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Quantitative grafting of peptide onto the nontoxic biodegradable waterborne polyurethanes to fabricate peptide modified scaffold for soft tissue engineering

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Abstract Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) peptide has frequently been used in the biomedical materials to enhance adhesion and proliferation of cells. In this work, we modified the nontoxic biodegradable waterborne polyurethanes (WBPU) with GRGDSP peptide and fabricated 3-D porous scaffold with the modified WBPU to investigate the effect of the immobilized GRGDSP peptide on human umbilical vein endothelial cells (HUVECs) adhesion and proliferation. A facile and reliable approach was first developed to quantitative grafting of GRGDSP onto the WBPU molecular backbone using ethylene glycol diglycidyl ether (EX810) as a connector. Then 3-D porous WBPU scaffolds with various GRGDSP content were fabricated by freeze-drying the emulsion. In both of the HUVECs adhesion and proliferation tests, enhanced cell performance was observed on the GRGDSP grafted scaffolds compared with the unmodified scaffolds and the tissue culture plate (TCP). The adhesion rate and proliferation rate increased with the increase of GRGDSP content in the scaffold and reached a maximum with peptide concentration of 0.85 μmol/g based on the weight of the polyurethanes. These results illustrate the necessity of the effective control of the GRGDSP content in the modified WBPU and support the potential utility of these 3-D porous modified WBPU scaffolds in the soft tissue engineering to guide cell adhesion, proliferation and tissue regeneration.

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1 Introduction

In vascular tissue engineering, designing of natural or synthetic grafts to support adhesion and proliferation of seeded cells presents a promising approach to the generation of small diameter (<6 mm) blood vessels [1]. However, the grafts have not provided a patency when applied to small diameter blood vessels due to the incomplete cover of endothelial cells on the graft surface and the subsequent myointimal hyperplasia [1, 2]. Arg-Gly-Asp (RGD) containing peptides have been frequently used as cell adhesive peptide to promote various cell attachment and proliferation [3–11] by specific interaction with integrin cell receptors since RGD was identified as an independent cell attachment site in fibronectin in the earlier 1980s [12]. It has also been reported that the soluble RGD peptide can inhibit cell adhesion because it is the antagonist on attachment of cells, which further confirmed that RGD was the recognition sequence for integrin [13]. Therefore, efficient immobilization of the RGD peptide on the surface of materials is important.

As one of the most intensively investigated RGD containing peptides for cell adhesion and proliferation, Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) has been grafted onto the surface of materials to promote cell adhesion and proliferation during in vitro cell culture and used as an artificial ECM protein to induce specific cellular responses and new tissue formation [10, 14–16]. The previous studies also revealed that 0.02 wt% of peptide in the film on the surface of the substrate material is enough to induce skin fibroblast cell adhesion [10] and 0.2 pmol/cm^2 density of GRGDSP

on the surface of an interpenetrating polymer network is sufficient to encourage the adhesion and spreading of endothelial cells [14]. Thus, the content of immobilized peptides containing RGD sequence would affect the cell adhesion and proliferation on the polymer surfaces.

Additionally, 3-D porous scaffolds with interconnect pore network play a key role in the soft tissue engineering field on regeneration of damaged or diseased tissues. These scaffolds with controllable porosity and pore size can mimic the physical structure of natural extracellular matrix to guide cell in-growth and facilitate the inflow of nutrients and the elution of metabolic waste in the scaffolds [17]. Several methods have been used to fabricate 3-D porous scaffold with polyurethane, such as electrospinning [18], solvent casting/salt leaching [19], phase inversion [20] and thermally induced phase separation [21]. In our previous work, we have developed a series of 3-D porous polyurethane scaffolds with various pore diameters and porosities by freeze-drying [22].

To better understand the effect of GRGDSP peptides on the adhesion and proliferation of cells cultured in the scaffolds matrix, 3-D porous scaffold fabricated with GRGDSP grafted materials is an ideal candidate for the cell culture tests. However, the exact content of peptide, which is extremely low in the modified materials, is difficult to determine. The usually used peptide density determination methods, such as, ninhydrin method [23], amino acid analysis [15] and ^{125}I radiolabelling [24] are complicated and unsuitable for detecting the bulk density of GRGDSP peptide in the modified WBPU. The objective of this research is to develop a reliable process to control the content of GRGDSP in the modified WBPU and to study the effect of GRGDSP content on the adhesion and proliferation of HUVECs on the 3-D porous scaffolds. Firstly, WBPU containing –COOH was reacted with equivalent molar amount of EX810 in solution based on molar amount of –COOH in WBPU. And then, ethylenediamine perfluorocaprylate (EDAPFC) containing fluorinated chain synthesized with ethylenediamine and perfluorocaprylic acid (PFCA) was employed to confirm the feasibility of reaction between epoxy group of EX810 modified WBPU and amino group, and ensure that the epoxy groups remained on the modified WBPU are enough for the grafting of GRGDSP peptide molecules added in the later experiment, since EDAPFC is the same as GRGDSP containing an amino group, which has high reactive activity with epoxy group. FTIR and XPS were used to analyses the products of EX810 modified WBPU and EDAPFC grafted WBPU, respectively. Finally, GRGDSP was reacted with EX810 modified WBPU and a series of GRGDSP modified WBPU with different amount of peptide content were obtained, and the exact content of GRGDSP grafted onto WBPU can be determined. The 3-D porous scaffolds were fabricated with WBPU emulsion and GRGDSP modified WBPU emulsion by freeze-drying. The scaffolds obtained with mean pore-size of 40.9 μm and pore size distribution from 10.1 to 172.3 μm [22] were then used for cell culture to investigate the effect of GRGDSP content on the adhesion and proliferation of HUVECs. The results of cell culture tests revealed that the HUVECs cultured on the GRGDSP grafted scaffolds displayed enhanced viability compared with the cells seeded on the unmodified scaffolds, which demonstrated that the presence of GRGDSP peptide is beneficial to the adhesion and proliferation of HUVECs on the scaffolds.

2 Materials and methods

2.1 Materials

PFCA was obtained from Jiangsu Chemical Company (China). GRGDSP (99.5% purity) was bought from Calbiochem. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT reagent) and ethylene glycol diglycidyl ether (EX810) (50 wt% solution) were purchased from Sigma-Aldrich Chemical Company. Other chemicals and solvents were used as received.

2.2 Methods

2.2.1 EX810 modified WBPU

WBPU emulsion was synthesized based on isophorone diisocyanate (IPDI), 1,4-butandiol (BDO), poly (ethylene glycol) (PEG, molecular weight 1450), poly (ε-caprolactone) (PCL, molecular weight 2000) and L-lysine as described in our previous work [25]. The feed molar ratio of IPDI, PCL, PEG, BDO and L-lysine in this work is 3: 0.75: 0.25: 0.85: 0.85. WBPU emulsion was added into EX810 solution and then reacted at 60°C with 0.1 wt% tetrabutylammonium hydrogen sulfate (TBAHS) as the phase transfer catalyst. Equivalent molar amount of –COOH reacted with EX810 in this reaction. The reaction was monitored by Fourier transform infrared (FTIR) spectrometer.

2.2.2 Immobilization of EDAPFC on EX810 modified WBPU

2.2.2.1 Synthesis of EDAPFC PFCA (1 g, 2.4 mmol) dissolved in tetrahydrofuran was added into the ice-bath cooled solution of Boc-ethyldiamine (0.46 g, 0.29 mmol) in tetrahydrofuran. The mixture was stirred at 0°C for 1 h

subsequently at room temperature for 24 h. And then 10 ml HCl/EtOAc was added, the mixture was stirred overnight at room temperature to deprotect the Boc group [26]. The product was precipitated by adding petroleum ether (30–60°C), then washed with saturated sodium chloride solution and dried in vacuum to yield 0.51 g EDAPFC (43.4%). The EDAPFC obtained was characterized by nuclear magnetic resonance spectroscopy (^{1}H NMR) with Varian^{unity} Inova-400 spectrometer (400 MHz) in MeOD. ^{1}H NMR (MeOD, 400 MHz) δppm: 3.24(4H, m, –CH_2–CH_2–), 3.01(2H, t, NH_2–), 3.51(1H, t, –NH–).

2.2.2.2 EDAPFC grafted on EX810 modified WBPU ED APFC (0.19 g, 0.4 mmol) dissolved in tetrahydrofuran was added into EX810 modified WBPU emulsion (22.7 g, 13.65 wt%). The mixture was stirred at 40°C for 12 h, and then at 60°C for 2 h to obtain unpurified EDAPFC grafted WBPU. The mixture was precipitated with diethyl ether for three times to remove the unreacted EDAPFC completely and the product was named as purified EDAPFC grafted WBPU. The unpurified and purified EDAPFC grafted WBPU were dropped on the cover glass slides and dried at 60°C for 24 h and then 60°C under vacuum for XPS analysis.

2.2.3 Immobilization of GRGDSP on EX810 modified WBPU

A series of GRGDSP peptide modified WBPU were obtained by adding GRGDSP (0.09, 0.17, 0.85 and 1.70 μmol/g based on the amount of EX810 modified WBPU) into EX810 modified WBPU emulsion and reacted at 45°C for 12 h, then at 60°C for 2 h under magnetic stirring. The result products were named PUR005, PUR01, PUR05 and PUR1, respectively.

2.2.4 Fabrication of 3-D interconnected porous scaffolds and characterization of the porous structure

3-D porous scaffolds were fabricated by freeze-drying the WBPU emulsion and GRGDSP modified WBPU emulsion. 0.5 ml WBPU emulsion was added into a 1.5 cm diameter cylindrical plastic mold to form the scaffold with thickness around ~2.8 mm. The molds with emulsion were kept in refrigerator at 4°C for 4 h, and then pre-freezing at −25°C for 24 h before freeze-drying. The frozen emulsion in cylindrical plastic mold was lyophilized in a freeze-dryer (Boyikang, Beijing) for 24 h to dry the sample completely. All the scaffold samples were fabricated using emulsion (16 wt%) to obtain porous scaffolds with mean pore-size of 40.9 μm and pore size distribution between 10.1 and 172.3 μm [22].

2.2.5 Human umbilical vein endothelial cells culture

HUVECs provided by Internal Medicine Laboratory of HuaXi Medicinal Center, Sichuan University were cultured in complete Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin at 37°C and 5% of CO_2/95% air and kept at approximately 90% relative humidity in culture bottles. The medium was renewed every 2 days.

Cells in increased logarithmic phase were rinsed with sterilized phosphate-buffer saline (PBS) and then incubated in 0.25% trypsin for 3 min. Trypsinization was stopped by adding DMEM medium with 10% fetal bovine serum. Cells were centrifuged and resuspended in DMEM with 10% FBS and then diluted into certain concentration. Before cell seeding, a series of disinfection steps were taken to ensure a contamination-free environment for cell growth. The WBPU scaffolds were firstly washed with ethanol for 15 min, and then immersed in 75% ethanol overnight to remove the impurities and low molecular weight compound. After being immersed in 75% ethanol, the samples were then immersed in sterilized PBS for 3 h and replace the solution with fresh sterilized PBS every 15 min. Then the disinfected samples with the same size of culture well were placed into each well of the culture plate individually to exactly cover the bottom of the wells. These samples were then immersed in DMEM medium overnight before cell culture.

2.2.6 Endothelial cell adhesion rate assay

The cell adhesion rate was tested based on Williamson et al.'s work [27]. Briefly, 1×10^5 cells in 1 ml of serum-containing medium were added into each well of 24-well TCP containing WBPU scaffold and GRGDSP modified WBPU scaffold. 1×10^5 cells seeded in the well of TCP was used as control. Cells were allowed to adhere for 60 min with lid removed before analysis. Culture conditions were maintained in incubator at 37°C and 5% of CO_2/95% air and kept at approximately 90% relative humidity. After 60 min, the culture medium was aspirated out and the samples were washed gently with PBS to remove the non-adherent cells. And then the samples were incubated with 0.5 mg/ml methyl thiazolyl tetrazolium (MTT) in culture medium. After incubating at 37°C for 4 h, purple formazan crystal salts were formed. The solution in the well was removed and 1 ml of DMSO was added. The culture plates were mildly shaken for 15 min, and then 200 μl solution from each well was transferred to a 96 wells plate for test. 6 replicates were tested for each sample. The optical density (O.D.) was obtained at 490 nm using a Microplate Reader. The O.D. values of cells on the material samples (D_1) were

compared with the O.D. value of 1×10^5 cells (D_0) to obtain the adhesion rate of each sample. The adhesion rate of endothelial cells (R_s) on WBPU materials was calculated using the Eq. 1:

$$R_s = D_1/D_0 \times 100\% \quad (1)$$

2.2.7 Endothelial cell viability

Endothelial cells suspension from culture flask was calculated and diluted to 1×10^6 cell/ml. 50 μl of cells suspension were added into each well of 24-well TCP with scaffold samples. The same amount of cells was seeded in TCP without any scaffold sample as a control. Cells were allowed to adhere for 60 min and then 950 μl DMEM medium with 10% fetal bovine serum was added to each well for cell proliferation for certain period of time before analysis. Culture conditions were maintained in incubator at 37°C and 5% of CO_2/95% air and kept at approximately 90% relative humidity. The culture medium was changed every 2 days. After culture, the medium was aspirated out and the scaffolds were washed with sterile PBS to remove the non-adherent cells. After 1, 2, 4 and 6 days' culture, the samples for proliferation analysis were tested with MTT assay as described previously to measure the amount of the cells. 6 repeats were conducted to acquire an average value for each sample. The O.D. values of endothelial cells seeded on scaffold (D_1') were compared with the O.D. value of the control endothelial cells seeded in the TCP well without scaffolds (D_0') to obtain the proliferation rate of each sample [28, 29]. The proliferation rate of endothelial cells (P_s) on WBPU materials were calculated using the Eq. 2:

$$P_s = D_1'/D_0' \times 100\% \quad (2)$$

The samples for endothelial morphology observation were fixed with 4 wt% paraformaldehyde PBS solution in 24-well plate at room temperature. After 1 h, the samples were washed twice with sterile PBS for further morphology observation. FEI/Philips XL-30 field Environmental Scanning Electron Microscope (ESEM) was used to obtain high-resolution images of the cell morphology.

2.2.8 Characterization

2.2.8.1 Fourier transform infrared spectroscopy Infrared spectra of WBPU and EX810 grafted WBPU thin films covered on KBr discs were obtained with the Nicolet-560 spectrophotometer between 4,000 and 600 cm^{-1} in the resolution of 4 cm^{-1}. Fifty scans were averaged for each sample.

2.2.8.2 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° corresponding to 5 nm of analyzed depth was used. The area of the analytical X-ray spot on the sample surface is about 250 micron. 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. Fitting was then realized with XPSPEAK 1.0 spectrometer software.

2.2.9 Statistical analysis

All experimental results were expressed as mean ± the standard deviation of the mean. Three repeats for each test were carried out for the adhesion assay and proliferation experiment. The significance of difference was determined with ANOVA followed by LSD test. Statistical significance was determined and accepted at $P < 0.05$.

3 Results and discussion

3.1 Synthesis of EDAPFC and EX810 modified WBPU

The synthesis routes of EDAPFC and EX810 modified WBPU are shown in Fig. 1a and b, respectively. FTIR spectrometer was employed to monitor the reaction between epoxy groups of EX810 and carboxyl groups (–COOH) of the WBPU. Figure 2a shows the FTIR spectra of WBPU, EX810 modified WBPU after 2 and 4 days' reaction. Figure 2b, c and d present the amido (N–H) and hydroxyl (O–H) stretching region, carbonyl (C=O) stretching region of WBPU and stretching region of epoxy group of EX810, respectively.

In the N–H and O–H stretching region, as shown in Fig. 2, a broad peak corresponding to the N–H stretching around 3,550 cm^{-1} and the O–H stretching around 3,600 cm^{-1} was observed [30–32]. The O–H stretching in the range 3,700–3,500 cm^{-1} and centered at about 3,600 cm^{-1} decreases in the EX810 grafted WBPU, owing to the decreasing of carboxyl groups in WBPU. In the carbonyl stretching region, the carbonyl stretching band in the 1,800–1,600 cm^{-1} region is overlapped by the stretching band around 1,730, 1,700, and 1,650 cm^{-1} due to the absorption of the free carbonyl (in PCL and urethane), hydrogen-bonded carbonyl (in urethane) and free carbonyl (in urea), hydrogen-bonded carbonyl (in urea) and carboxyl group (Fig. 2), respectively [31–34]. In the FTIR spectra of EX810 modified WBPU, a decreasing absorbrance is also observed in the 1,680–1,610 cm^{-1} region centered at around 1,650 cm^{-1} because of the decrease amount of carboxyl groups in WBPU. The result is coincident with the changing trend of O–H stretching, which further verifies the reaction between the epoxy groups of EX810 and –COOH

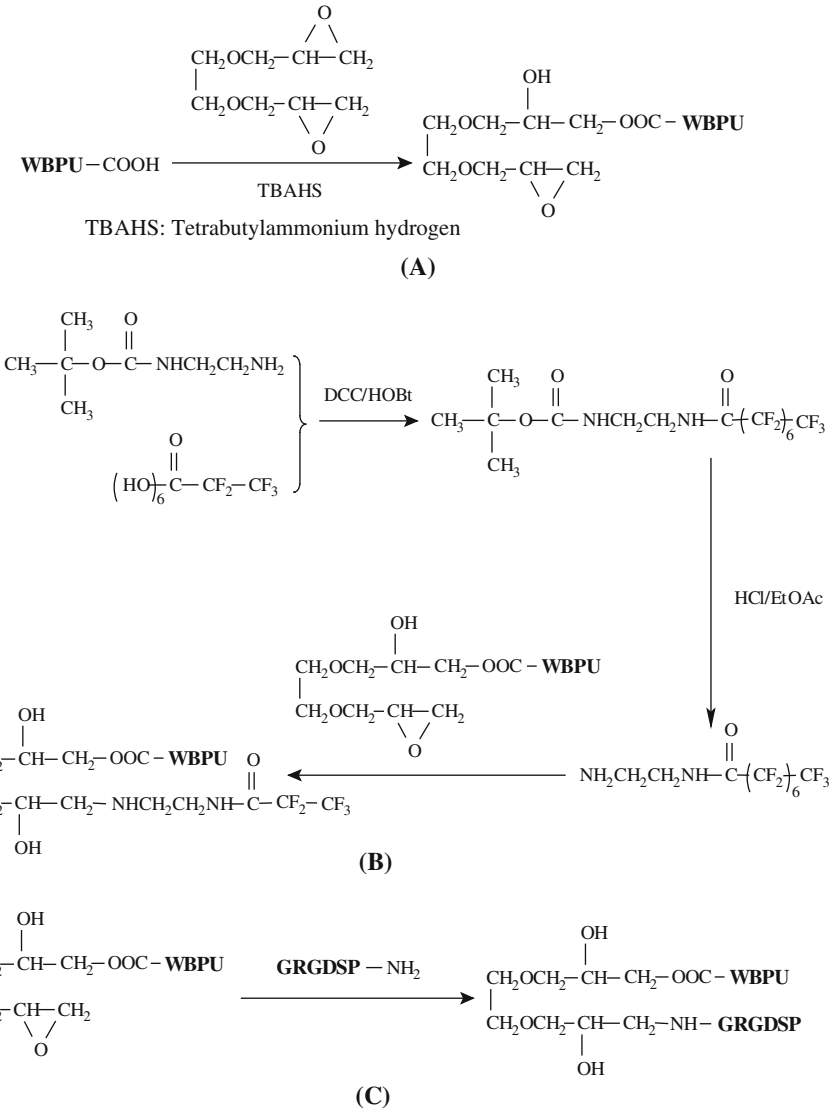

Fig. 1 The scheme of synthesis of GRGDSP grafted WBPU. **a** Synthesis of EX810 modified WBPU; **b** synthesis of EDAPFC and grafting of EDAPFC in WBPU; **c** grafting of GRGDSP in WBPU

in the WBPU. Additionally, in the epoxy stretching region, the stretching of epoxy group characterization bond at 855–840 and 909 cm^{-1} is strengthened in the spectrum of EX810 modified WBPUs, which implies that epoxy groups are available in the EX810 modified WBPUs emulsion. These results demonstrated that EX810 could be grafted on the WBPU with tetrabutylammonium hydrogen sulfate (TBAHS) as the phase transfer catalyst. Moreover, there were epoxy groups in the EX810 modified WBPU sample for subsequent reaction.

3.2 Covalent grafting GRGDSP into EX810 modified WBPU

It is a hard technical challenge for us to characterize the amount of GRGDSP grafted into WBPU. Fortunately, our previous studies have demonstrated that the long fluorocarbon side chains attached to polyurethane chains could largely migrate onto the surfaces of the polymer due to their lower surface free energies. Particularly, the ratio of surface and theoretical bulk fluorine content ($F_{surface}/F_{bulk}$) of long fluorocarbon chains end-capped with CF_3 group is about 10–20 because the surface free energy of CF_3 group is only one-sixth to that of CF_2 group [35, 36]. To confirm the epoxy groups remaining in the EX810 modified WBPU are enough for grafting GRGDSP into WBPU, EDAPFC containing free amino group and end-capped with CF_3 group was applied to react with EX810 modified WBPU, as model reaction. The reaction scheme of EDAPFC with EX810 modified WBPU was shown in Fig. 1b. The surface element composition of EDAPFC modified WBPU before and after purification was investigated by XPS spectra (Fig. 3).

The C 1s peaks of unpurified EDAPFC modified WBPU can be fairly resolved into three component peaks: a hydrocarbon (–C–C–C–) peak at 284.78 eV, an ether

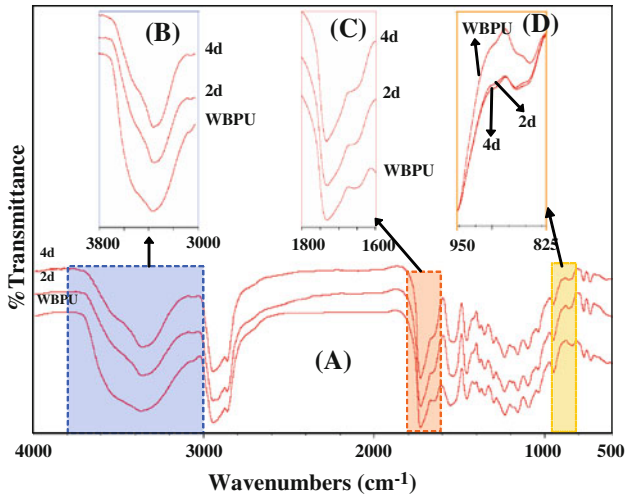

Fig. 2 FTIR spectra of WBPU and EX810 modified WBPU (**a**); amido (N–H) and hydroxyl (O–H) stretching region (**b**); carbonyl (C=O) stretching region of WBPU (**c**) and stretching region of epoxy group in EX810 (**d**). 2d–EX810 modified WBPU after 2 days' reaction; 4d–EX810 modified WBPU after 4 days' reaction

(–C–O–C–) peak at 286.19 eV and a broad peak centered around 290.76 eV overlapped by ester (–COO–) peak in urethane and PCL, $–CF_2–$ and $–CF_3$ peak. However, in the purified product, besides hydrocarbon (–C–C–C–) sub-peak at 284.78 eV and an ether (–C–O–C–) sub-peak at 286.19 eV, the weakened $–CF_2–$, $–CF_3$ sub-peak at 291.65 eV and a sub-peak of ester (–COO–) at 288.49 eV have also been observed due to the free EDAPFC removed from modified WBPU [37], suggesting that the EDAPFC can be covalently bonded to EX810 modified WBPU via epoxy group.

To quantificationally investigate EDAPFC content in modified WBPU, atomic percentage (%) of fluorine, nitrogen, oxygen and carbon on the surfaces of unpurified and purified EDAPFC modified WBPU are calculated from the XPS survey scan spectra, as listed in Table 1. In the purified EDAPFC modified WBPU, the fluorine content on the surface was decreased from 34.81 to 12.06%, while the carbon, oxygen and nitrogen content increased. The extraction of excessive EDAPFC in the modified WBPU emulsion resulted in decrease of EDAPFC content and increase of WBPU component on the surface of the film. These results indicated that EDAPFC was successfully grafted on EX810 modified WBPU, which proved the amino group containing compound could be easily grafted into the EX810 modified WBPU. Therefore, based on the fluorine content on unpurified fluorinate WBPU surface and on purified WBPU surface (Table 1), we can roughly calculate around 34.65% of EDAPFA grafted on the WBPU, indicating that the epoxy group available content in 1 g EX810 modified WBPU is about 0.045 mmol.

Table 1 Atom percentage (%) of oxygen, fluorine, nitrogen and carbon in EDAPFC modified WBPU products before and after purification obtained by XPS analysis

Element	Unpurified product (at)%[a]	Purified product (at)%[a]
O 1s	18.37	19.15
F 1s	34.81	12.06
N 1s	1.88	2.39
C 1s	28.49	48.92

[a] (*at*)% atom percentage (F, O, N and C)

In this work, the GRGDSP peptide can be immobilized on WBPU through EX810 as previously described. To modify WBPU with GRGDSP, different dosage of GRGDSP (0.09, 0.17, 0.85 and 1.70 μmol/g based on the weight of WBPU) reacted with EX810 modified WBPU to obtain a series of modified WBPU containing various

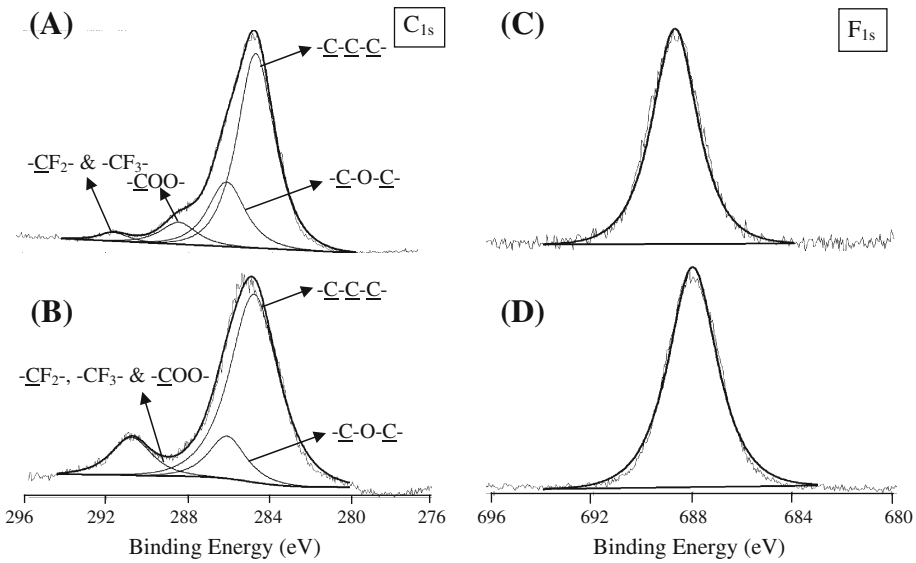

Fig. 3 XPS spectra of EDAPFC modified WBPU before and after purification. **a** Carbon in the purified product; **b** carbon in the unpurified product; **c** fluorine in the purified product; **d** fluorine in the unpurified product

amount of GRGDSP. The feed molar amount of GRGDSP is much less than the molar amount (not less than 0.045 mmol/g) of epoxy group available for grafting, which ensure that all the GRGDSP added can be completely grafted in the WBPU.

3.3 HUVECs cultured on GRGDSP modified WBPU scaffolds

To study the effect of GRGDSP on the cell adhesion on WBPU scaffolds, HUVECs were seeded on the surfaces of GRGDSP modified WBPU scaffolds, WBPU scaffolds, and TCP. Figure 4 shows the endothelial cell adhesion rate on the surfaces of GRGDSP modified WBPU scaffolds, WBPU scaffold and TCP. After culture for 60 min in the DMEM culture medium, 35% of HUVECs adhered to the surface of the TCP, while 67% of the HUVECs adhered to the WBPU scaffold sample and more than 75% of HUVECs adhered to all the GRGDSP modified WBPU scaffolds samples. The statistical analysis results revealed significant difference ($P < 0.05$) between TCP and all WBPU scaffolds.

The higher adhesion rate was observed on WBPU scaffolds than that on the TCP due to the good hydrophilicity of WBPU materials [38], the higher roughness and more space available for HUVECs adhesion. Also, our previous research has demonstrated that the polymer scaffolds with 3-D interconnected pores can evidently enhance the HUVECs adhesion rate [22]. On the other hand, the adhesion rate on the surface of GRGDSP modified WBPU scaffolds is higher than that on the WBPU scaffold, which indicates that the GRGDSP peptide modified WBPU can further promote the adhesion of HUVECs to the surfaces of scaffolds. This result also further confirmed that GRGDSP peptide added has been grafted on the WBPU, since the soluble GRGD had shown inhibition on the cell adhesion and caused low adhesion rate in the previous study [11]. The endothelial cell adhesion rate increased with the increasing of GRGDSP content in the modified WBPU, and the maximum value (~90%) was obtained on the PUR05 containing 0.85 μmol/g GRGDSP. Yet, no significant increase of the adhesion rate can be observed with more GRGDSP (>0.85 μmol/g) grafted into WBPU. This result is coincident with the Sawyer et al.'s [9] research result that high RGD coating concentration does not inhibit or increase cell attachment.

The proliferation rate of HUVECs on the surfaces of GRGDSP modified WBPU scaffolds and WBPU scaffold obtained by MTT assay was shown in Fig. 5. It's observed that the GRGDSP can markedly promote the proliferation of HUVECs on the scaffolds. Thus, the growth of HUVECs on the GRGDSP modified WBPU scaffolds are superior to that on the WBPU scaffold during the whole culture period (6 days) (Fig. 5). With the peptide content less than 0.85 μmol/g in the modified WBPU scaffold, the proliferation rate of HUVECs increased with the increasing of GRGDSP content in the scaffolds. And in the same manner as the endothelial cell adhesion rate discussed above, the highest proliferation rate reached 160% on 0.85 and 1.70 μmol/g GRGDSP grafted into the modified WBPU scaffolds, showing that the further increase of GRGDSP content in the WBPU scaffolds hardly improve the HUVECs proliferation rate on these scaffolds. Similarly, Neff et al. [24] have demonstrated that the optimal density for fibroblasts proliferation at 48 h post incubation was

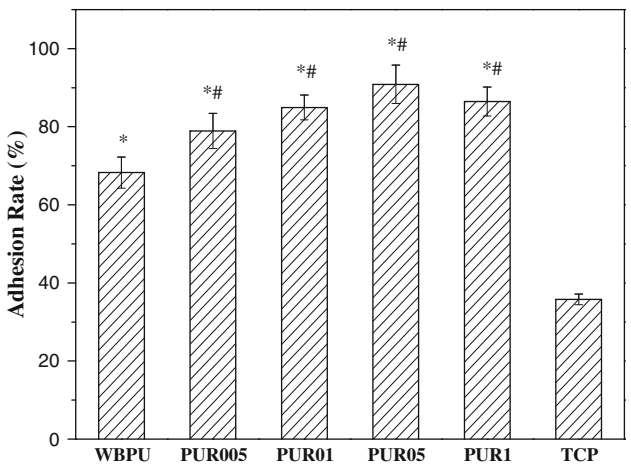

Fig. 4 Adhesion rate (%) of HUVECs on the TCP, WBPU scaffold and GRGDSP modified WBPU scaffolds. Data represents the mean of six samples ± standard deviation. $^{*}P < 0.05$ compared to TCP; $^{\#}P < 0.05$ compared to WBPU

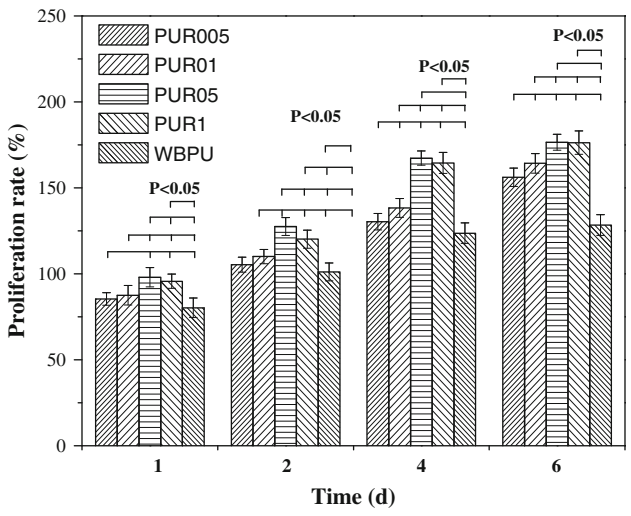

Fig. 5 Proliferation rate of HUVECs after 1, 2, 4, 6 days' culture on the WBPU and GRGDSP modified WBPU scaffolds. Data represents the mean of six samples ± standard deviation

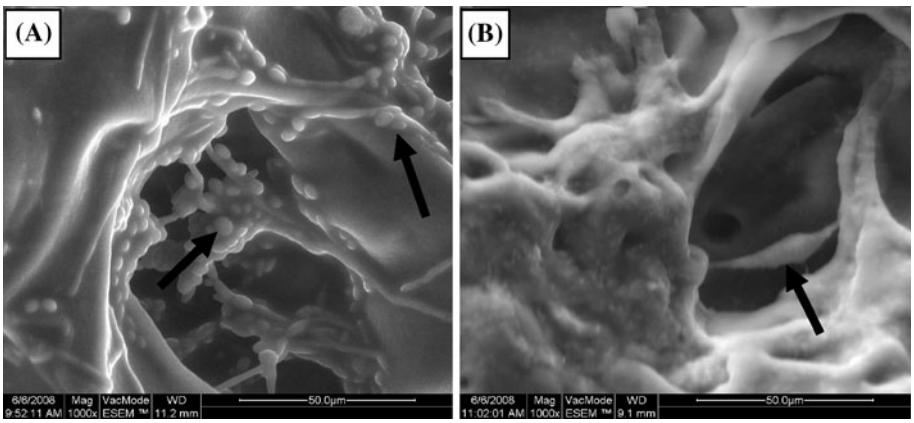

Fig. 6 The ESEM images of HUVECs after cultured for 1 day (**a**) and 6 days (**b**) on scaffold PUR05. The *size bar* corresponds to 50 μm. The HUVECs in the pictures are shown by the *arrows*

about 8000 GRGDSY peptides/μm^2 on PS. Garland et al. [39] also reported that the HUVECs density on RGD modified polymer surfaces increased with increasing RGD concentration up to a bulk peptide concentration of around 5 μmol/g and reached a plateau. Since there are several impact factors, including cellular lines, peptides, techniques used for modification, means of density quantification and so on, affect the results of cellular proliferation rate, the optimal concentration of peptide on the polymer surfaces is different in various culture systems. In this study, the results indicate that the PUR05 with 0.85 μmol/g of GRGDSP is more suitable for endothelial cell proliferation on these modified WBPU scaffolds.

The Fig. 6 shows the ESEM images of endothelial cells after cultured for 1 and 6 days on scaffold PUR05. After 1 days' culture, the endothelial cells were visible and spread out on the surface of the scaffold. After 6 days' culture, the amount of HUVECs notably increased and the cells combined together to form a tissue-like surface on the scaffold. The surface configuration of the scaffold changed and some of the pores were covered by the cells.

4 Conclusions

In this study, a novel convenient method was firstly developed to quantitatively graft GRGDSP on the WBPU through EX810 with two terminal epoxy groups, and a series of 3-D porous scaffolds containing various GRGDSP content were fabricated by freeze-drying to investigate the effect of GRGDSP on the adhesion and proliferation of HUVECs in vitro. The results of HUVECs adhesion and proliferation on the GRGDSP modified WBPU scaffolds confirm that 0.85 μmol/g of GRGDSP grafted into WBPU is more advantageous for the endothelialization of scaffolds. The promotion effect of GRGDSP on the adhesion and proliferation of HUVECs suggests that the modified polyurethane scaffolds may be effective as a tissue regeneration substrate used in soft tissue engineering.

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References

1. Xue L, Greisler HP. Biomaterials in the development and future of vascular grafts. J Vasc Surg. 2003;37(2):472–80.
2. Ratcliffe A. Tissue engineering of vascular grafts. Matrix Biol. 2000;19(4):353–7.
3. Gumusderelioglu M, Turkoglu H. Biomodification of non-woven polyester fabrics by insulin and RGD for use in serum-free cultivation of tissue cells. Biomaterials. 2002;23(19):3927–35.
4. Massia SP, Hubbell JA. Covalent surface immobilization of ARG-GLY-ASP-containing and TYR-ILE-GLY-SER-ARG-containing peptides to obtain well defined cell-adhesive substrates. Anal Biochem. 1990;187(2):292–301.
5. Nakaoka R, Tsuchiya T, Nakamura A. Neural differentiation of midbrain cells on various protein-immobilized polyethylene films. J Biomed Mater Res A. 2003;64A(3):439–46.
6. Olbrich KC, Andersen TT, Blumenstock FA, Bizios R. Surfaces modified with covalently-immobilized adhesive peptides affect fibroblast population motility. Biomaterials. 1996;17(8):759–64.
7. Sharifpoor S, Labow RS, Santerre JP. Synthesis and characterization of degradable polar hydrophobic ionic polyurethane scaffolds for vascular tissue engineering applications. Biomacromolecules. 2009;10(10):2729–39.
8. Conconi MT, Ghezzo F, Dettin M, Urbani L, Grandi C, Guidolin D, et al. Effects on in vitro and in vivo angiogenesis induced by small peptides carrying adhesion sequences. J Pept Sci. 2010; 16(7):349–57.
9. Sawyer AA, Hennessy KM, Bellis SL. Regulation of mesenchymal stem cell attachment and spreading on hydroxyapatite by RGD peptides and adsorbed serum proteins. Biomaterials. 2005; 26(13):1467–75.
10. Loschonsky S, Shroff K, Worz A, Prucker O, Ruhe J, Biesalski M. Surface-attached PDMAA-GRGDSP hybrid polymer monolayers that promote the adhesion of living cells. Biomacromolecules. 2008;9(2):543–52.
11. Massia SP, Hubbell JA. Covalently attached Grgd on polymer surfaces promotes biospecific adhesion of mammalian-cells. Ann NY Acad Sci. 1990;589:261–70.

12. Pierschbacher MD, Ruoslahti E. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. Nature. 1984;309(5963):30–3.
13. Staubli U, Chun D, Lynch G. Time-dependent reversal of long-term potentiation by an integrin antagonist. J Neurosci. 1998; 18(9):3460–9.
14. Patel S, Tsang J, Harbers GM, Healy KE, Li S. Regulation of endothelial cell function by GRGDSP peptide grafted on interpenetrating polymers. J Biomed Mater Res A. 2007;83A(2): 423–33.
15. Lateef SS, Boateng S, Hartman TJ, Crot CA, Russell B, Hanley L. GRGDSP peptide-bound silicone membranes withstand mechanical flexing in vitro and display enhanced fibroblast adhesion. Biomaterials. 2002;23(15):3159–68.
16. Li JH, Ding MM, Fu Q, Tan H, Xie XY, Zhong YP. A novel strategy to graft RGD peptide on biomaterials surfaces for endothelization of small-diameter vascular grafts and tissue engineering blood vessel. J Mater Sci Mater Med. 2008;19(7): 2595–603.
17. Langer R, Vacanti JP. Tissue engineering. Science. 1993; 260(5110):920–6.
18. Demir MM, Yilgor I, Yilgor E, Erman B. Electrospinning of polyurethane fibers. Polymer. 2002;43(11):3303–9.
19. Fujimoto K, Minato M, Miyamoto S, Kaneko T, Kikuchi H, Sakai K, et al. Porous polyurethane tubes as vascular graft. J Appl Biomater. 1993;4(4):347–54.
20. Kowligi RR, Maltzahn WWV, Eberhart RC. Fabrication and characterization of small-diameter vascular prostheses. J Biomed Mater Res. 1988;22(S14):245–56.
21. Saad B, Matter S, Ciardelli G, Uhlschmid GK, Welti M, Neuenschwander P, et al. Interactions of osteoblasts and macrophages with biodegradable and highly porous polyesterurethane foam and its degradation products. J Biomed Mater Res. 1996;32(3): 355–66.
22. Jiang X, Yu F, Wang Z, Li J, Tan H, Ding M, et al. Fabrication and characterization of waterborne biodegradable polyurethanes 3-dimension porous scaffolds for vascular tissue engineering. J Biomater Sci Polym Ed. 2010;21:1637–52.
23. Ito Y, Inoue M, Liu SQ, Imanishi Y. Cell-growth on immobilized cell-growth factor. 6. Enhancement of fibroblast cell-growth by immobilized insulin and or fibronectin. J Biomed Mater Res. 1993;27(7):901–7.
24. Neff JA, Tresco PA, Caldwell KD. Surface modification for controlled studies of cell-ligand interactions. Biomaterials. 1999; 20(23–24):2377–93.
25. Jiang X, Li JH, Ding MM, Tan H, Ling QY, Zhong YP, et al. Synthesis and degradation of nontoxic biodegradable waterborne polyurethanes elastomer with poly(epsilon-caprolactone) and poly(ethylene glycol) as soft segment. Eur Polym J. 2007;43(5): 1838–46.
26. Dabrowska E, Pietka-Ottlik M, Palus J. Bis(2-aminophenyl) diselenide derivatives with amino acids moieties as potential antivirals and antimicrobials. Phosphorus Sulfur Silicon Relat Elem. 2008;183(4):1082–6.
27. Williamson MR, Black R, Kielty C. PCL-PU composite vascular scaffold production for vascular tissue engineering: attachment, proliferation and bioactivity of human vascular endothelial cells. Biomaterials. 2006;27(19):3608–16.
28. She ZD, Jin CR, Huang Z, Zhang BF, Feng QL, Xu YX. Silk fibroin/chitosan scaffold: preparation, characterization, and culture with HepG2 cell. J Mater Sci Mater Med. 2008;19(12): 3545–53.
29. Zhu XH, Lee LY, Jackson JSH, Tong YW, Wang CH. Characterization of porous poly(D,L-lactic-co-glycolic acid) sponges fabricated by supercritical CO_2 gas-foaming method as a scaffold for three-dimensional growth of hep3B cells. Biotechnol Bioeng. 2008;100(5):998–1009.
30. Zhang S, Lv H, Zhang H, Wang B, Xu Y. Waterborne polyurethanes: spectroscopy and stability of emulsions. J Appl Polym Sci. 2006;101(1):597–602.
31. Marcos-Fernandez A, Abraham GA, Valentin JL, San Roman J. Synthesis and characterization of biodegradable non-toxic poly(ester-urethane-urea)s based on poly(epsilon-caprolactone) and amino acid derivatives. Polymer. 2006;47(3):785–98.
32. Srichatrapimuk VW, Cooper SL. Infrared thermal-analysis of polyurethane block polymers. J Macromol Sci Phys. 1978; B15(2):267–311.
33. Coleman MM, Skrovanek DJ, Hu JB, Painter PC. Hydrogen-bonding in polymer blends. 1. FTIR studies of urethane ether blends. Macromolecules. 1988;21(1):59–65.
34. Tang YW, Labow RS, Santerre JP. Enzyme-induced biodegradation of polycarbonate polyurethanes: dependence on hard-segment concentration. J Biomed Mater Res. 2001;56(4):516–28.
35. Tan H, Guo M, Du RN, Xie XY, Li JH, Zhong YP, et al. The effect of fluorinated side chain attached on hard segment on the phase separation and surface topography of polyurethanes. Polymer. 2004;45(5):1647–57.
36. Tan H, Liu J, Li JH, Jiang X, Xie XY, Zhong YP, et al. Synthesis and hemocompatibility of biomembrane mimicing poly(carbonate urethane)s containing fluorinated alkyl phosphatidylcholine side groups. Biomacromolecules. 2006;7(9):2591–9.
37. Diki T, Erich SJF, Ming W, Huinink HP, Thune PC, van Benthem RATM, et al. Fluorine depth profiling by high-resolution 1D magnetic resonance imaging. Polymer. 2007;48(14):4063–7.
38. Hsu SH, Chen WC. Improved cell adhesion by plasma-induced grafting of L-lactide onto polyurethane surface. Biomaterials. 2000;21(4):359–67.
39. Fussell GW, Cooper SL. Endothelial cell adhesion on RGD-containing methacrylate terpolymers. J Biomed Mater Res A. 2004;70A(2):265–73.