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Antimicrobial surfaces grafted random copolymers with REDV peptide beneficial for endothelialization

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Abstract
Polycarbonate urethane (PCU) elastomeric materials have been developed for
vascular prosthesis applications, because of their excellent mechanical and physical properties. However, thrombosis and inflammation often limit their usage as small-diameter vascular grafts. Herein, we focused on the design and functionalization of PCU elastomer with enhanced hemocompatibility, rapid endothelialization and antimicrobial property. Atom transfer radical polymerization (ATRP) technique was utilized to graft random copolymers of N-(2-hydroxypropyl)methacrylamide (HPMA) and eugenyl methacrylate (EgMA) onto PCU surface, and subsequently the cysteine-terminated CREDV peptide sequence was directly linked onto the surface by thiol-ene chick reaction to prepare a series of REDV peptide functionalized surfaces. The surface chemical compositions of the modified surfaces were quantified by X-ray photoelectron spectroscopy (XPS), and the hydrophilicity was evaluated by water contact analysis and water uptake. The surface hemocompatibility was verified by the platelet adhesion assays, and the results demonstrated that platelet adhesion was significantly reduced on the modified surface. More importantly, the functionalized surfaces with high hydrophilicity and cell specifically adhesive REDV peptide could selectively enhance the adhesion and proliferation of human umbilical vein endothelial cells (HUVECs) but suppressed these behaviors of human arterial smooth muscle cells (HASMCs). Moreover, these surfaces showed excellent antibacterial property, which is beneficial from the EgMA moieties of the copolymers. The successful fabrication of multifunctional surfaces with excellent hemocompatibility, rapid endothelialization, and good antimicrobial activity through a feasible route could be an attractive platform for tissue engineering application.
Keywords: hemocompatibility, poly(N-(2-hydroxypropyl)methacrylamide), endothelialization, peptide, surface modification, antimicrobial, polyurethanes

1. Introduction

Cardiovascular diseases have become one of the leading causes for death in the world, and the design and application of cardiovascular implants such as stents and vascular grafts have attracted more attention to treat these diseases.\textsuperscript{1-3} Polyurethane elastomers have been widely applied in cardiovascular implants such as cardiac pacemakers, left ventricular assist device, total artificial heart, small caliber vascular grafts and so on, due to their good biocompatibility, satisfied mechanical and physical properties.\textsuperscript{4, 5} However, the risks of inadequate hemocompatibility, restenosis, thrombosis and infections limit their ultimate success in clinical long-term implantations.\textsuperscript{6-8} When the artificial cardiovascular grafts are implanted, their surfaces are in direct contact with blood and often recognized as the foreigners which are likely to activate the blood coagulation system and cause inflammatory reactions.\textsuperscript{9} The triggered coagulation cascades (namely protein conformation transformation, platelet adhesion and activation, clot formation and dysfunction of fibrinogen) disturb the endothelialization and finally lead to restenosis and thrombosis.\textsuperscript{10}

It has been postulated that the surface wettability and the overall neutral charge are the essential properties for efficient resistance to protein adsorption.\textsuperscript{11, 12} To reduce the interactions of blood components, especially the plasma proteins and platelets, with the
exposed material surface and to improve the hemocompatibility of the polycarbonate urethane (PCU), we modified PCU with poly(ethylene glycol), zwitterionic polymers, silk-fibroin, heparin, and gelatin by blend electrospinning or surface grafting techniques.13-29

Poly[N-(2-hydroxypropyl) methacrylamide] (PHPMA) and its copolymers have been widely applied for the delivery of drugs, genes and adenoviruses because of their good water-solubility, stability in aqueous, biocompatibility, nontoxicity and nonimmunogenicity.30-32 Furthermore, PHPMA can effectively resist protein adhesion in a single plasma protein condition, moreover it shows unprecedented low fouling in undiluted blood plasma. Importantly, PHPMA is comparable or even better than the commonly considered ultra-low fouling polymers.33, 34

Besides hemocompatibility problem, restenosis and thrombosis could also happen on artificial vascular grafts when smooth muscle cells (SMCs) undergo rapid and unregulated adhesion and proliferation over endothelial cells (ECs) and when re-endothelialization is delayed.35 As ECs form the innermost layer of the entire vascular systems in vivo, they play a key role in forming nonthrombogenic surface.6 Rapid endothelialization is beneficial to form a confluent, robust layer of functional ECs, therefore, it provides a potential strategy to succeed in the long-term treatment of cardiovascular diseases.36 Based on these understanding, many methods have been developed to promote endothelialization of biomaterial surfaces.37, 38 The extracellular matrix (ECM) proteins, collagen, fibronectin and tropoelastin have been used to enhance the adhesion and migration of ECs.6, 39, 40 Furthermore, many researchers have
investigated various peptide sequences derived from ECM, such as RGD, REDV, YIGSR, CAG and so on. These peptide sequences have been used to modify cardiovascular implant surfaces to directly interact with cell receptors and promote the adhesion, migration and proliferation of ECs. Among them, RGD is so far the most widely employed peptide sequence for modifying artificial vascular scaffolds. Numerous studies have demonstrated that RGD peptide can enhance the adhesion, spreading and proliferation of ECs on the functionalized surfaces. However, the RGD modified surfaces often show increased platelet adhesion, and the adhered platelets have spread morphology. On the other hand, REDV peptide is a well-known EC selectively adhesive peptide, which can specifically bind to the abundant receptors on ECs. But SMCs rarely have the specific receptors for REDV adhesion. The selective adhesion of ECs over SMCs is attracting more attention to use REDV peptide on the surface modification of artificial vascular grafts and gene carriers. Besides, other investigators have immobilized antibodies, growth factors, proteins and genes to improve the interaction of implants with ECs in order to accelerate endothelialization.

Infections become one of the main causes of patient morbidity and mortality. Especially, infections caused by surface-bound bacterial cells are a major problem in clinical surgeries and implantation operations. What’s more, infections can also trigger the inflammatory reactions and coagulation cascades narrowing the luminal volume and resulting in restenosis and thrombosis. So the antimicrobial and anti-inflammatory should be taken into consideration in designing and preparing artificial vascular
implants. The commonly utilized antimicrobial agents include quaternary ammonium compounds, small molecular weight antibiotics, and plant-derived antimicrobial agents.\textsuperscript{8,63-65} One example is eugenol, which has been popular for its analgesic and anti-inflammatory properties, antimicrobial activity, antipyretic activity and antianaphylactic properties.\textsuperscript{66,67} The acrylate derivate of eugenyl methacrylate (EgMA) can covalently link eugenol to macromolecular chains without decreasing its natural properties.\textsuperscript{68, 69} EgMA based copolymers have been demonstrated to have high inhibition effect for bacterial growth.\textsuperscript{68}

An ideal cardiovascular implant should possess the excellent hemocompatibility, directing the fate of vascular cells, and anti-infective and anti-inflammatory properties.\textsuperscript{54} Here we developed a biomimetic multifunctional surface by grafting hydrophilic HPMA, antimicrobial EgMA, and EC adhesive peptide REDV. Firstly, we used activators regenerated by electron transfer atom transfer radical polymerization (ARGET ATRP) technique to graft the random copolymers of HPMA and EgMA with different molar ratios onto PCU surface. Then cysteine-terminated peptide CREDV was immobilized on the terminated allyl group of EgMA via thiol-ene click reaction. By varying the molar ratios of hydrophilic HPMA and EC selective REDV peptide, the optimized biomimetic multifunctional surface can be obtained with excellent hemocompatibility and antimicrobial properties, which benefits for selective endothelialization on the PCU surface. This modification process is relatively convenient and effective, and can be used to create multifunctional surface for other biomaterials.
2. Materials and methods

2.1. Materials

Eugenol (99%), methacryloyl chloride (98%), 1-aminopropan-2-ol (92%), \(\alpha,\alpha\)-dimethoxy-\(\alpha\)-phenylacetophenone (DMPA, 99%), 5,5’-dithio-bis(2-nitrobenzoic acid) (DTNB, 98%) and ascorbic acid (99%) were obtained from Tianjin heowns Biochemical Technology Co., Ltd. Cysteine-terminated peptide CREDV was purchased from GL Biochem. (Shanghai) Ltd. PCU (Chronoflex C, \(M_n = 110\) kDa) was purchased from Cardio International Incorporated, USA. 2, 2’-Bipyridine (bpy, 99%), Cu(II)Br\(_2\) (99.999%), 2-bromoisobutryl bromide (BIBB, 98%) and ethyl-2-bromoisobutyrate (EBIB, 98%) were purchased from Sigma-Aldrich and used as received. Fluorescein diacetate (FDA) was obtained from Sigma-Aldrich. The orange cell-tracker dye 5-(and 6)-(((4-chloromethyl)-benzoyl)-amino)-tetramethyl-rhodamine (CMTMR) was purchased from Molecular Probes. All other chemicals and solvents were of analytical grade. The solvents were dried and purified by conventional procedures and distilled before use.

2.2. Modification and functionalization of PCU-film

2.2.1. Preparation of PCU-g-poly(HPMA-co-EgMA) surfaces by ARGET ATRP

HPMA and EgMA monomers were synthesized and purified using previously reported methods, respectively.\(^{32,66}\) The PCU-film was prepared and immobilized BIBB initiator according to our previous method.\(^{63,70}\) Firstly, PCU-films was prepared by solution casting and cut into 3 cm \(\times\) 3 cm pieces. Then they were treated with hexamethylene diisocyanate with DBTDL as the catalyst at 50 °C for 90 min. After
immersing in water for 12 h, the amino group terminated PCU-films were prepared. Next, 5 pieces of the amino group terminated PCU-films were put in 50 mL dry n-hexane with pyridine (1 mL) as the catalyst and the mixture was cooled to -5 °C. In the nitrogen atmosphere, 1.5 mL BIBB initiator (in 10 mL n-hexane) was added drop-wise under constant stirring for 1 h. The reaction was further stirred at 25 °C for 6 h. The obtained films were cleaned subsequently with n-hexane, ethanol and pure water for 12 h. Then the films were dried in vacuum at 50 °C for 24 h.

The preparation of PCU-g-poly(HPMA-co-EgMA) surface was carried out by a ARGET ATRP procedure: a total 10 mmol of HPMA and EgMA mixtures with different molar ratios (4/0, 3/1, 2/2, 1/3, 0/4) were dissolved in 15 mL mixed solvent (DMSO: H₂O, v/v = 1/3) containing CuBr₂ (6.7 mg, 0.03 mmol), bpy (31.2 mg, 0.2 mmol) and EBIB (14.7 µL, 0.1 mmol). The mixture was purged with nitrogen for 1 h under continuous stirring. Then a piece of the initiator immobilized PCU-film was dropped into the reaction tube at 30 °C. Afterwards, ascorbic acid (44 mg, 0.25 mM in 1 mL H₂O), which was purged with nitrogen for 10 min, was added drop-wise by degassed syringe at 20-30 min intervals for 4 h. The samples were rinsed and incubated in H₂O to remove the unconnected reagents for 10 h. After dried in vacuum oven overnight, a series of surface modified PCU-films were obtained, namely PCU-g-poly(HPMA4-co-EgMA0), PCU-g-poly(HPMA3-co-EgMA1), PCU-g-poly(HPMA2-co-EgMA2), PCU-g-poly(HPMA1-co-EgMA3) and PCU-g-poly(HPMA0-co-EgMA4), where the numbers indicated HPMA and EgMA molar ratios in feed. They were abbreviated as H, H3E1, H2E2, H1E3 and E surfaces, respectively. Besides, PCU blank was also used as
a control group in the following studies.

2.2.2. Immobilization of REDV peptide by click reaction

Cysteine-terminated peptide CREDV (57 mg, 0.080 mmol), DMPA (2.1 mg, 0.0082 mmol) and a piece of allyl group terminated PCU film were added in 10 mL DMF in a transparent glass plate. The click reaction was carried out at 30 °C in nitrogen atmosphere for 30 min under exposure to a 365 nm UV lamp (300 W) with a distance of 30 cm. After 15 min, the light source was turned off, and the surface was reversed towards light. Then turn on the light for another 15 min to create both side homogenous surfaces. After the completion of reaction, the film was cleaned ultrasonically with phosphate buffered saline (PBS, pH = 7.4) to remove the physi-sorbed peptide, dried in vacuum at 30 °C overnight and stored at -20 °C. REDV peptide functionalized surfaces with ID names of H3E1-REDV, H2E2-REDV, H1E3-REDV and E-REDV were obtained from H3E1, H2E2, H1E3 and E surfaces, respectively.

2.3. Characterizations

2.3.1. Surface chemical composition

The surface chemical compositions of the PCU-g-poly(HPMA-co-EgMA) surfaces (H, H3E1, H2E2, H1E3 and E surfaces) and the PCU blank were studied by PHI-1600 X-ray photoelectron spectroscopy (XPS) with a Mg Kα X-ray source at 2 × 10⁻⁸ Torr. Low-resolution survey scans were performed at 187.85 eV with a step of 0.8 eV and high-resolution survey scans were done at pass energy of 29.35 eV with a step of 0.25 eV. Core-level signals were obtained at a photoelectron take-off angle of 45°, and C 1s spectrum bands were deconvoluted into sub-peaks by means of the XPS PEAK41
2.3.2 Quantification of REDV peptide immobilized

The concentrations of CREDV peptide immobilized on the modified PCU-films were determined by Ellman’s method. Briefly, the CREDV stock solution (100 µL, 0.08 mmol) was diluted in 2.5 mL of 0.5 M PBS (pH = 8.0) and reacted with 2.5 mL of Ellman’s reagent (DTNB, 0.02 mmol in pH = 8.0 PBS). The absorbance of the reaction mixture at 412 nm (A412s) was continuously determined until reaching the constant value with PBS as a control. In parallel, the CREDV stock solution (100 µL, 0.08 mmol) was diluted in 5 mL of 0.5 M PBS (pH = 8.0) but without DTNB, and measured the absorbance to obtain A412r as the reagent blank. In another control, 0.02 mmol DTNB was diluted in 5 mL 0.5 M PBS (pH = 8.0) and its absorbance was determined as A412c. The sulfhydryl group concentration in the CREDV solution was calculated according to the following formula with the standard value $\varepsilon_{412} = 14150 \text{ M}^{-1}\text{cm}^{-1}$:

$$[\text{SH}] = \frac{(A412s-A412r - A412c)}{(\varepsilon_{412} \times 1 \text{ cm})}$$

Where: A412s is the absorbance of CREDV solution in the presence of DTNB, A412r is the absorbance of CREDV solution without DTNB, and A412c is the absorbance of DTNB solution. The results were obtained from three parallel experiments. The immobilized CREDV peptide on surfaces was calculated from the sulfhydryl group concentrations of the solutions before and after the surface modification and the collected physical adsorbed peptide after washing with PBS.

2.3.3 Water contact angle (WCA) measurement

Wettability changes of the modified PCU-films, namely the PCU-g-poly(HPMA-
co-EgMA) surfaces (H, H3E1, H2E2, H1E3, and E surfaces), the REDV peptide functionalized surfaces (H3E1-REDV, H2E2-REDV, H1E3-REDV and E-REDV surfaces), and the PCU blank were characterized by static WCA measurement using the sessile drop technique of 3 µL distilled water at room temperature on a Kruss Easy Drop goniometer (Kruss, Hamburg, Germany) equipped with a digital photoanalyzer. Contact angles were obtained as an average from six measurements. Three specimens of each film were tested at two different locations, and then calculated the average contact angle from them.

2.3.4. Water uptake test

Water uptake (WU) of the PCU-g-poly(HPMA-co-EgMA) surfaces (H, H3E1, H2E2, H1E3, and E surfaces), the REDV peptide functionalized surfaces (H3E1-REDV, H2E2-REDV, H1E3-REDV, and E-REDV), and the PCU blank control were characterized. The pre-weighed samples ($w_0$) were incubated in PBS (pH 7.4) at 37 °C for 24 h. Then after taken out and dried with filter paper, they were re-weighed as $w_1$. The percent of WU was calculated according to the following formula:

$$WU = \frac{w_1 - w_0}{w_0} \times 100\%$$

WU values were calculated from three parallel experiments.

2.4. Platelet adhesion test

The hemocompatibility of the modified PCU-films, namely H, E, H3E1-REDV, H2E2-REDV, H1E3-REDV, and E-REDV, as well as the PCU blank was detected by the means of platelet adhesion experiment. Fresh blood was taken from a healthy human volunteer (Tianjin Hospital of Armed Police Forces, Tianjin, China), and stored in
disposable vacuum blood collection tube (EDTA-2K, Guangzhou Improve Medical Technology Co., Ltd.) for this study. The blood was centrifuged at 1500 rpm, 37 °C for 15 min to prepare platelet-rich plasma (PRP). The PCU-films were placed in a 24-well tissue culture plates and immersed in PBS (pH 7.4) for 12 h, and then incubated in PRP at 37 °C for 2 h. After rinsing with PBS three times to remove any non-adhered platelets, the films were kept in 2.5 wt% glutaraldehyde in PBS to fix the adhered platelets for 30 min. Then the samples were dehydrated with a series of graded alcohol-water solutions (50, 70, 80, 90 and 100%) for 30 min per step, and dried under vacuum at 25 °C. All films were coated with gold for scanning electron microscopy (SEM) examination (S-4800, HI-9053-0003). Platelet attachment was quantified by acquiring 6 random images from three parallel experiments and evaluated by ImageJ software.

2.5. Cell culture

The human umbilical vein endothelial cells (HUVECs) were purchased from Allcells Biomart. (Shanghai) and cultured in high glucose DMEM supplemented with 10% FBS in an incubator (37 °C, 5% CO₂). The cell culture medium for human arterial smooth muscle cells (HASMCs) was RPIM 1640 (Gibco, USA) supplemented with 10% FBS and 100 U/mL penicillin and 100 μg/mL streptomycin. Change with fresh culture medium every other day until the cells have reached 90% confluence. Then the cells were trypsinized to subculture according to the standard techniques. The cell densities for all the experiments were precisely calculated by a hematocytometer and all samples were sterilized with UV radiation for 30 min before the cell studies.

The adhesion, spread and proliferation of HUVECs and HASMCs on different
PCU-films, namely the PCU blank, H, E, H3E1-REDV, H2E2-REDV, H1E3-REDV, and E-REDV surfaces, were investigated by the FDA assay for observation. Briefly, cells were seeded at a density of $1 \times 10^4$ cells per well in 200 μL complete medium on different PCU-films in 96-well tissue culture plates and the medium were replaced every other day. At the pre-determined time points (1-, 3- and 7-day), these samples in the tissue culture plates were analyzed by FDA assay method. Briefly, FDA solution (5 mg/mL in acetone) was added into the medium, and cells were further cultured for 15 min. Then they were washed with D-Hanks for three times. The adhered cells were stained with FDA and photographed by fluorescence microscopy (Fluorescence Olympus U-RFLT50 and microscopy Olympus DP72). Six random pictures were taken from three parallel experiments at 20× magnification. The cell density and surface coverage on different surfaces were calculated by Image-Pro Plus software.

MTT assay was used here to investigate the cell metabolic activity (viability) of HUVECs and HASMCs on the modified PCU-films. 20 μL MTT (5 mg·mL$^{-1}$ in 0.01 M, pH 7.4 PBS) solution was added into each well at the pre-determined time points (3-day). After 4 h incubation at 37 °C, the medium was removed and 150 μL DMSO was added to dissolve the insoluble formazan crystals with 10 min shaking. The absorbance of 490 nm was determined by using a microplate reader (Bio-Rad, IMARK™), and the cell viability as a percentage relative to the untreated control cells was calculated from OD490. All the results were obtained from four parallels.

2.6. Co-culture of HUVECs and HASMCs

The competitive adhesion of HUVECs and HASMCs was performed according to
the protocol. Briefly, after the cells were completely detached from the cell-culture flasks, the cell suspensions were centrifuged at 1000 rpm for 10 min and washed with D-Hanks twice. The cells were re-suspended in fresh media with cell-tracker dyes (FDA and CMTMR) added for 30 min, then HUVECs and HASMCs were stained green and red, respectively. The stained cells were centrifuged, resuspended and adjusted to a concentration of $2 \times 10^5$ cells·mL$^{-1}$. The two types of cells with the same volume of cell suspensions were mixed together forming the finally concentration of $1 \times 10^5$ cells·mL$^{-1}$ for each cell, and seeded onto the modified PCU-films in 24-well tissue culture plates at the density of $1 \times 10^5$ cells per well. The competitive adhesion of the two types of cells was observed by a fluorescent microscope after 2 h incubation (Fluorescence Olympus U-RFLT50, microscopy Olympus DP72). All experiments were performed at least twice and 6 images were taken for each sample at each channel. The Image-Pro Plus software was used to determine the number of cells.

2.7. Surface antibacterial assay

The antibacterial activity of the modified PCU-films, namely H, E, H3E1-REDV, H2E2-REDV, H1E3-REDV, and E-REDV, as well as the PCU blank was qualitatively and quantitatively evaluated in vitro against Bacteria inocula (E. coli). E. coli were precultured overnight in Luria-Bertani (LB) liquid medium and subcultured in a fresh medium until the mid-log phase (OD$_{600} = 0.5$, where OD$_{600}$ is the optical density of a sample measured at a wavelength of 600 nm). The nutrient agar was prepared from LB liquid medium containing 1.5% agar. After autoclaved for 30 min, the agar solution was poured into each of 100 mm diameter Petri dishes for the
In the qualitative method, LB-agar plates were prepared and allowed to dry. Then 1 μL of the diluted strain suspension ($1 \times 10^8$ CFU/mL) were dropped in eight different places on the surface of each agar plate and left to absorb. Afterward, disc samples (6.0 mm diameter) which were first sterilized with UV radiation for 30 min, were placed on top of the bacterial spots. The bacterial spot without films was used as blank control. The plates were incubated at 37 °C for 24 h. Colony formation was visually inspected for each polymer disk. Three replicates were tested for each sample.

In the quantitative method, the sub-cultured inoculum ($E. coli$) was diluted to the final concentration of $1 \times 10^6$ CFU·mL$^{-1}$. 30.0 μL of the bacterial suspension was dropped on the surface of each film and incubated at 25 °C for 3 h. After incubation, 10.0 μL of the inoculum was withdrawn and spread on agar plates, the forming colonies were imaged and counted by Image-Pro Plus software. The remaining bacterial suspension and samples were immersed in 2.0 mL fresh LB medium and further incubated overnight for optical density measurement by OD600 using Cary Eclipse fluorescence spectrometer. Three parallels were tested for each sample.

2.8. Statistical analysis

Data were represented as mean ± SD (standard deviation of the mean value) unless indicated otherwise and compared by one-way ANOVA tests via the Origin 8.0 software (MicroCal, USA). $P$-value less than 0.05 was considered statistically significant.

3. Results
In order to specifically enhance endothelialization on biomaterial surfaces, we prepared surface modified PCU-films with multifunctions including resistance against non-specific adsorption of proteins, specific recognition of ECs as well as antibacterial properties. The PCU-films were firstly grafted by the random copolymers of HPMA and EgMA with different monomer ratios via a very versatile method of living polymerization ARGET ATRP (Scheme 1). Hydrophilic monomer HPMA was applied here to improve the hemocompatibility and resistance against the non-specific adhesion of proteins, platelets and blood cells. Meanwhile, EgMA monomer acted mainly as the antimicrobial moieties and its polymers provided many pendant double bonds for covalently linking cysteine-terminated CREDV peptide by photo-initiated thiol-ene click chemistry. By adjusting the monomer ratios of HPMA and EgMA in feed, we prepared a series of poly(HPMA-co-EgMA) and poly(HPMA-co-EgMA)-REDV modified PCU surfaces with selective adhesion for ECs and antimicrobial activity.

**Scheme 1** Surface grafting of HPMA and EgMA copolymers by ATRP at different monomer ratios, and the terminated allyl groups were functionalized with cysteine-terminated CREDV peptide by photo-initiated thiol-ene click chemistry.

3.1. Surface modification of PCU-films
HPMA and EgMA were grafted onto PCU-films with the monomer ratios of 4:0, 3:1, 2:2, 1:3 and 0:4 by ARGET ATRP. Ascorbic acid was used as a strong reducing agent in the reaction with the aim to quickly convert Cu (II) to Cu (I). Thus low concentration of Cu species was needed.\textsuperscript{77}

**Table 1 XPS chemical compositions of different PCU surfaces**

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<th>Sample ID</th>
<th>Chemical structure</th>
<th>C 1s (%)</th>
<th>O 1s (%)</th>
<th>N 1s (%)</th>
<th>C/N</th>
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<td>PCU blank</td>
<td>PCU</td>
<td>69.4</td>
<td>28.5</td>
<td>2.1</td>
<td>33.0</td>
</tr>
<tr>
<td>H</td>
<td>PCU-g-PHPMA (4:0)</td>
<td>68.5</td>
<td>16.4</td>
<td>15.1</td>
<td>4.5</td>
</tr>
<tr>
<td>H3E1</td>
<td>PCU-g-poly(HPMA3-co-EgMA1) (3:1)</td>
<td>69.0</td>
<td>17.9</td>
<td>12.8</td>
<td>5.4</td>
</tr>
<tr>
<td>H2E2</td>
<td>PCU-g-poly(HPMA2-co-EgMA2) (2:2)</td>
<td>69.3</td>
<td>19</td>
<td>11.2</td>
<td>6.2</td>
</tr>
<tr>
<td>H1E3</td>
<td>PCU-g-poly(HPMA1-co-EgMA3) (1:3)</td>
<td>69.7</td>
<td>19.5</td>
<td>10.7</td>
<td>6.5</td>
</tr>
<tr>
<td>E</td>
<td>PCU-g-PeGMA (0:4)</td>
<td>71.2</td>
<td>18.4</td>
<td>9.5</td>
<td>7.4</td>
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Surface chemical compositions of different PCU-films were analyzed by XPS and the results were summarized in Table 1. The basic peaks of C 1s, O 1s and N 1s indicated C, O and N as major elements. In the C 1s narrow spectrum of PCU blank (Fig. 1), C=H, C=N and O=C=O peaks were found at 284.7 eV, 286.4 eV and 289.7 eV, respectively. After grafting of PHPMA (H), the C 1s narrow scan of H surface showed C=H, C=O and C=N peaks at 284.1 eV, 285.2 eV and 286.4 eV, respectively. The high intensity of C=O peak confirmed the successful grafting of PHPMA onto PCU surface. C=H, C=O, C=N and O=C=O peaks of H2E2 surface were found at 284.7 eV, 285.5 eV, 286.3 eV and 289.4 eV, respectively.
**Fig. 1** The C 1s narrow spectra of PCU blank, H, E and H2E2 surfaces. H, E and H2E2 surfaces were the abbreviation of PCU-g-poly(HPMA4-co-EgMA0), PCU-g-poly(HPMA0-co-EgMA4) and PCU-g-poly(HPMA2-co-EgMA2), respectively.

Considering REDV peptide as an EC adhesive peptide, we conjugated cysteine-terminated CREDV peptide with the pendant double bonds on the modified PCU-films by photo-initiated thiol-ene click chemistry. The immobilized REDV peptide amount on the modified surfaces was determined by Ellman’s method\(^{73}\): the total sulfhydryl group (-SH) concentration of the stock peptide solution was first determined. Then after click reaction with different PCU surfaces, the remaining -SH group concentration was subtracted from the total to obtain the immobilized REDV concentration as well as number of peptide units per unit area (cm\(^{-2}\)) as given in Table 2.
Table 2 Immobilized REDV concentrations on the modified surfaces

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>REDV peptide amount</th>
<th>REDV peptide amount</th>
</tr>
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<tr>
<td></td>
<td>nmol·cm⁻²</td>
<td>Number of REDV·cm⁻² × 10⁻¹⁵</td>
</tr>
<tr>
<td>H3E1-REDV</td>
<td>9.5 ± 0.5</td>
<td>5.7 ± 0.3</td>
</tr>
<tr>
<td>H2E2-REDV</td>
<td>11.4 ± 1.3</td>
<td>6.8 ± 0.9</td>
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<tr>
<td>H1E3-REDV</td>
<td>12.8 ± 0.7</td>
<td>7.7 ± 0.4</td>
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<tr>
<td>E-REDV</td>
<td>15.6 ± 1.2</td>
<td>9.3 ± 0.7</td>
</tr>
</tbody>
</table>

E-REDV surface had relatively high REDV concentration (15.6±1.2 nmol·cm⁻²), which was because of high EgMA content in the grafted copolymer as the bridge for the immobilization of REDV peptide. The more EgMA molecules on the surface, the relatively more reaction points for REDV peptide to be conjugated.

3.3. Hydrophilicity of modified PCU-films

The surface hydrophilicity of modified PCU-films was investigated by WCA and WU measurements. It’s generally considered that a relatively lower WCA and a higher WU indicate better hydrophilicity of materials. Among the modified surfaces and control surface, PCU blank showed the highest hydrophobicity with a high WCA of about 104° (Fig. 2). When the surface was modified by grafting homopolymer PHPMA as well as its copolymers, the surface hydrophilicity was increased significantly. Especially, the H surface, having many hydrophilic PHPMA brushes, had the lowest WCA, while EgMA moieties reduced the surface hydrophilicity compared with the H
control, because of the presence of relatively hydrophobic eugenyl residues. The REDV peptide functionalized PCU-films showed the same tendency, furthermore their hydrophilicity was relatively higher than their parent surfaces, respectively. This is due to linking hydrophilic REDV peptide to EgMA moieties in poly(HPMA-co-EgMA) copolymers on surfaces.

Fig. 2 WCA of different modified PCU-films. H, H3E1, H2E2, H1E3 and E surfaces were the abbreviation of PCU-g-poly(HPMA4-co-EgMA0), PCU-g-poly(HPMA3-co-EgMA1), PCU-g-poly(HPMA2-co-EgMA2), PCU-g-poly(HPMA1-co-EgMA3), and PCU-g-poly(HPMA0-co-EgMA4), respectively. H3E1-REDV, H2E2-REDV, H1E3-REDV and E-REDV surfaces were peptide functionalized surfaces PCU-g-poly(HPMA3-co-EgMA1)-REDV, PCU-g-poly(HPMA2-co-EgMA2)-REDV, PCU-g-poly(HPMA1-co-EgMA3)-REDV, and PCU-g-poly(HPMA0-co-EgMA4)-REDV, respectively. PCU blank was used as the control group. (Error bars represent mean ± SD)
The WU of different modified PCU-films was evaluated after 24 h incubation in PBS (pH 7.4) at physiological temperature (37 °C) (Fig. 3). The WU values of H (WU = 10.2%) and H3E1 (9.8%) surfaces were relatively high because they had high content of hydrophilic HPMA on the modified surfaces. After peptide functionalization, the modified surfaces showed even higher WU values, for example, H3E1-REDV adsorbed 16.1% water. H2E2-REDV significantly increased its surface hydrophilicity with a 15.5% WU, while before peptide functionalization, i.e., H2E2, had a low WU of 4.58%. The water adsorption capacity increased with the increasing of hydrophilic peptide content on the surfaces. On the other hand, relative hydrophobic EgMA moieties were responsible for the decrease of WU from H3E1 to H1E3, and from H3E1-REDV to H1E3-REDV surfaces. This reasonable tendency was associated to the increase of the hydrophobicity with this monomer because it contains a hydrophobic aromatic ring.68
Fig. 3 WU (%) of different modified PCU-films after incubated at 37 °C for 24 h. H, H3E1, H2E2, H1E3 and E surfaces were the abbreviation of PCU-g-poly(HPMA4-co-EgMA0), PCU-g-poly(HPMA3-co-EgMA1), PCU-g-poly(HPMA2-co-EgMA2), PCU-g-poly(HPMA1-co-EgMA3) and PCU-g-poly(HPMA0-co-EgMA4), respectively. H3E1-REDV, H2E2-REDV, H1E3-REDV and E-REDV surfaces were peptide functionalized surfaces of PCU-g-poly(HPMA3-co-EgMA1)-REDV, PCU-g-poly(HPMA2-co-EgMA2)-REDV, PCU-g-poly(HPMA1-co-EgMA3)-REDV, and PCU-g-poly(HPMA0-co-EgMA4)-REDV, respectively. PCU blank was used as the control group. (Error bars represent mean ± SD)

3.4. *In vitro* hemocompatibility test

As platelet adhesion plays an important role in the formation of thrombosis and inflammation, the hemocompatibility of different modified PCU-films was assessed via platelet adhesion assay. If the interactions between platelets and biomaterial surfaces
are very weak, few platelets will be adhered and the adhered platelets may maintain their discoidal shape with small spreading area. Conversely, if the interactions are too strong, platelets will be activated with spreading and aggregated morphologies.

The platelet adhesion on different modified PCU-films was investigated by SEM and the adhered platelets were quantified by manual counting and summarized in Fig. 4. We could see clearly large quantities of platelets adhered on the PCU blank, and most of the adhered platelets gathered and deformed, which indicated that these platelets were activated. In contrast, the H surface adhered platelets with the lowest number and the adhered platelets kept the discoidal shape. The REDV peptide functionalized surfaces showed significantly lower platelet adhesion than the PCU control. Furthermore, the number of adhered platelets on the modified surfaces decreased with increasing of hydrophilic HPMA contents on the surfaces. These results demonstrated that surface hydrophilicity plays an important role in inhibiting platelet adhesion and the modification lead to the improvement of surface hemocompatibility.
Fig. 4 Platelet adhesion on different PCU-films after in contact with PRP for 2 h, the SEM micrographs (A) and the statistical result (B), where the H, E, H3E1-REDV, H2E2-REDV, H1E3-REDV, and E-REDV were the sample ID of PCU-g-poly(HPMA4-co-EgMA0), PCU-g-poly(HPMA0-co-EgMA4), PCU-g-
poly(HPMA3-co-EgMA1)-REDV, PCU-g-poly(HPMA2-co-EgMA2)-REDV, PCU-g-poly(HPMA1-co-EgMA3)-REDV, and PCU-g-poly(HPMA0-co-EgMA4)-REDV surfaces. PCU blank was used as the control group. The REDV peptide functionalized surfaces showed relatively lower platelet adhesion than the PCU blank. (Error bars represent mean ± SD)

From the above observations, it can be concluded that PHPMA modified PCU-film could significantly decrease platelet adhesion, but too strong repelling ability would also negatively affect the adhesion of cells on biomaterial surfaces. So we prepared a series of poly(HPMA-co-EgMA) modified PCU-films with different ratios of HPMA and REDV in the copolymers. In the following cell experiments, we will investigate the effects of these modified surfaces on EC adhesion and growth to find the optimum ratio of PHPMA and REDV.

3.5. Adhesion, spreading and proliferation of HUVECs and HASMCs on modified surfaces

The surface chemical structures and hydrophilic properties significantly influence the adhesion, spreading and proliferation of ECs and further affect endothelialization. The preferential cell behaviors of HUVECs and HUASMCs on different modified PCU-films were evaluated by FDA fluorescence staining method at different culture time (1-, 3- and 7-day). Fluorescence micrographs, cell density results and surface coverage of HUVECs on different modified surfaces were shown in Fig. 5. The average number of cells was converted to cell density. The PCU blank, H and E surfaces were used as controls.
It can be found that HUVEC numbers on all surfaces increased along with the culture time clearly. At the same culture time, H2E2-REDV and H1E3-REDV surfaces adhered more cells than other surfaces. These moderate hydrophilic surfaces were beneficial for cell growth, furthermore, REDV peptides were prone to adhere HUVECs. Conversely, the H surface had the lowest number of HUVECs owing to its high surface hydrophilicity. On the first day, HUVEC adhesion on the REDV peptide modified surfaces was enhanced except for H3E1-REDV surface. This is also due to the antifouling of HPMA and a relatively low REDV peptide content on the modified surface. After 3-day culture, the number of HUVECs appeared obviously different, which could further be confirmed from the statistical results (Fig. 5B). After 7-day culture, we could find the significant increase of HUVEC number on all REDV modified surfaces, especially on H2E2-REDV and H1E3-REDV surfaces. Furthermore, the H1E3-REDV surface had the highest cell coverage area of 39.8% which could be attributed to the interplay of the wettability of HPMA and the cell adhesion of REDV peptide (Fig. 5C). These results confirmed that the REDV peptide modified surfaces were highly beneficial for HUVEC adhesion and proliferation.
**Fig. 5** Adhesion and proliferation of HUVECs on different PCU-films for 1-, 3- and 7-day culture. Fluorescence micrographs (A), cell density results (B), and surface coverage (%) (C) varied with different PCU-films and culture time, where the H, E, H3E1-REDV, H2E2-REDV, H1E3-REDV and E-REDV surfaces were the sample ID of PCU-g-poly(HPMA4-co-EgMA0), PCU-g-poly(HPMA0-co-EgMA4), PCU-g-poly(HPMA3-co-EgMA1)-REDV, PCU-g-poly(HPMA2-co-EgMA2)-REDV, PCU-g-poly(HPMA1-co-EgMA3)-REDV, and PCU-g-poly(HPMA0-co-EgMA4)-REDV, respectively. The statistic result demonstrated that H2E2-REDV and H1E3-REDV surfaces showed relatively higher cell density and cell coverage for HUVECs than PCU blank control, H and E groups. (Error bars represent mean ± SD)

The results of HASMCs on different PCU-films were shown in Fig. 6. On the first day of culture, PCU blank and E control surfaces were found to have relatively more cells than other surfaces. For REDV peptide modified surfaces, HASMC number on the surfaces only slightly increased with the decrease of HPMA moiety. HASMC adhesion on these surfaces mainly depends on the surface hydrophilic and hydrophobic balance, but not on REDV peptide. Because REDV peptide can only specific adhere ECs, but it is not beneficial for HASMC adhesion and growth. In order to understand the effects of REDV modified surfaces on the proliferation of HUVECs and HASMCs,
we calculated the cell density curves and shown in Fig. 7 (A)-(E). For PCU blank, the growth tendency of HUVECs and HASMCs was similar, both cells showed an increased tendency in cell number during the culture period. While, for REDV modified surfaces, HUVECs proliferated more rapidly but HASMCs increased with a mild growth. The cell number on the first day culture confirmed the specifically selective REDV peptide for HUVEC adhesion, because the number of HUVECs on the REDV modified surfaces was much higher than that of HASMCs. After 7-day culture, the proliferation of HUVECs and HASMCs on different PCU-films was significantly different. The ratios of HUVECs to HASMCs on PCU blank, H3E1-REDV, H2E2-REDV, H1E3-REDV and E-REDV surfaces were 0.91, 1.70, 2.19, 2.21 and 1.37, respectively (Fig. 7(F)). The density of HUVECs on these REDV modified surfaces increased faster than that of HASMCs, especially for H2E2-REDV and H1E3-REDV surfaces. All of these results further confirmed the adhesion selectivity of REDV peptide for HUVECs. The copolymer and REDV peptide functionalized surfaces showed the enhanced effect on the proliferation of HUVECs.
Fig. 6 Adhesion and proliferation of HASMCs on different PCU-films for 1-, 3- and 7-day culture. Fluorescence micrographs (A), cell density results (B), and surface coverage (%) (C) varied with different PCU-films and culture time, where the H, E, H3E1-REDV, H2E2-REDV, H1E3-REDV and E-REDV surfaces were the sample ID of PCU-g-poly(HPMA4-co-EgMA0), PCU-g-poly(HPMA0-co-EgMA4), PCU-g-poly(HPMA3-co-EgMA1)-REDV, PCU-g-poly(HPMA2-co-EgMA2)-REDV, PCU-g-poly(HPMA1-co-EgMA3)-REDV, and PCU-g-poly(HPMA0-co-EgMA4)-REDV, respectively. The REDV peptide functionalized surfaces showed relatively lower cell density and cell coverage for HASMCs than the PCU blank group. (Error bars represent mean ± SD)
Fig. 7 Cell density curves of HUVECs and HASMCs on different PCU-films after 1-, 3-, 7-day culture (A)-(E) and the density ratios of HUVECs/HASMCs on the 7th day (F), where the H3E1-REDV, H2E2-REDV, H1E3-REDV, and E-REDV were the sample ID of PCU-g-poly(HPMA3-co-EgMA1)-REDV, PCU-g-poly(HPMA2-co-EgMA2)-REDV, PCU-g-poly(HPMA1-co-EgMA3)-REDV, and PCU-g-poly(HPMA0-co-EgMA4)-REDV, respectively. PCU blank was used as the control group. REDV peptide functionalized surfaces, especially H2E2-REDV and H1E3-REDV surfaces, were more favorable for the growth and proliferation of HUVECs than HASMCs while PCU blank control showed the opposite results. (Error bars represent mean ± SD)

The morphologies of HUVECs and HASMCs on the modified PCU-films after 3-day culture were shown in supplementary (Fig. S1). Both HUVECs and HASMCs
appeared to spread, flatten and grow on PCU blank. While, on the REDV modified surfaces, HUVECs formed bridges, spread well, appeared multi-angular and robust. However, HASMCs did not spread well and shrank on these surfaces, which could also be verified by the surface coverage (HUVECs 32.8% (Fig. 5(C)) v.s. HASMCs 26.1% (Fig. 6(C)) on H2E2-REDV surface). These results demonstrated that the REDV modified surfaces, especially H2E2-REDV and H1E3-REDV, selectively promoted the adhesion, spreading and proliferation of HUVECs.

For the rapid endothelialization, biomaterial surfaces should have good adhesion, spreading and proliferation abilities for ECs, furthermore the adhered ECs should also maintain their viability and biofunctions. Here we used MTT assay to assess the cell viability of HUVECs and HASMCs after 3-day culture. The cell viability data of the blank control was defined as 100%. As shown in Fig. 8, all surfaces except the H control showed high cell viabilities for both HUVECs and HASMCs. This was because of the high hydrophilicity of HPMA on H surface. For REDV modified PCU, the viability of these two kinds of cells was similar except that the H2E2-REDV surface exhibited statistically difference between HUVECs and HASMCs, showing that H2E2-REDV surface could selectively promote HUVEC growth.
Fig. 8 HUVECs and HASMCs relative viability after 3-day culture, where the H, E, H3E1-REDV, H2E2-REDV, H1E3-REDV and E-REDV surfaces were the sample ID of PCU-g-poly(HPMA4-co-EgMA0), PCU-g-poly(HPMA0-co-EgMA4), PCU-g-poly(HPMA3-co-EgMA1)-REDV, PCU-g-poly(HPMA2-co-EgMA2)-REDV, PCU-g-poly(HPMA1-co-EgMA3)-REDV, and PCU-g-poly(HPMA0-co-EgMA4)-REDV, respectively. H2E2-REDV, H1E3-REDV and E-REDV groups maintained both cells growth above 70%. However, H and H3E1-REDV groups exhibited poor cell relative viability (less than 70%) because of the high hydrophilicity of HPMA moieties. Only the H2E2-REDV surface exhibited statistically difference between HUVECs and HASMCs, further indicating this surface beneficial for selective enhance HUVEC growth. (Error bars represent mean ± SD)

3.6. Co-culture of HUVECs and HASMCs

In order to investigate the effect of REDV peptide and HPMA on the competitive adhesion of HUVECs and HASMCs, we performed the co-culture of HUVECs and HASMCs in the presence of modified PCU-films. The two kinds of cells were pre-
stained with different cell-tracker dyes prior to their mixture, i.e. HUVECs by FDA with green fluorescence, and HASMCs by CMTMR with red fluorescence. The initial cell adhesion on different PCU-films was imaged after 2 h co-culture. The fluorescence micrographs were shown in Fig. 9(A). Five different positions from three parallel experiments were imaged for each sample and two different channels were taken for the same position. The cell number was calculated and converted to the amount of these two kinds of cells on different PCU-films (Fig. 9(B)). The ratios of HUVECs to HASMCs adhered on PCU blank, H, E, H3E1-REDV, H2E2-REDV, H1E3-REDV, and E-REDV surfaces were 0.94, 0.68, 0.95, 1.76, 3.11, 1.72 and 1.25, respectively (Fig. 9(C)). The PCU blank had approximately similar amount of HUVECs and HASMCs, while both kinds of cells decreased significantly on the H surface because of its high hydrophilicity. The REDV modified surfaces attached more HUVECs than HASMCs, and the tendency was significant especially for H2E2-REDV surface with 3.11 (ratio of HUVECs to HASMCs). The REDV modified surfaces enhanced the initial competitive adhesion of HUVECs over HASMCs, preferential for rapid endothelialization.
**Fig. 9** Co-culture of HUVECs and HASMCs on REDV functionalized PCU-films after 2h incubation. Fluorescence micrographs (A) were taken with a Fluorescence Olympus U-RFLT50 and microscopy Olympus DP72. HUVECs were stained green and HASMCs were red. Cell adhesion numbers (B) were obtained by manually counting and the ratio of HUVEC/HASMC density (C) was calculated from (B), where H, E, H3E1-REDV, H2E2-REDV, H1E3-REDV and E-REDV surfaces were PCU-g-poly(HPMA4-co-EgMA0), PCU-g-poly(HPMA0-co-EgMA4), PCU-g-poly(HPMA3-co-EgMA1)-REDV, PCU-g-poly(HPMA2-co-EgMA2)-REDV, PCU-g-poly(HPMA1-co-EgMA3)-REDV, and PCU-g-poly(HPMA0-co-EgMA4)-REDV, respectively. The REDV peptide functionalized surfaces, especially H2E2-REDV, showed higher adhesion for HUVECs than HASMCs. While the PCU blank, H and E surfaces showed opposite results. (Error bars represent mean ± SD)

### 3.7. Antimicrobial performance of the modified PCU-films

The antimicrobial activity of different modified PCU-films was qualitatively and quantitatively studied against *E. coli* by the direct contact method. The qualitative results were shown in Fig. 10. When the samples were in direct contact with bacteria, only the samples containing EgMA were capable of inhibiting the bacterial growth, or
at least delaying colony formation on them. The inhibitory effect was enhanced with the increasing of EgMA moieties of poly(HPMA-co-EgMA) on the surfaces as could clearly observed from H3E1-REDV (obvious colony), H2E2-REDV (almost no colony), H1E3-REDV (almost no colony), and E-REDV (almost no colony). It is due to the hydrophobic property together with the 4-allyl group of EgMA enabling these surfaces with the inhibitory capacity of colony growing.\textsuperscript{68} In contrast, for the PCU blank and the H control, the bacterial growth inhibition was not observed and the colonies were formed both beneath and around them.

**Fig. 10** Qualitative evaluation of antimicrobial effects of different PCU-films by *E. coli* colony formation tests after 24 h incubation at 37 °C, where H, E, H3E1-REDV, H2E2-REDV, H1E3-REDV and E-REDV surfaces were PCU-g-poly(HPMA4-co-EgMA0), PCU-g-poly(HPMA0-co-EgMA4), PCU-g-poly(HPMA3-co-EgMA1)-REDV, PCU-g-poly(HPMA2-co-EgMA2)-REDV, PCU-g-poly(HPMA1-co-EgMA3)-REDV, and PCU-g-poly(HPMA0-co-EgMA4)-REDV, respectively. The PCU blank, H and E
surfaces, as well as blank group without films were used as the controls.

To quantitatively evaluate the antimicrobial performance of different PCU-films, the samples were incubated with $3 \times 10^4$ CFU of *E. coli* for 3 h. Then 10.0 µL of the inoculum was withdrawn and spread on agar plates, the forming colonies were counted and the CFU count results were shown in Fig. 11(A). Significantly low colony number was observed on the agar plate for culture on E, H2E2-REDV, H1E3-REDV and E-REDV surfaces. While bacterial cells grew quickly on the PCU blank and H control surfaces to have colony count of $2.9 \times 10^8$ and $2.8 \times 10^8$ CFU/mL, respectively. This indicated that EgMA provided the modified PCU-films with antibacterial property. When the samples were immersed in bacterial suspension for 12 h, the optical density (OD600) of the suspension was used to characterize the bacterial concentration. PCU blank and H control could not inhibit bacteria growth (Fig. 11(B)), but E, H2E2-REDV, H1E3-REDV and E-REDV surfaces showed high antibacterial activity with low OD600 values. These results confirm that the antimicrobial activity of EgMA is retained after immobilization. The H2E2-REDV, H1E3-REDV, and E-REDV surfaces possessed good antimicrobial property.
Fig. 11 Antimicrobial activities of different PCU-films against *E. coli* after 3 h incubation by CFU counting (A) after incubating of bacterial suspension with different PCU-films and optical density (OD600) measurements of LB medium after overnight incubation with different PCU-films containing remaining bacterial suspension after performing CFU counting (B). H, E, H3E1-REDV, H2E2-REDV, H1E3-REDV and E-REDV surfaces were PCU-g-poly(HPMA4-co-EgMA0), PCU-g-poly(HPMA0-co-EgMA4), PCU-g-poly(HPMA3-co-EgMA1)-REDV, PCU-g-poly(HPMA2-co-EgMA2)-REDV, PCU-g-poly(HPMA1-co-EgMA3)-REDV, and PCU-g-poly(HPMA0-co-EgMA4)-REDV, respectively. H2E2-REDV, H1E3-REDV and E-REDV surfaces demonstrated good antimicrobial activity compared with E surface. (Error bars represent mean ± SD)

4. Discussion

Nowadays vascular stents and grafts have been usually applied for the treatment of coronary and peripheral artery diseases. However, restenosis, thrombosis, and bacterial infection often cause implant failure. Therefore, in order to design and develop an ideal vascular implant, mechanical and biological factors have become major concerns. First of all, excellent flexibility and strength of biomaterials are the basic requirements for vascular grafts. They must tolerate the force imposed by the blood flow, avoid the recoil and support vascular remodeling. Secondly, biomaterials should possess superior hemocompatibility and antithrombosis. When the implant surface is in direct contact with blood, plasma proteins are prone to be adsorbed on the exposed surface. The adsorption and denaturation of some plasma proteins can cause the subsequent adhesion
and activation of platelets, which trigger the coagulation cascade and inflammatory reactions, finally resulting in restenosis and thrombosis. Thirdly, the surface should be beneficial for endothelialization. The entire natural vascular system is protected by a non-thrombogenic lining of ECs, so EC layer plays an important role in keeping high patency of artificial vascular grafts. Last but not the least, antibacterial properties should also be taken into consideration because infections caused by the adhered bacterial cells during the operation are a major problem in clinical application.

PCU materials have an elastic modulus of 1.3 MPa which is four times stronger than the coronary artery. The compliance value of PCU was tested to be about 8.1 percent per mmHg $\times 10^{-2}$, similar to that of the artery (8.0 and 1.2 percent per mmHg $\times 10^{-2}$, respectively). For the burst pressure, porous polyurethane grafts was found to be 1850-2050 mmHg, much greater than other grafts. Owing to their excellent mechanical and physical properties, PCU materials have been widely applied for vascular application and relevant fields. However, the long-term application is still limited due to insufficient hemocompatibility, potential thrombosis and restenosis. Surface modification, as one of the simple and convenient method to improve surface properties, was used here to overcome these severe problems. First of all, the copolymers of hydrophilic HPMA and antimicrobial EgMA were used to modify PCU surfaces with the aim to improve both hemocompatibility and antibacterial properties. As a robust method, ARGET ATRP technique was applied here to prepare the stable modified surface with controllable properties. Then, photo-initiated thiol-ene click chemistry was used to conjugate cysteine-terminated CREDV peptide to the pendant
double bonds on poly(HPMA-co-EgMA) modified PCU films to improve the selective adhesion and proliferation of ECs. The thiol-ene click reaction also provided a convenient and stable method for conjugation of biological moieties. The hydrophilic poly(HPMA-co-EgMA) copolymers could increase the surface wettability which was demonstrated by the decreased WCA (Fig. 2). Surface wettability is related to the fouling resistance. H surface has highly hydrophilic PHPMA, which can inhibit the platelet adhesion confirmed by the lowest number of adhered platelets. Although high wettability can effectively prevent protein adsorption on biomaterial surfaces, it can also lead to decreased cell adhesion. For example, both ECs and SMCs cannot adhere and proliferate well on H surface. We adjusted the surface hydrophilicity by grafting poly(HPMA-co-EgMA) copolymers with different ratios of HPMA and EgMA. The moderate hydrophilicity is beneficial for cell adhesion and migration. Furthermore, cell adhesive peptides have been usually used to modify biomaterial surfaces in order to improve cell adhesion. Among cell adhesive peptides, REDV peptide can selectively adhere ECs. Thus we used CREDV peptide to modify the surfaces for improving EC adhesion and hemocompatibility. Besides, the surface modified PCU maintained its intrinsic mechanical properties, such as the high elastic modulus and burst pressure.

In order to improve the long-term patency of artificial vascular grafts, endothelialization on graft surface is often enhanced by various approaches. The endothelialization processes on artificial vascular grafts involve many complex processes, such as EC adhesion, migration and proliferation, which are regulated by numerous signals. The interactions between ECs and SMCs in blood vessel wall may
control the growth and function of blood vessels. The adhesion, spread, migration and proliferation of ECs must compete and interact with other cells. The growth and proliferation of various cells may interfere with ECs, which could further affect the rapid endothelialization, especially SMCs. To mimic the properties of native tissues for promoting endothelialization, Ji et al.\textsuperscript{53, 74, 80} have modified stent with hydrophilic components, such as poly(ethylene glycol), phosphorylcholine or polycarboxybetaine and EC specific adhesion peptide REDV. The functionalized stents can effectively enhance the competitive growth of ECs over SMCs. In our study, the adhesion and proliferation of HUVECs and HASMCs were investigated in solo-culture system for 1-, 3- and 7-day and co-culture system for 2 h to study the effects of HPMA and REDV peptide on endothelialization (especially initial cell adhesion). Our results have demonstrated that poly(HPMA-co-EgMA)-REDV modified surfaces enhanced the adhesion and proliferation of HUVECs but suppressed the behaviors of HASMCs. Especially for poly(HPMA-co-EgMA)-REDV with HPMA and EgMA molar ratio of 1:3, the ratio of HUVECs to HASMCs could reach 2.21 on the 7th day in solo-culture, while initial adhesion of HUVECs is also relatively higher after 2 h co-culture. REDV peptide can specifically bind to $\alpha_4\beta_1$ integrin, which is abundant on ECs whereas scarce on SMCs. It specifically adsorbs ECs rather than SMCs. The selective adhesion and proliferation of HUVECs is beneficial from synergetic effects of hydrophilic HPMA and REDV peptide on the surfaces.

For addressing bacterial infection after operation, one strategy is to prepare the ultra-low fouling surfaces of implants with the aim to effectively prevent bacterial
adhesion, and another involves using active moieties to kill the attached bacterial cells.\textsuperscript{8} PHPMA modified biomaterial surfaces show ultra-low fouling property, especially, they can effectively prevent protein adsorption when contacted with blood. But PHPMA is powerless to kill bacteria once they are adhered on the implant surface.\textsuperscript{34} Recently, the antimicrobial and anti-inflammatory properties of eugenol have brought much attention.\textsuperscript{66, 67} The acrylate derivate of EgMA and its homopolymer or copolymers also showed superior antibacterial properties. In this study, we combined these two strategies together by using the copolymers of poly(HPMA-co-EgMA) to modify PCU surface. HPMA as the antifouling moiety and EgMA as the antimicrobial moiety can provide the surface with both antifouling and antimicrobial properties. The antibacterial assay results have demonstrated that the EgMA immobilized surfaces have effectively antibacterial activity, consistent with the previous reports.\textsuperscript{68, 69} More importantly, poly(HPMA-co-EgMA)-REDV modified surfaces possess multifunctions, such as excellent hemocompatibility, specific adhesion and proliferation of ECs, as well as antibacterial properties. These multifunctions are beneficial for vascular graft biomaterials.

\section*{5. Conclusion}
We have developed a multifunctional surface with hydrophilic HPMA, antibacterial EgMA and EC adhesive REDV peptide on PCU to selectively promote endothelialization. Firstly, different monomer ratios of HPMA and EgMA were grafted onto PCU-film by ATRP technique to mediate the surface hydrophilicity. Secondly, cysteine-terminated CREDV peptide was conjugated with the pendant double bonds on
the modified PCU-film by photo-initiated thiol-ene *click-chemistry*. By varying HPMA and EgMA ratios in feed, we found that the high amount of HPMA (such as H, H3E1, H2E2 and their corresponding REDV peptide modified surfaces) could effectively enhance hemocompatibility. REDV peptide enhanced the competitive growth of HUVECs over HASMCs on the hydrophilic surfaces. Furthermore, antibacterial EgMA could inhibit the bacterial induced infection. The successful fabrication of multifunctional biomaterial surfaces with excellent hemocompatibility, endothelialization, as well as effective antimicrobial activity, through a feasible route could be an attractive platform for tissue engineering application.

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**References**

Multifunctional surfaces have been created by surface modification and click reaction. These surfaces possess excellent hemocompatibility and endothelialization, as well as effective antimicrobial activities.