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A novel strategy to graft RGD peptide on biomaterials surfaces for endothelization of small-diamater vascular grafts and tissue engineering blood vessel

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Abstract To improve the performance of small-diamater vascular grafts, endothelization of biomaterials surfaces and tissue engineering are more promising strategies to fabricate small-diamater vascular grafts. In this study, a Gly–Arg–Gly–Asp–Ser–Pro (GRGDSP) peptide was grafted on the surfaces of poly(carbonate urethane)s (PCUs), with photoactive 4-benzoylbenzoic acid (BBA) by UV irradiation. The photoactive peptides (BBM-GRGDSP) were synthesized with classical active ester of peptide synthesis. The modified surfaces of PCU with the photoactive RGD peptides were characterized by water contact angle measurement and X-ray Photoelectron Spectroscopy (XPS), which results suggested that the peptides were successfully grafted on the PCU surfaces. The effect of these modified surfaces on endothelial cells (ECs) adhesion and proliferation was examined over 72 h. PCU surfaces coupled with the synthetic photoactive RGD peptides, as characterized with phase contrast microscope and the metabolic activity (MTT) assay enhanced ECs proliferation and spreading with increasing concentration of RGD peptides grafted on their surfaces. Increased retention of ECs was also observed on the polymers surfaces under flow shear stress conditions. The results demonstrated that GRGDSP peptides grafted on the surfaces of polymers with photoactive 4-benzoylbenzoic acids could be an efficient method of fabrication for artificial small-diamater blood vessels. The modified polymer is expected to be used for small-diamater vascular grafts and functional tissue engineered blood vessels to improve ECs adhesion and retention on the polymer surfaces under flow shear stress conditions.

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1 Introduction

Cardiovascular disease is the major cause of death in the world each year, especially, coronary artery disease and peripheral vascular disease [1]. The need for improved small diameter (≤6 mm) blood vessels is critical owing to the increase in vascular replacement surgeries. Adequate healthy autograft vessels (usually internal mammary artery or saphenous vein) for bypass conduits are lacking in many patients; therefore, the artificial vascular graft must be used. Synthetic materials such as microporous polyurethanes, expanded poly(tetrafluoroethylene) (ePTFE) and Dacron [poly(ethylene teraphthalate)] have been successfully used as large-diameter (>6 mm) vascular substitutes [2–4]. Currently, these materials for small-diameter applications such as coronary artery bypass grafting have been largely unsuccessful due to rapid occlusion caused by thrombosis and intimal hyperplasia [4–7]. To overcome those problems, numerous strategies have been investigated to improve the performance of small-diamater vascular grafts, including endothelization on these biomaterials surfaces [8], tissue engineering fabrication [9], and chemical modification [10, 11]. In these methods, endothelization of biomaterials surfaces and tissue engineering are more promising to fabricate small-diamater vascular grafts [12–14]. However, these biomaterials support poor adhesion and growth of endothelial cells (ECs). And the retention of the EC is still a major challenge for the seeded grafts in dynamic conditions [15]. To promote endothelialization on biomaterial surfaces, modification of the

chemical structure [16], surface modification by plasma treatment [17], and coating or grafting adhesive proteins or bioactive peptides [18, 19], were employed. Especially, the RGD peptide sequence plays a crucial role in mediating cell attachment and subsequent spreading. Many researches about the interactions of RGD-containing peptide and cell receptors (i.e. integrins) improve the development of long-term endothelial cell attachment and growth on surfaces of biomaterials [20–24]. Photochemical methods known simple and nontoxic strategy of surface modification have also been applied to graft different antithrombotic chemicals (e.g. albumin, heparin and fibronection) to polyurethane (PU) [25, 26] and RGD-peptide to PU surfaces with azide groups [27]. However, very few papers have examined benzophenone as the photoactive crosslinker for RGD peptides grafted on biomaterials surfaces. Benzophenone has been suggested to be potentially a more efficient crosslinker than aryl azides in protein probe field due to its inability to react with water, a key competitor of the photoaffinity labeling reaction and preferentially reacting with fairly unreactive C–H bonds [28, 29]. Hence, it will be advantageous for RGD peptide grafted on biomaterials surfaces with this method in endothelization of small-diamater vascular grafts and tissue engineering fields. In this paper, poly(carbonate urethane)s designed by Pinchuk et al. to remove the susceptible ester and ether linkages in the soft segments, which have shown excellent resistance to hydrolysis, environmental stress cracking (ESC), and metal ion oxidation (MIO) as long-term biostable cardiovascular biomaterials, were employed as matrices [30]. The 4-Benzoylbenzoic acid coupling GRGDSP (Gly–Arg–Gly–Asp–Ser–Pro) peptides, containing the cell adhesive sequence of fibronectin RGDS sequence, and grafted on the surfaces of the poly(carbonate urethane)s by UV irradiation, were investigated. The relationship between the peptide density grafted and ECs adhesion and growth, the adhesion strength and the retention of ECs on the surface of poly(carbonate urethane)s were roughly evaluated as well.

2 Experimental methods

2.1 Materials

H-GRGDSP-OH was purchased from Calbiochem (99.5% purity). 4-benzoylbenzoic acid (BBA) was purchased from Aldrich (99% purity). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT reagent) was purchase from Sigma Chemical Company. Dulbecco's Modified Eagle's Medium (DMEM) and trysin were purchased from Gibco BRL Co. Methylenebis(phylene isocyanates) (MDI) and 1,4-butanediol (BDO) were distilled under vacuum. 1,6-hexanediol (HD), 1,5-pentanediol (PD), sodium ethoxide, diethyl carbonate (DEC), N-hydroxysuccinimide and dicyclohexylcarbodiimide (DCC) were used as received.

2.2 Preparation of materials

The synthesis methods of polycarbonate diol and poly(carbonate urethane)s have been reported previously in detail [31]. The PCU was based on a 3:1.65:1.35 molar ratio of methylene diphenylene diisocyanate (MDI), 1, 4-butanediol (BDO) and polycarbonate diol (poly(1,6-hexyl-1,5-pentyl carbonate)diol, PHPC, MW = 1,058). The PCU films with thickness 0.2 mm was obtained by spreading THF solution of PCU (10%) onto a glass plate and evaporated the solvent, then were dried for 24 h at 60°C in the oven and for additional 24 h at 70°C in the vacuum oven. Then the PCU films were cut into small pieces (1 × 1 cm) for the next experiments.

2.3 The synthesis of 4-Benzoylbenzoic acid N-Hydroxysuccinimide Ester (BBM)

About 1.13 g (5 mmol) of 4-benzoylbenzoic acid and 0.60 g (5.25 mmol) of N-hydroxysuccinimide were dissolved in 50 ml of acetonitrile. And then, 1.13 g (5.5 mmol) of dicyclohexylcarbodiimide (DCC) was added into the solution at room temperature. After being stirred for 15 h at room temperature, the solution was filtered, the solvent was evaporated under vacuum, and the residue was recrystallized from ethyl acetate [29]. Yield: 1.01 g (3.1 mmol, 62%), TCL R_f 0.35 (hexan/ethyl acetate 1/1)

2.4 BBM-GRGDSP synthesis

A total of 25 mg H-GRGDSP-OH (Calbiochem®,USA) was dissolved in 1 ml water, and the pH value was adjusted to 7–8 with saturated solution of sodium bicarbonate. BBM (21 mg) dissolved in 2 ml 1,4-dioxan was added into the peptide solution, and the mixture was stirred for 24 h at room temperature. The 1, 4-dioxan was evaporated under vacuum, and the residual solution was extracted with ethyl ether three times and precipitated with acetonitrile. The precipitate was washed twice with ethyl ether and dried under vacuum at room temperature to yield 73% of BBM-GRGDSP. The route of synthesis was illustrated in Fig. 1. APCIms (negative) m/z theoretical 795 g/mol, observed 794 g/mol (100%).

2.5 Photochemical grafting

A total of 10 mg BBM-GRGDSP was dissolved in 1 ml phosphate buffered saline (PBS pH = 7.2), then diluted

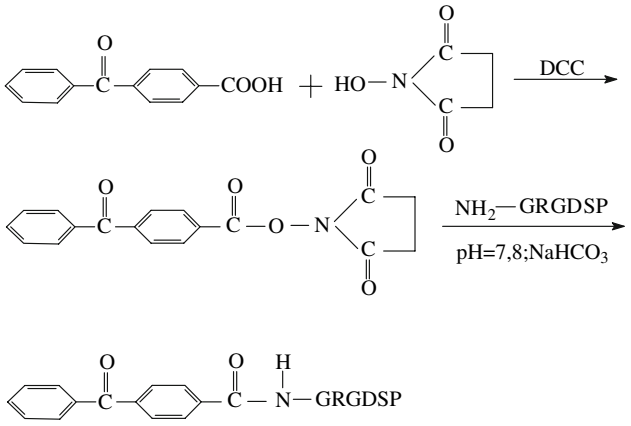

Fig. 1 The route of BBM-GRGDSP synthesis

with PBS from 0.3 mg/ml to 2 mg/ml concentration series. The solution (100 μl/cm^2) was coated on the PCU film, and incubated in the dark for 6 h at 37°C. The PCU surface was UV irradiated for 8 min with wavelength ≥320 nm. The films were fully rinsed with distilled water to remove unreacted reagents, and then dried at room temperature. These samples were named with respect to the amount of peptide coated on each square centimeter of PCU. For example, PCU200 means 200 μg peptides coated on one square centimeter PCU film grafted. The reaction scheme is illustrated in Fig. 2.

2.6 Characterization

2.6.1 Instrumentation

The mass spectra were obtained on an HP1100-LC/MSD with atmosphere pressure chemical ionization (positive mode). Amino acid analysis was performed on a Hitch 835–50 Amino Acid Analyzer. The sample was hydrolyzed in 6 N HCl at 110°C for 48 h. Contact angles were measured with a KRÜSS DSA100 Drop Shape Analysis System and 3 μl of distilled water at 25°C, and the results reported are the mean values for five replicates.

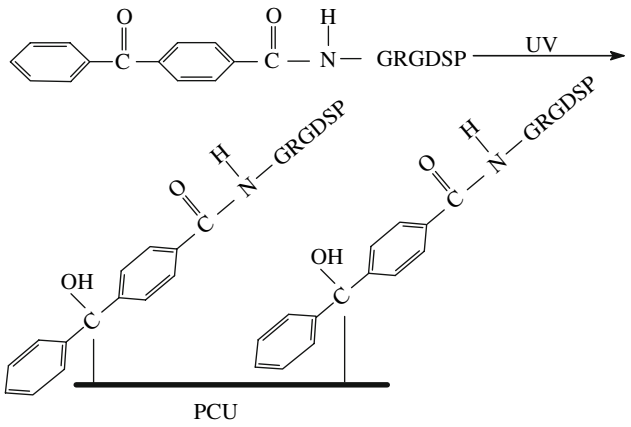

Fig. 2 The reaction schemes of the photochemical immobilization GRGDSP on PCU surface

2.6.2 X-ray photoelectron spectroscopy

X-ray Photoelectron Spectroscopy (XPS) was performed on a Kratos XSAM-800 Spectrometer. A magnesium anode at 20 kV 10 mA, and a take-off angle of 30° was used. The relative atomic percentage of each element on the surface was estimated from the peak areas using atomic sensitivity factors specified for the XSAM-800. The high survey (i.e., 0–1100 eV with pass energy of 50 eV and 1 eV of spot size) and high-resolution scans (i.e., 20 eV of pass energy and 0.1 eV of spot size) were performed for determining O/C and N/C ratio, and fraction of carbon functional groups, respectively. It is noted that C1s spectra bands were deconvoluted into sub-peaks by processing with the XPSPEAK1.0 spectrometer software.

2.7 Cell culture

Human umbilical vein endothelial cells (HUVECs) were provided by Internal Medicine Laboratory of HuaXi Medicinal Center, Sichuan University. The cells were cultured in DMEM with 10% fetal bovine serum at 37°C with 5% CO_2/95% air and kept at approximately 90% relative humidity for 2–3 days.

Cells were harvested for experiments by incubating in 0.5 mg/ml trysin and 0.25% EDTA in Hank's balanced salt solution for 3 min. Trypsinnization was stopped by the addition of DMEM with 10% fetal bovine serum. Cells were collected by centrifugation and resuspended in DMEM with 10% fetal bovine serum.

PCU-GRGDSP film (1 × 1 cm^2) with different concentrations of grafted GRGDSP were fitted into the surfaces of a 24 well tissue culture polystyrene plate (Becton Dickinson & Company). After sterilization with UV light overnight, 8 × 10^4 cells were seeded on each well and allowed to attach for the desired time in an incubator at 37°C with 5% CO_2/95% air. The incubator was kept at approximately 90% relative humidity.

The films were subsequently rinsed with Hank's balance salt solution to remove nonadhesive cells, while the adhesive cells were fixed with a solution of 3% paraformaldehyde for 3 h. The morphology of cells was observed by a phase contrast microscope (Olympus-CK2, Japan). The average cell areas were quantified by computer software (Nikon&Spot image collecting system, Image-Pro plus). Approximately 20–40 cells were analyzed per sample. Results were expressed as mean ± standard deviation.

Films (0.45 × 0.45 cm^2) and 96 well tissue culture plates were sterilized with UV light overnight. A total of 1×10^4 cells were separately seeded onto the wells and incubated at 37°C and 5% CO_2/95% air. At least three replicates were used for each experimental data. After 24, 48 and 72 h, the relative cellular growth was assayed using the metabolic activity (MTT) assay. Each well received 20 µl of MTT solution. After 4 h of incubation at 37°C, 100 µl of dimethyl sulfoxide solution was added to dissolve formazan crystals. The absorbance of the formazan solution obtained from different material samples was measured at 570 nm by an enzyme-linked immunosorbent assay microplate reader (Bio-RAD Model 550). Assuming the cellular growth on the tissue culture polystyrene surfaces (TCPS) to be 1.0, the relative cellular growth on PCU and modified PCU film surfaces was calculated. The mean value of triplicate samples for each film was calculated with the standard deviation, and statistical error was calculated by the Student's *t*-test ($p < 0.05$).

2.8 Retention of endothelial cells under flow shear stress

After ECs were cultured on PCU200 and PCU films 24 h, films with cells were exposed to laminar flow in a parallel plate flow chamber, and flushed with the DMEM (with 10% fetal bovine serum and 1% antibiotics) for 2 h under 2 Pa wall shear stress condition [15, 32]. The cells remaining on the films were trypsinized, centrifuged, and resuspended for cell counting by using a hemacyto-meter combined with phase contrast microscope. The cells coverage on films was photographed.

3 Results and discussion

3.1 BBM-GRGDSP synthesis and characterization

The photoactive BBM-GRGDSP was obtained with classical active ester method in peptide chemistry without further purification. Mass spectroscopy (negative mode) and amino acid analysis were employed to confirm that the photoactive peptides were successfully synthesized. In Mass spectra (Fig. 3), the main relative abundance peaks (m/z) are at 794.4 and 795.3. These results suggest that the molecular weight of the main resultants is 795, which coincides with the molecular weight of BBM-GRGDSP. The results of the amino acid analysis (Table 1) show that the resultants only contain five kinds of amino acid, which are glycine (Gly), arginine (Arg), aspartic (Asp), serine (Ser) and proline (Pro). Moreover, the ratios of these amino acids (based on the average of glycine, arginine and aspartic acid detected, i.e. 30.510 nmol, 15.039 nmol and 15.188 nmol, respectively) were in good agreement with the theoretical ratios in the composition of BBM-GRGDSP. However, the average of serine and proline detected (9.472 nmol and 4.087 nmol, respectively) was lower than their theoretical ratios in the photoactive peptides. This is because serine and proline are prone to

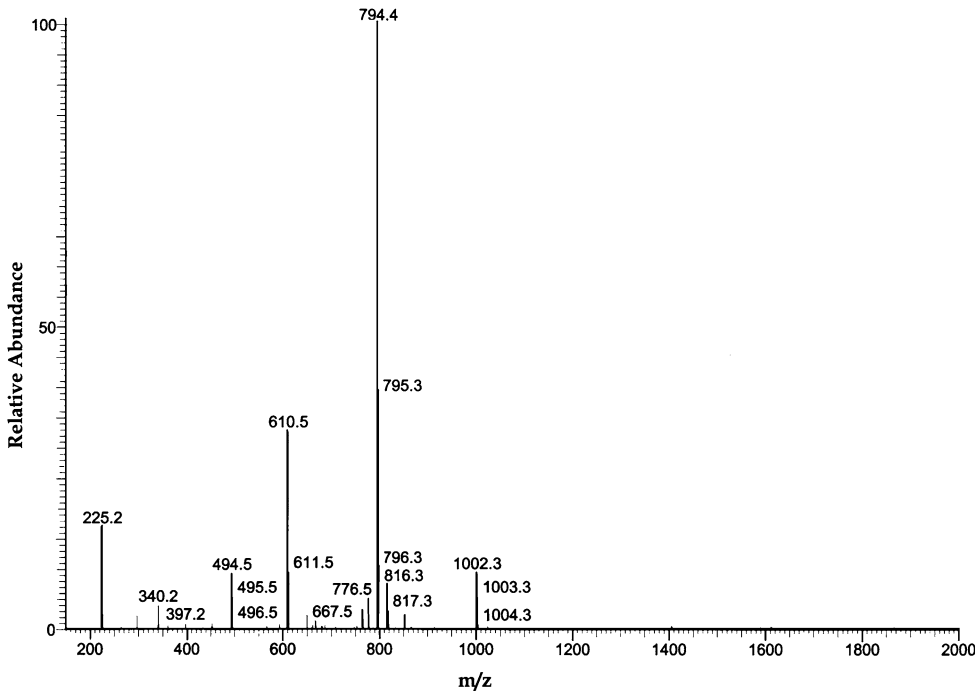

Fig. 3 Mass spectrum of BBM-GRGDSP (negative model)

Table 1 The results of the amino acid analysis

Amino acid	Time (min)	N mol
Asp (D)	11.34	15.039
Ser (S)	12.85	9.472
Pro (P)	14.73	4.087
Gly (G)	18.42	30.510
Arg (R)	43.89	15.188

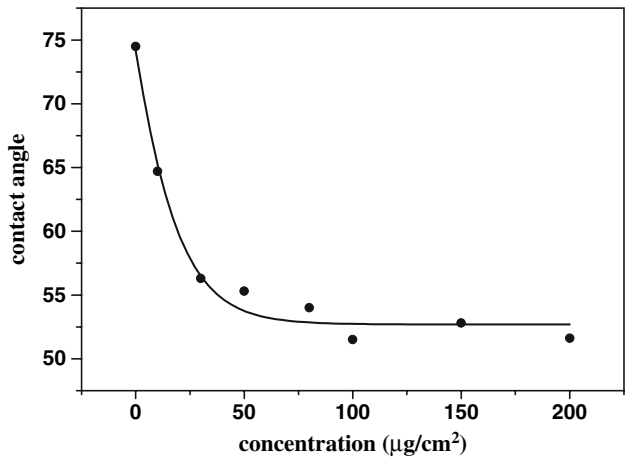

Fig. 4 Water contact angle on the surfaces of PCU grafted with various concentrations of BBM-GRGDSP

cyclization or decarboxylation under high concentration hydrochloric acid and high temperature condition. Further investigation in the future is necessary.

3.2 Surface properties and structure

The surface microheterogeneity sensitivity of contact angle measurement is approximately 0.5–1.0 nm on the polymer surface [33]. In order to study the photoactive peptides grafted on the surfaces of poly(carbonate urethane)s with various concentration of peptides solution, the water contact angle was measured. The results are shown in Fig. 4. The contact angles of these samples decrease with increasing amount of grafted peptide on the PCU surfaces. The contact angle nearly levels off with a constant value of 50° ± 2° once the amount of the peptides grafted on PCU surfaces is over 100 μg/cm^2. The hydrophobic aromatic ring in the structure of photoactive peptides improves their hydrophobic property [25]. These results suggest that the photoactive peptides were grafted on the surfaces of PCU.

Table 2 Atomic percentages and atomic proportions on the surfaces of PCU and PCU200

Samples	Atomic proportions (mole percent,%)				
	C1s	N1s	O1s	N/C	O/C
PCU	74.3	2.1	23.6	0.03	0.32
PCU200	75.3	4.2	20.4	0.06	0.27

Table 3 XPS C1s curve fitting of PCU and PCU200

Samples	C1s composition (mole percent, %)				
	C–C	*C*–O(*C*–N)	–HN*C*OO	–O*C*OO–	–HN*C*OC–
PCU	67.0	26.3	1.9	4.8	–
PCU200	67.6	20.0	1.9	4.3	6.3

To further examine the PCU surfaces grafted the photoactive peptides, atomic percentages on the 5 nm depths (take-off angle: 30°) of PCU200 and PCU uppermost surfaces were detected with XPS. Table 2 shows that both nitrogen molar percentage and N/C ratio on the PCU-200 surfaces are twice that on the PCU surfaces. The C1s spectra of PCU and PCU-200 were deconvoluted into sub-speaks. An extra peak appeared at 287.6 eV in the PCU-200 spectra compare with that of PCU, at which the characteristic peak ascribes to the carbonyl carbon of amide group in the peptide (–HN*C*OC–) (Fig. 5). The relative molar fraction of

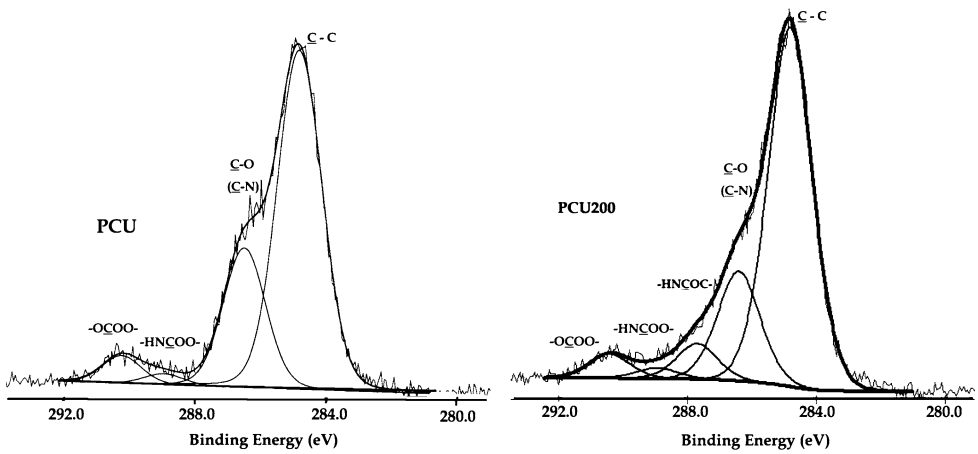

Fig. 5 Representative C1s curve fitting of PCU and PCU200, takeoff angle 30°

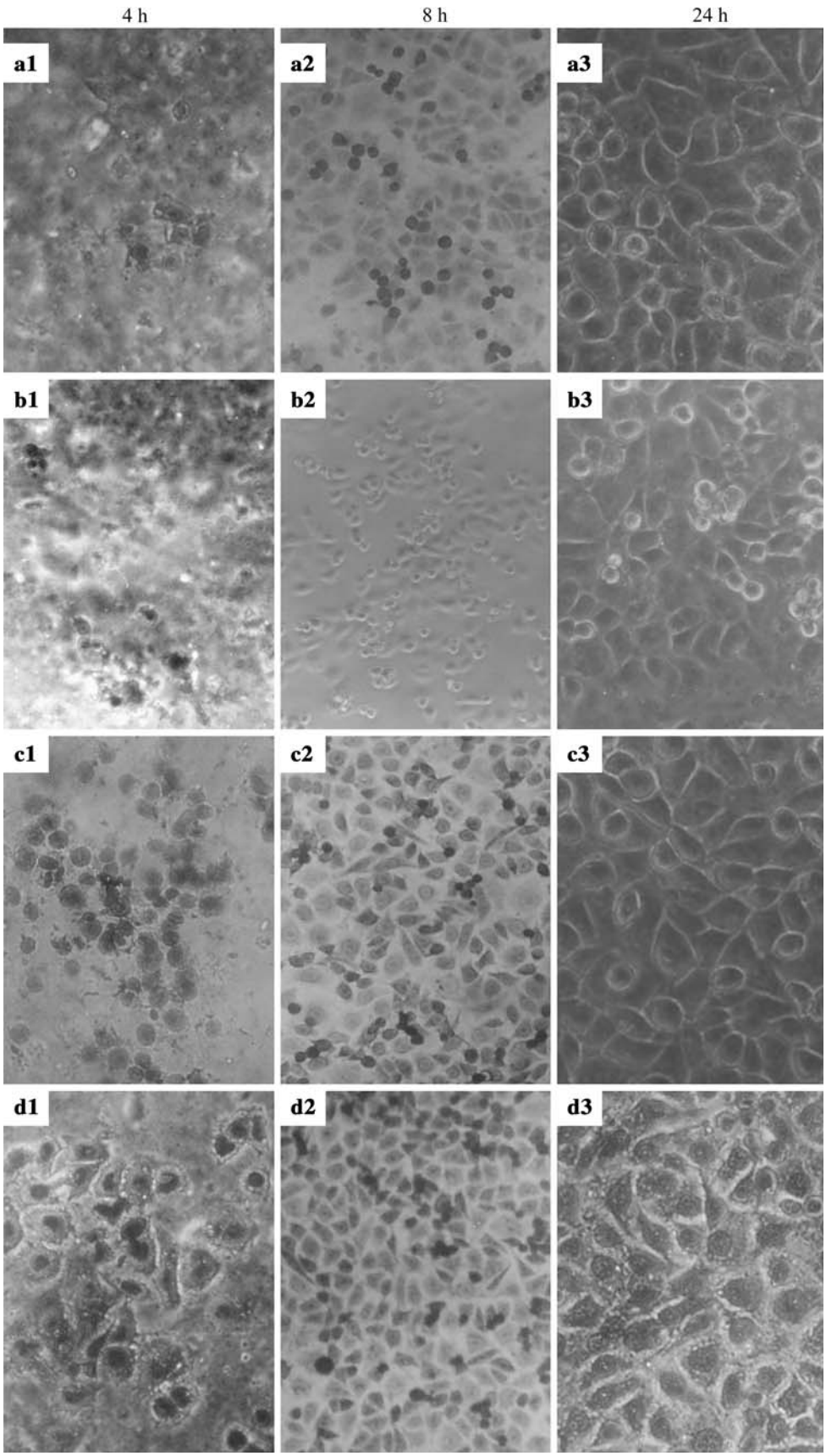

Fig. 6 The adherent cell images of phase converse microscope on PCU and modified PCU surfaces at 4, 8 and 24 h cultured, (**a**) PCU, (**b**) PCU30, (**c**) PCU100 and (**d**) PCU200

Table 4 Area analysis of adherent cell on PCU and modified PCU surfaces after 8 h incubation

Sample	Single cell average area (μm^2) (Mean ± SD)
PCU	809 ± 136
PCU30	783 ± 178
PCU100	1438 ± 479
PCU200	1722 ± 374

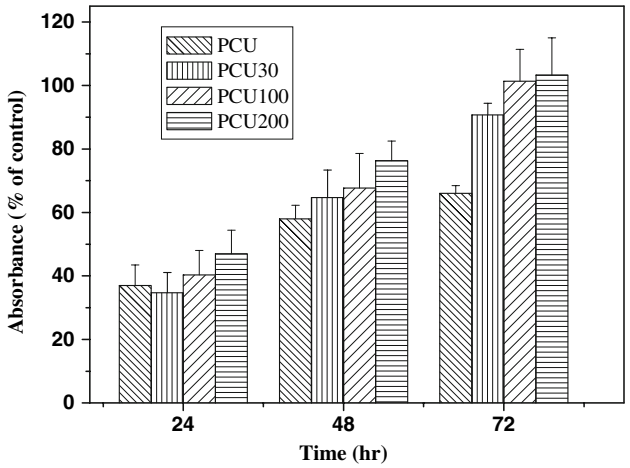

Fig. 7 The viability endothelial cell growth on PCU and modified PCU surfaces after 24 h, 48 h and 72 h cultured, Cell population on tissue culture polystyrene is 100%. The mean value of triplicate samples for each film was calculated with the standard deviation, and statistical error was calculated by the Student's *t*-test ($p < 0.05$)

peptide functional groups (–HN*C*OC–) in the high-resolution scan for C1s analysis increased from 0 to 6.3% for PCU and PCU-200, respectively (Table 3). In addition, the other peaks of PCU-200 and PCU at 284.8, 286.36, 288.65 and 290.46 eV corresponded to *C*–C, *C*–O (*C*–N), –HN*C*OO and –O*C*OO–, respectively (Fig. 5). These functional groups of C–O and –OCOO– represent polycarbonate soft segments in PCU structures. Their carbon atomic proportions on the surfaces of PCU-200 were lower than that of PCU (Table 3), which indicated that the photoactive peptides were mainly grafted into polycarbonate chains. Therefore, these results showed that the peptides were successfully grafted on the PCU surfaces.

3.3 Endothelial cell adhesion and growth

The morphology of adhered, viable HUVECs at 4, 8 and 24 h after cell seeding is presented in Fig. 6 for PCU and modified PCU with various concentrations of photoactive peptides. After incubated with polymer films for 4 h, the HUVECs began to adhere to PCU and modified PCU films. The number of HUVECs adhered to the films increases with increasing photoactive peptide concentrations grafted on surfaces of PCU. For example, there are more cells adhered to the surfaces of PCU200 films than that of the other films (Fig. 6 a_1,b_1,c_1,d_1). With incubation time increasing, more and more cells were attached to the surfaces of all films, and the surfaces of these films were almost completely covered with adherent ECs after 24 h of incubation. Furthermore, it was investigated that the concentration of photoactive peptides grafted on the surfaces of PCU film influenced on spread of adherent ECs after 8 h incubation. The single adherent EC area was enlarged with increasing concentration of photoactive peptides grafted on the surfaces of PCUs (Table 4). The cell area on surfaces of PCU200 exceeds that of PCU and PCU30. These results suggest that the higher concentration of photoactive peptides grafted on PCU surfaces helps promote ECs adhesion and spreading on the surfaces of PCU. However, it should be noted that the number of adhesive cells and the cell area on PCU30 surfaces are both smaller than PCU's (Fig. 6 a_1, b_1, a_2, b_2 Table 4). This indicates that lower concentration of RGD peptides grafted on PCU surfaces is not desirable for ECs to adhere to these surfaces and spread at an early stage [34].

Proliferation of ECs on the surfaces of PCU with or without GRGDSP attachment was measured together by MTT assay over time (Fig. 7). The number of corresponding samples tested increased with increasing time. The proliferation rate of the PCU with high concentration GRGDSP attached was faster than that of PCU with low concentration peptides attachment. Therefore, PCU200 had relatively higher cell numbers than the other samples at three points in time. Similarly, the number of cells on PCU30 was significantly lower compared to that of unmodified PCU at 24 h. This is in agreement with the results of cell adhesion. The PCU30 had a higher cell number than PCU after 48 h. These results show that the proliferation of adherent ECs on PCU surfaces can be promoted by increasing the concentration of photoactive peptides grafted on PCU surfaces. It is also verified that photoactive peptides grafted on polymer surfaces is an efficient strategy to enhance cells adhesion, spreading and proliferation on their surfaces.

3.4 Retention of endothelial cells

Endothelization of biomaterials surfaces for small-diamater vascular grafts must be continuously exposed to hemodynamically imposed mechanostress, including static pressure, circumferential stress, and shear stress in vivo because of blood flow generating shear stress. For the development of functional tissue engineered small-diamater blood vessels, providing efficient mass transfer from the

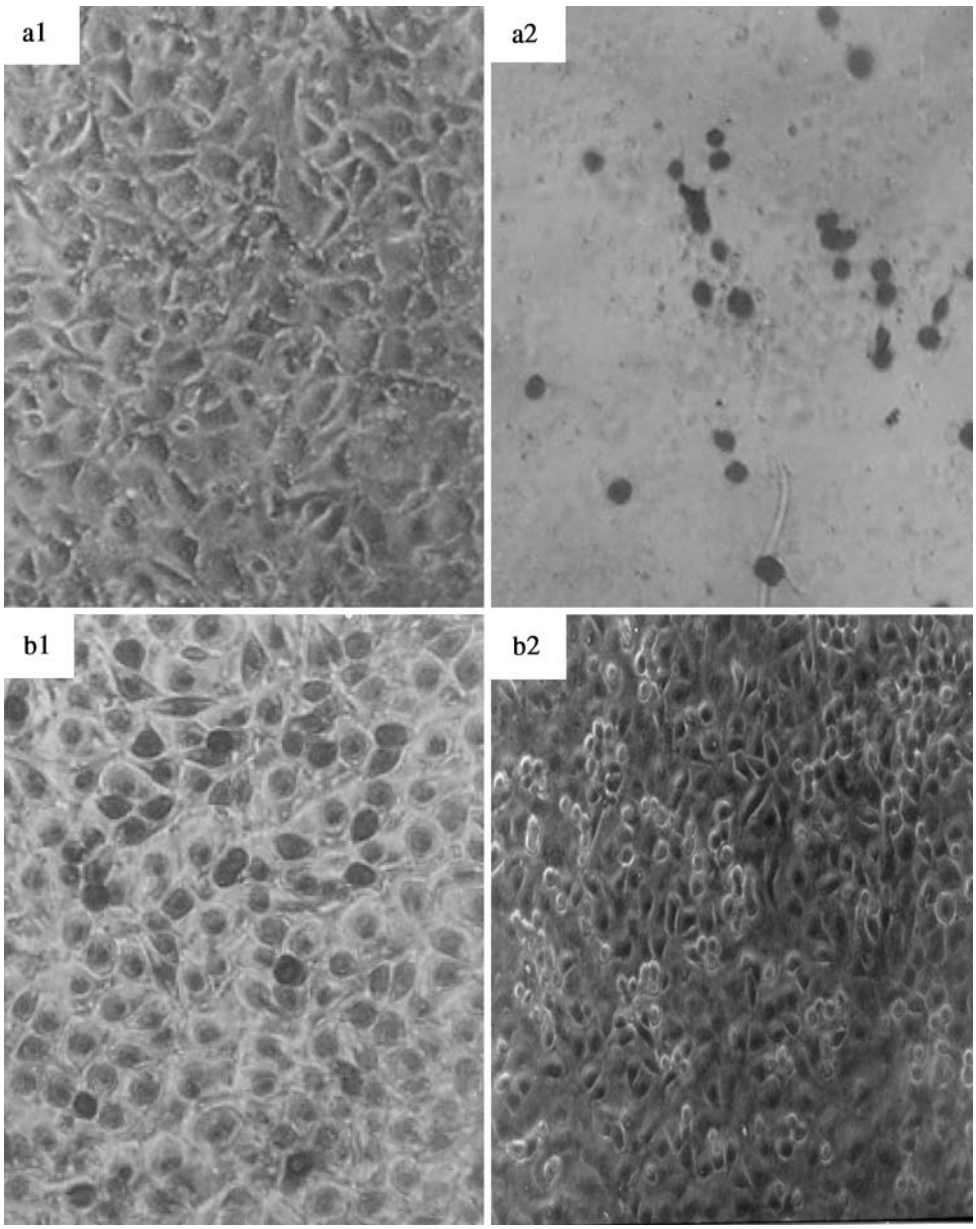

Fig. 8 The adherent cell images of phase converse microscope on PCU and PCU200 before and after imposition of a flow shear stress

Table 5 Retention results after 2 h of a flow shear stress for PCU and PCU200 incubated in vitro for 24 h

Samples	Cells before flushed	Cells after flushed	% Retention
PCU	1.0×10^5	1.0×10^3	1
PCU200	2.6×10^5	2.4×10^5	92

medium to the tissue surface. It is critical to have a low shear stress environment and expose the developing tissue to physical stimuli. Therefore, the retention of endothelial cells on graft biomaterials surfaces or scaffold materials surfaces should be high enough to resist the shear stress described above. In this study, the adherent strength of ECs on the surfaces of PCU grafted GRGDSP was roughly characterized with a primary perfusion experiment in vitro. The maximum number of ECs adhered to PCU200 surfaces can be retained after flow loading for 2 h (Fig. 8). The retention rate of the ECs is approximately 92% (Table 5). Conversely, only 1% adherent ECs on PCU surfaces is left after encountering 2 Pa of shear stress for 2 h (Fig. 8, Table 5). These primary results show that GRGDSP peptides can greatly increase the retention of ECs on the surface of PCU to load the shear stress of blood flow. It is confirmed that GRGDSP peptides grafted on the surfaces of polymers with photoactive 4-benzoylbenzoic acid by UV irradiation will be an efficient method for small-diamater vascular grafts and functional tissue engineered small-diamater blood vessels to retain ECs on the polymer

surfaces under flow shear stress conditions. Moreover, in order to regulate endothelial cell attachment to the inner surfaces grafted with the peptides of small-diamater vascular grafts, a rotatory cultivation method of endothelial cell will be employed. It has been verified that the small-diamater tube surfaces were only covered with a confluent monolayer endothelial cell through a rotatory cultivation method [35].

4 Conclusions

In summary, photoactive RGD peptides have been prepared (BBM-GRGDSP) with an active ester of peptide synthesis. The results of water contact angle measurement and XPS indicated that BBM-GRGDSP were successfully grafted on the surfaces of the poly(carbonate urethane)s by UV irradiation. Thus the proliferation and spreading of adherent ECs on modified PCU surfaces can be promoted by increasing the concentration of photoactive peptides grafted on PCU surfaces. The retention of ECs on the modified surface of PCU increases greatly compared to that on the surface of PCU under flow shear stress conditions. These results suggest that that GRGDSP peptides sequence grafted on the surfaces of polymers with photoactive 4-benzoylbenzoic acids should be an efficient method for small-diamater vascular grafts and functional tissue engineered small-diamater blood vessels.

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