLA−PGL/NHS in DMF with the help of TEA. ^{1}H NMR, FT-IR, amino acid analysis, and XPS analysis were used to confirm the structures of PEG−PLA−PGL/RGD and its precursors. Addition of 5 wt % PEG−PLA−PGL/RGD into a PLGA matrix significantly improved the surface wettability of the blend films, and the AFM and ESEM measurements showed that PEG−PLA−PGL/RGD could be mixed with PLGA well, although phase separation occurred. The cell adhesion and cell spread on the PEG−PLA−PGL/RGD-containing film was enhanced compared to those on pure PLGA film. This improvement was dependent on the RGD content in the copolymer. Therefore, by changing the copolymer composition and by changing the RGD-grafting ratio, the amount of grafted RGD and, consequently, the cell adhesion,

cell spread, and cell proliferation could be adjusted. This RGD-grafted copolymer is expected to be used as biodegradable scaffolds in cell and tissue engineering. Further investigations are in progress.

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References and Notes

(1) Gombotz, W. R.; Pettit, D. K. *Bioconjugate Chem.* **1995**, *6*, 332−351.
(2) Ryser, H. G. P.; Shen, W. C. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 3867−3870.
(3) Zeng, J.; Xu. X. Y.; Chen, X. S.; Liang, Q. Z.; Bian, X. C.; Yang, L. X.; Jing, X. B. *J. Controlled Release* **2003**, *92*, 227−231.
(4) Reed, A. M.; Gilding, D. K. *Polymer* **1981**, *22*, 494−498.
(5) Gilding, D. K.; Reed, A. M. *Polymer* **1979**, *20*, 1459−1464.
(6) Epple, M.; Rueger, J. M. *Nachr. Chem. Technol. Lab.* **1999**, *47*, 1405−10.
(7) Thull, R. *Materialwiss Werkst.* **2001**, *32*, 949−52.
(8) Langer, R. *Acc. Chem. Res.* **2000**, *31*, 94−101.
(9) Rong, G. Z.; Deng, M. X.; Deng, C.; Tang, Z. H.; Piao, L. H.; Chen, X. S.; Jing, X. B. *Biomacromolecule* **2003**, *4*, 1800−1804.
(10) Deng, M. X.; Wang, R.; Rong, G. Z.; Sun, J. R.; Zhang, X. F.; Chen, X. S.; Jing, X. B. *Biomaterials* **2004**, *25*, 3553−3558.
(11) Yuan, M. L.; Wang, Y. H.; Li, X. H.; Xiong, C. D.; Deng, X. M. *Macromolecules* **2000**, *33*, 1613−1617.
(12) Salem, A. K.; Cannizzaro, S. M.; Davies, M. C.; Tendler, S. J. B.; Roberts, C. J.; Williams, P. M.; Shakesheff, K. M. *Biomacromolecules* **2001**, *2*, 575−580.
(13) Barrera, D. A.; Zylsrta, E.; Lansbury, P. T.; Langer, R. *J. Am. Chem. Soc.* **1993**, *115*, 11010−11011.
(14) Pierschbacher, M. D.; Ruoslahti, E. *Nature* **1984**, *309*, 30−34.
(15) Fussell, G. W.; Cooper, S. L. *Biomaterials* **2004**, *25*, 2971−2978.
(16) Jo, S.; Shin, H.; Mikos, A. G. *Biomacromolecules* **2001**, *2*, 255−261.
(17) Schmedlen, R. H.; Masters, K. S.; West, J. L. *Biomaterials* **2002**, *23*, 4325−4332.
(18) Magnusson, I.; Batich, C.; Collins, B. R. *J. Periodontol.* **1988**, *59*, 1−7.
(19) Freed, L. E.; Marquis, J. C.; Nohria, A.; Emmanual, J.; Mikos, A. G.; Langer, R. *J. Biomed. Mater. Res.* **1993**, *27*, 11−23.
(20) Desai, N. P.; Hubell, J. A. *J. Biomed. Mater. Res.* **1991**, *25*, 829−843.
(21) Merrill, E. W. *J. Biomater. Sci. Polym. Ed.* **1993**, *5*, 1−11.
(22) Cima, L. G. *J. Cell. Biochem.* **1994**, *56*, 155−161.
(23) Drumheller, P. D.; Hubbell, J. A. *J. Biomed. Mater. Res.* **1995**, *29*, 207−215.
(24) Daly, W. H.; Poché, D. *Tetrahedron Lett.* **1988**, *29*, 5859−5862.
(25) Deng, C.; Rong, G. Z.; Tian, H. Y.; Tang, Z. H.; Chen, X. S.; Jing, X. B. *Polymer* **2005**, *46*, 653−659.
(26) Hong, Z. K.; Zhang, P. B.; He, C. L.; Qiu, X. Y.; Liu, A. X.; Chen, l.; Chen, X. S.; Jing, X. B. *Biomaterials* **2005**, *26*, 6296−6304.
(27) Gümüsderelioğlu, M.; Türkoğlu, H. *Biomaterials* **2002**, *23*, 3927−3935.
(28) Pierschbacher, M. D.; Ruoslahti, E. *J. Biol. Chem.* **1987**, *262*, 17294−8.
(29) Hern, D. L.; Hubbell, J. A. *J. Biomed. Mater. Res.* **1998**, *39*, 266−276.

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