Surface Modification of Polycarbonate Urethane with Zwitterionic Polynorbornene via Thiol-ene *Click-Reaction* to Facilitate Cell Growth and Proliferation

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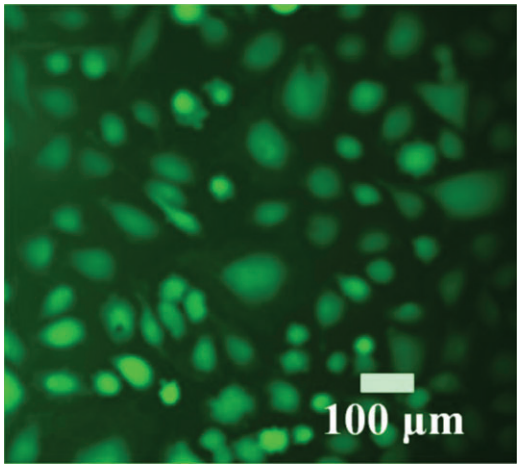
Herein, we grafted the zwitterionic polynorbornene onto polycarbonate urethane (PCU) film surface by a convenient route of thiol-ene click-chemistry. The PCU film surface was first treated with hexamethylene-1,6-diisocynate and subsequently with two different thiol agents (L-cysteine and β-marcaptoethanol) in the presence of di-n-butyltin dilaurate (DBTDL) to immobilize sulfhydryl groups onto the surface. Here, DBTDL acted as selective catalyst for the reaction between surface-tethered isocyanates and amine/hydroxyl groups in thiol agents over that of free thiol groups. In the next step, zwitterionic polynorbornene (poly(NSulfoZI)) having functionalizable double bonds was grafted onto these surfaces by photo-initiated thiol-ene click-reaction. The modified surfaces were characterized by water contact angle and XPS analysis. Moreover, the cytocompatibility of these surfaces was investigated by model endothelial cells, EA.hy926, for 1, 3, and 7 d culture times, which showed enhanced cell adhesion and growth. Therefore, the poly(NSulfoZI) functionalized PCU surface using l-cysteine as thiol agent could be a good candidate for tissue engineering material application.



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1. Introduction

Surface modification of biomaterials with hydrophilic polymers or bioactive agents is an effective strategy to create anti-thrombogenic surfaces. Although heparin coating has significantly anticoagulant efficiency, it usually suffers from leaching of the physically absorbed anticoagulant, its non-uniform substitution, and variation in activities.[1] The ideal scaffold biomaterials should promote cell adhesion, but not induce a chronic immune response or inflammatory reaction after implantation. In addition, these biomaterials should also possess mechanical properties tailored for the specific tissue type.[2]

Polycarbonate urethanes (PCUs) have many advantages for elastic biomaterial applications because of favorable mechanical properties, chemical properties, bio-durability, tolerance in the body during the healing process, and controlled degradation rate, that allows the retention of physical properties throughout the remodeling period.[3–7] But their exclusive use in tissue engineering is limited because PCUs are unsatisfactory for cell adhesion and proliferation. Additionally, PCUs exhibit hydrophobic nature and bioinert properties. In order to address these problems, PCUs have been modified by hydrophilic polymers.[8–11]

Recently, poly(ethylene glycol) methacrylate (PEGMA) has been grafted on model substrates, such as silicon, gold, and other metals, via surface-initiated atom transfer radical polymerization (si-ATRP). [12–14] Compared with above model substrates, polymer surface is more difficult to achieve well-defined brushes with high graft density via si-ATRP, partially due to the difficulty of forming high density of ATRP-initiator.[15] Furthermore, the immobilized poly(ethylene glycol) (PEG) brushes decompose in the presence of oxygen and transition metal ions that exist in most biochemically relevant solutions.[16] Besides hydrophilic PEG, zwitterionic polymers have been usually used to improve surface hydrophilicity and anti-fouling properties. In our previous studies, we have modified PCU surface by zwitterionic phosphorylcholine or sulfoammonium zwitterionic polymers via photopolymerization and si-ATRP.[17,18] In order to obtain high grafting density, PEGMA was first grafted onto the PCU surface by si-ATRP to provide hydrophilic flexible PEG spacer and abundant reactive sites, subsequently introduced bromide-initiators by the reaction between 2-bromoisobutyryl bromide and OH group of PEG, and finally grafted with 2-methacryloyloxyethyl phosphorylcholine or 3-dimethyl (methacryloyloxyethyl) ammonium propane sulfonate. Grafting zwitterionic polymers with a flexible hydrophilic PEG spacer is a very effective method to increase grafting density and surface hydrophilicity.[19,20] Furthermore, the zwitterionic polymer-modified surfaces exhibit lipid-like biomimetic features, which is beneficial for resistance to non-specific protein adsorption, preventing platelet adhesion, and clot formation.[21,22]

The thiol-ene *click-reaction* is quite feasible for surface modification, which requires mild experimental conditions, low concentration of benign catalyst, and no cleanup. More importantly, this reaction is insensitive to ambient oxygen or moisture.[23–25] The use of thiol-ene *click-reaction* for the covalent immobilization of zwitterionic polymers could provide an important strategy for the modification of PCU surface. Zwitterionic polynorbornene (poly(NSulfoZI)),[26,27] which has many double bonds as well as zwitterions, can be used to modify PCUs via thiol-ene *click-reaction*. However, the synthesis of polymers containing multiple thiol groups is problematic due to unavoidable oxidative cross-linking.[28]

So far, several PCU surfaces have been designed and studied for cell adhesive properties containing either PEG, anionic, or other functional groups, but there was little focus on zwitterionic poly(NSulfoZI) modification. Herein, we grafted poly(NSulfoZI) onto thiol-terminated PCU surface using thiol-ene *click-reaction* (Scheme 1). The PCU film surface was first treated with hexamethylene-1,6-diisocynate and subsequently with l-cysteine/β-marcaptoethanol as a thiol agents in the presence of DBTDL to immobilize sulfhydryl groups onto the surface. DBTDL acted as selective catalyst for the reaction between surface-tethered isocyanates and amine/hydroxyl groups in comparison to free thiol groups. In the next step, poly(NSulfoZI) having functionalizable double bonds was grafted onto these surfaces. Poly(NSulfoZI)-modified surfaces were analyzed by XPS and water contact angle. In addition, the cytocompatibility of poly(NSulfoZI)-modified surfaces was evaluated by the adhesion and proliferation of endothelial cells (EA. Hy926) for one week culture time.

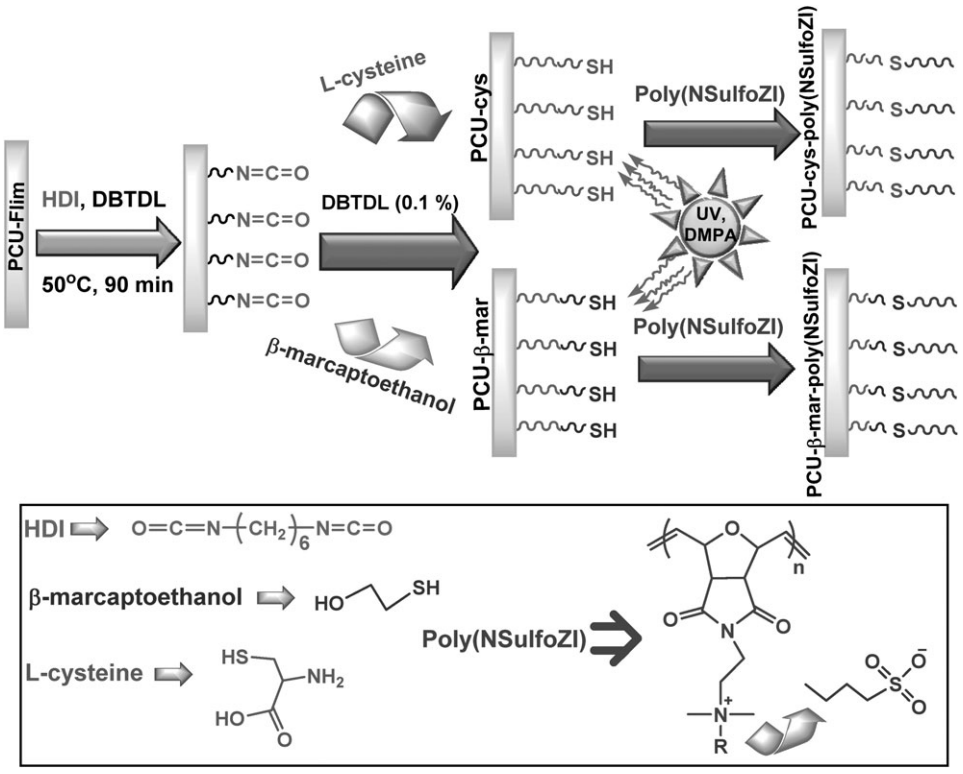
2. Experimental Section

2.1. Materials

β-Marcaptoethanol (98%), l-cysteine (98%), α,α-dimethoxy-α-phenylacetophenone (Irgacure 651) (DMPA) (99%), and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) were obtained from Tianjin Heowns Biochemical Technology Co., Ltd. Fluorescein diacetate (FDA) was obtained from Sigma–Aldrich. Di-n-butyltin dilaurate (DBTDL), hexamethylene-1,6-diisocyanate (HDI), 1,1,1,3,3,3-hexafluoroisopropanol (HFIP), and all other solvents of analytical grade were obtained from Tianjin Jiangtian Chemical Technology Company Ltd, China. PCU (Chronoflex C, $M_n = 110$ kDa) was purchased from Cardio International Incorporated, USA.

2.2. Polymer Synthesis

The zwitterionic polymer, poly(NSulfoZI), was synthesized according to our previously well-established protocol, using the same designed principle of ring-opening metathesis polymerization



Scheme 1. Different steps involved in PCU film surface modification using l-cysteine and β-marcaptoethanol as thiol agents, and subsequent photo-initiated thiol-ene *click-reaction* with zwitterion, poly(NSulfoZI).

(ROMP), and the structural characterization of the polymer has also been reported in the same literature.[21]

2.3. Film Casting and Curing

The PCU solution (10% w/w in N,N-dimethyl formamide) was mixed with HDI crosslinker (2.5%) at 50 °C for 90 min. The solution was then casted onto a glass petri dish (60 mm/25″) and heated at 70 °C until complete solvent evaporation, then cured at 90 °C for 7 h. Finally, the film was cleaned with ethanol, subsequently with water to remove un-crosslinked constituents, and then dried at 37 °C in a vacuum oven until constant weight.

2.4. Cell Culture

The human endothelial cell hybridoma line EA.hy926 cells were purchased from American Type Culture Collection and cultured in high glucose DMEM supplemented 10% FBS in 5% CO_2 atmosphere at 37 °C. The next day, the non-adherent cells were discarded and cultured the adherent cells to confluence, with medium being exchanged after every 3 d.

2.5. Modification of PCU Film Surface with Poly(NSulfoZI)

The PCU film surface (pre-cleaned, 2 × 2 cm^2) was first treated with HDI to create the terminal NCO groups, followed by treatment with l-cysteine or β-marcaptoethanol as thiol agents in the presence of DBTDL at 50 °C for 6 h, which incorporated terminal SH groups. The surfaces were ultrasonically cleaned with ethanol and rinsed with water. These thiol-immobilized surfaces would be represented hereinafter as PCU-β-mar and PCU-cys for β-marcaptoethanol and l-cysteine treatment, respectively. The immobilization of thiol reagent was confirmed by treating a test sample (0.5 × 0.5 mm^2) with DTNB 20 μL (5 mM stock solution) in 2 mL phosphate buffer solution (pH 7.4), which showed a quick color change from colorless to yellow due to the liberation of TNB^{2-} ion (UV/vis, $\lambda_{max} = 412$ nm). In the next step, poly(NSulfoZI) solution (dichloromethane/HFIP = 1:2 as mixture solvent) was spread onto these surfaces and exposed to a 365 nm UV lamp (20 W) for 10 min using DMPA as photo-initiator. These zwitterions-modified surfaces were denoted as PCU-β-mar-poly(NSulfoZI) and PCU-cys-poly(NSulfoZI), respectively. Finally, the obtained films were washed with ethanol and water, dried at 37 °C in a vacuum oven until constant weight, and then stored at −4 °C for further analysis.

2.6. Characterization of Zwitterion-Modified Surfaces

The contact angle was measured by the sessile drop method using a video contact angle instrument (Kruss Easy Drop goniometer, Germany) at room temperature. The contact angle values were calculated with the AutoFAST algorithm within the image analysis software.

Chemical composition was determined by PHI-1600 X-ray photoelectron spectroscopy (XPS) with an Mg Ka 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° 1 s spectra bands were deconvoluted into sub-peaks by means of the XPSPEAK41 spectrometer software.

At pre-determined times (1st, 3rd, and 7th day), the adherent cells on the different surfaces were stained with FDA and photographed by fluorescence microscopy (Fluorescence OLYMPUS U-RFLT50; microscopy Olympus DP72). Random pictures were taken at six different places of each sample.

2.7. Statistical Analysis

All experiments were performed at least three times. Quantitative data are presented as the mean ± S.D. Statistical comparisons were made with Student's t-test. *p*-Values (<0.05) were considered to be statistically significant.

3. Results

3.1. Preparation of Poly(NSulfoZI)-Modified PCU Surfaces

The grafting of poly(NSulfoZI) zwitterion onto the PCU film surface (Scheme 1), using two different thiol agents (l-cysteine and β-marcaptoethanol), was accomplished by a feasible reaction route. The reaction of HDI with surface amine groups is frequently used to introduce isocynate groups (NCO) onto material surface under anhydrous condition. We first treated PCU surface with HDI, subsequently reacted with l-cysteine or β-marcaptoethanol as a thiol agent in the presence of DBTDL as a catalyst, which showed high selectivity for hydroxyl/amine toward NCO groups over that of sulfhydryl, and hence created thiol-terminated surfaces.[29] The immobilized thiol concentration was determined by Ellman's method and given in section 3.3.[30] The photo-initiated thiol-ene *click-reaction* on these surfaces was proceeded using DMPA as a photo-initiator. Finally, we obtained poly(NSulfoZI)-grafted PCU film surfaces (Scheme 1).

3.2. XPS Analysis of Poly(NSulfoZI)-Modified PCU Film Surfaces

The surface chemical composition of poly(NSulfoZI)-modified PCU film surfaces was characterized by XPS analysis, and the results were shown in Figure 1 and Table 1. Figure 1 indicated that the characteristic band of S_{2p} was observed at 163.4 eV in XPS spectra of poly-(NSulfoZI)-modified PCU film surfaces, while PCU-blank surface did not show this peak. PCU-blank surface showed a small peak for Si_{2p}, which was assigned to leaching impurity whenever using the glass substrate during preparation.

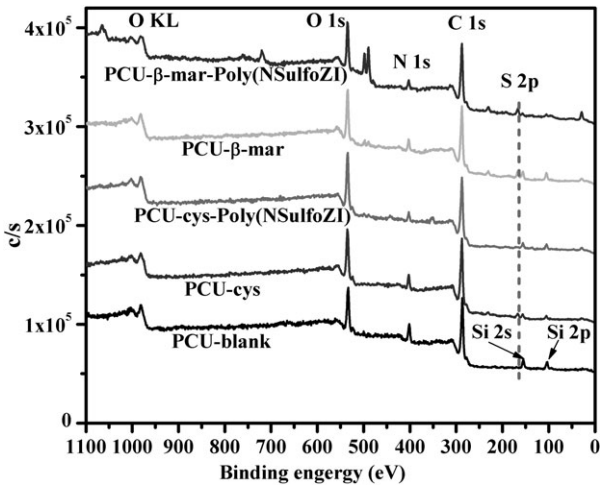


Figure 1. XPS spectra of poly(NSulfoZI)-modified PCU film surfaces using l-cysteine and β-marcaptoethanol as a thiol agent, where β-mar = β-marcaptoethanol, cys = l-cysteine.

The narrow scan S_{2p} XPS peak of PCU-β-mar-poly-(NSulfoZI) surface (Figure 2) indicated that the peak intensity of S_{2p} increased after modification. In case of PCU-cys-poly(NSulfoZI) surface modified through l-cysteine, the S_{2p} peak at 163.5 eV was masked, while the reason is still not clear. The peak at 168.3 eV was ascribed to S–O linkage in sulfate group of the zwitterions in both poly(NSulfoZI) modified PCU film surfaces. Moreover, the total percentage element (Table 1) showed an increase in oxygen/carbon at% (O 1s, C 1s) and decrease in nitrogen at% (N 1s), which also confirmed the successful zwitterion modification of the surfaces.

3.3. PCU Surface Immobilized Sulfhydryl Groups

The immobilized sulfhydryl concentration per unit area of surface ($\mu M \cdot cm^{-2}$), using either l-cysteine or β-marcaptoethanol as a thiol agent was determined by Ellman's method[30] using the formula: $[SH] = (A_{412s} - A_{412r} - A_{412b})/1\,cm \times \epsilon_{412}$, where the subscript s = sample, r = reagent only, and b = blank (PBS). The immobilized SH

Table 1. XPS surface chemical composition of poly(NSulfoZI)-modified PCU-film surfaces using l-cysteine and β-marcaptoethanol as thiol-agents.

Sample ID	C_{1s} [%]	O_{1s} [%]	N_{1s} [%]	S_{2p} [%]
PCU-blank	69.7	19.6	10.7	0.0
PCU-β-mar	67.8	21.0	7.9	3.2
PCU-cys	67.4	21.3	9.2	2.1
PCU-β-mar-Poly(NSulfoZI)	69.9	21.6	5.4	3.2
PCU-cys-Poly(NSulfoZI)	68.3	24.8	5.2	1.7

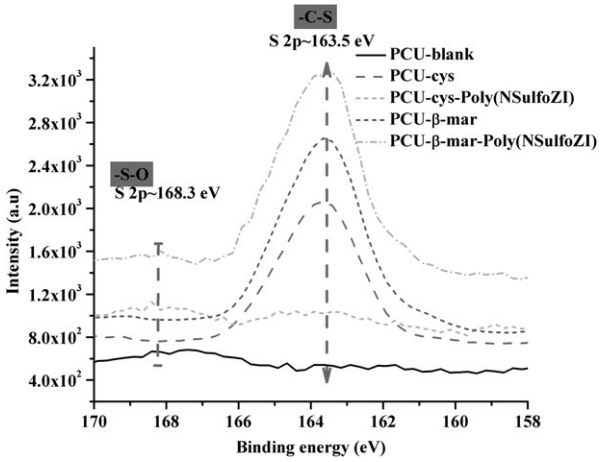


Figure 2. Narrow scan S_{2p} XPS peak of poly(NSulfoZI)-modified surfaces using two different thiol agents as l-cysteine and β-marcaptoethanol.

groups of PCU-cys and PCU-β-mar surfaces were found to be 30 ± 5 and $105 \pm 6\ \mu M\ cm^{-2}$, respectively.

3.4. Hydrophilicity

The PCU-blank surface exhibited highly hydrophobic characteristics with a water contact angle value of $106 \pm 6^\circ$. After thiol group immobilization, the surface exhibited hydrophilic behavior and the water contact angle values decreased to $87 \pm 4^\circ$ and $70 \pm 4.5^\circ$ for PCU-β-mar and PCU-cys surfaces, respectively (Figure 3). The surface hydrophilicity was further enhanced after zwitterion grafting with a water contact angle value of $64 \pm 3^\circ$ for PCU-β-mar-poly(NSulfoZI) surface and $57 \pm 2^\circ$ for PCU-cys-poly(NSulfoZI) surface. Hence, the thiol-ene *click-reaction* provided an effective approach to tune the hydrophilic features of PCU-film surface by zwitterionic poly(NSulfoZI).

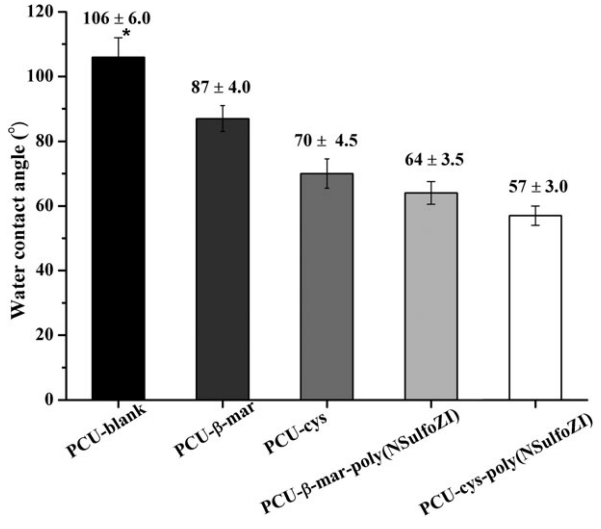


Figure 3. Water contact angle of different modified PCU film surfaces using β-marcaptoethanol and l-cysteine as sulfhydryl group agent. Error bars represent mean ± standard deviation (S.D) (*$p < 0.05$).

3.5. Cytocompatibility Assay

3.5.1. MTT Assay

In order to evaluate whether the designed materials were toxic or cytocompatible, the cytocompatibility of the poly(NSulfoZI)-modified surfaces was evaluated by MTT assay using endothelial cells (ECs, EA.hy926) culture for 1, 3, and 7 d (Figure 4). The results indicated that PCU-β-mar-poly(NSulfoZI) surface exhibited significantly less cell viability in comparison to its counterpart surface, i.e., PCU-cys-poly(NSulfoZI) surface. Moreover, this effect was more obvious after 3rd and 7th day culture time. This high cytotoxic effect of PCU-β-mar-poly(NSulfoZI) surface may arise from remained β-marcaptoethanol in the surface. β-Marcaptoethanol acted as an intermediate linker for covalent grafting of zwitterion onto the PCU-surface. While the rest of the other surfaces using l-cysteine as sulfhydryl group agent exhibited high relative cell viability (>90%) as manifested after 7th day culture time. This further indicated that high cytocompatibility of PCU-cys-poly(NSulfoZI) surface was mainly attributed to the biomimetic nature of zwitterionic poly(NSulfoZI).

3.5.2. EA.hy926 Adhesion and Proliferation

The adhesion and proliferation of ECs were investigated by FDA staining of the cultured cells after different time intervals (1st, 3rd, and 7th day) and analyzed by fluorescence

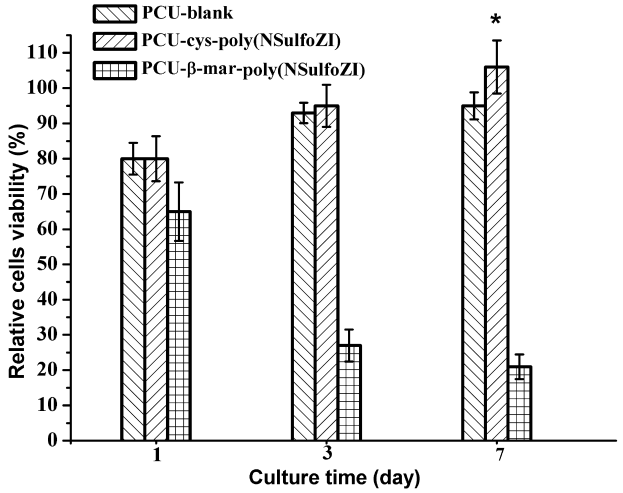


Figure 4. MTT assay of the modified PCU film surfaces for 1, 3, and 7 d. Error bars represent mean ± standard deviation (S.D) (*$p < 0.05$).

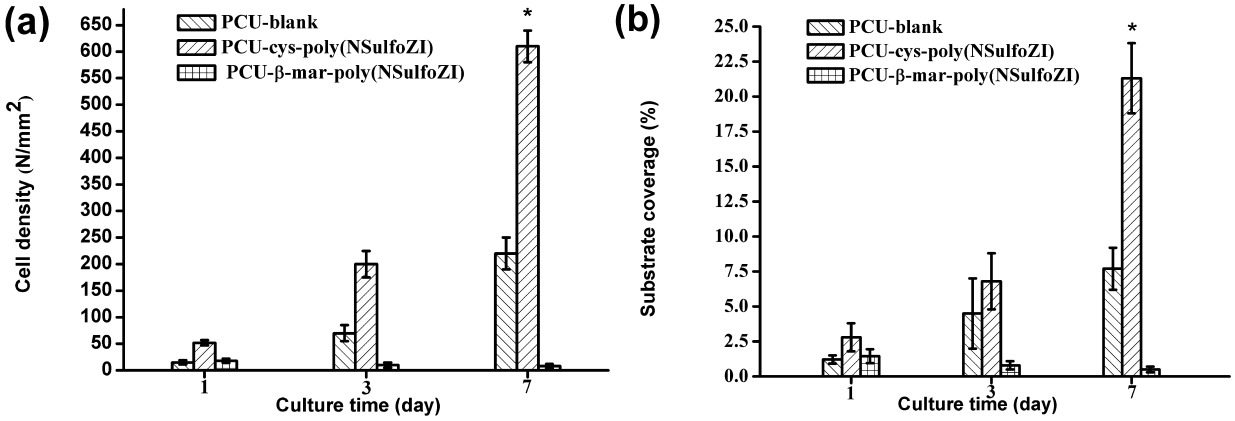


Figure 5. Quantitative representation of cells cultured on the two PCU modified surfaces at 1, 3 and 7 d time points. Error bars represent mean ± standard deviation (S.D) (*$p < 0.05$).

microscopy. The fluorescence images were processed using image-J software to obtain the quantitative results and shown in Figure 5. These microscopic images clearly showed the cell morphology and spreading behavior of the adhered ECs (Figure 6). At 1st day culture, there was quite no difference in ECs growth between PCU-β-mar-poly-(NSulfoZI) surface and PCU-blank surface. While PCU-cys-poly(NSulfoZI) surface showed relative more ECs number and well growth. After 3rd day culture, PCU-cys-poly-(NSulfoZI) surface and PCU-blank surface exhibited significantly high ECs adhesion and proliferation. Moreover, there was an apparent difference in cell morphology and shape. Although PCU-blank surface adhered appreciable number of cells after the 3rd day, these cells acquired rounded morphology in Figure 6. On the other hand, the enhanced growth and proliferation of ECs on PCU-cys-poly(NSulfoZI) surface were observed; furthermore, ECs adapted spindle shape structure as an indication of cell-spreading. But PCU-β-mar-poly(NSulfoZI) surface showed no adhered ECs after 3rd and 7th day culture (only a few cells attached at 1st day), which indicated that ECs died after culture. Moreover, ECs growth and proliferation were more pronounced on PCU-cys-poly(NSulfoZI) surface, which exhibited a good response towards cell growth particularly after 7th day culture.

The cell response on these surfaces was further evaluated by cell density ($N \cdot cm^{-2}$) at different culture time points (Figure 5a), which significantly reflected the surface behavior at 7th day culture time point. Furthermore, the cell spreading was characterized in terms of surface coverage of ECs (cell covered area %). If cells find more appropriate growth environment during culture, they will proliferate on the surface and cover more area. Surface coverage of ECs can directly support for the earlier statement of the expanded morphology (Figure 5b). The results showed that ECs covered more area of PCU-cys-poly(NSulfoZI) surface.

4. Discussion

The surface tailoring of elastomeric substrate for biomedical application has attracted significant attention in recent years because of the easily available grafting techniques as well as a variety of biomimetic synthetic materials. Currently, the use of PEG-derived non-fouling materials for long-term applications has been precluded because of their oxidization susceptibility in biochemical media.[31] On the other hand, biomaterial surface, which is coated or modified by zwitterionic polymers, can highly and efficiently resist non-specific protein adsorption even from undiluted blood plasma and serum and inhibit the long-term bacterial colonization on surface.[32] Among zwitterionic polymers, zwitterionic polynorbornene poly-(NSulfoZI) presents an emerging class of materials, which provide excellent hydrophilicity and non-fouling properties when immobilized onto the substrate surface.[21,27] These properties could mainly be attributed to their structural resemblance to the natural functional groups, such as phosphatidylcholines analogues to mammalian cell membranes, since they both present a bilayer structure, exposing the unique zwitterion functional group on the surface.[33]

PCUs have been widely used as biomaterials for the applications in drug release systems, scaffolds, catheters, and artificial vascular grafts. Especially, PCU artificial vascular grafts exhibit approximately similar mechanical properties and compliance of natural blood vessels. But, PCU surface is high hydrophobic, which is not beneficial for cell growth even through ECs are adhered onto it. In our present study, we found that the cultured ECs acquired a rounded morphology on PCU-blank surface, which was a sign of restricted cell growth on the surface. In order to increase the surface hydrophilicity and create a beneficial surface for cell adhesion and proliferation, we used zwitterionic polynorbornene poly(NSulfoZI) to modify PCU film surface. We first immobilized SH groups onto

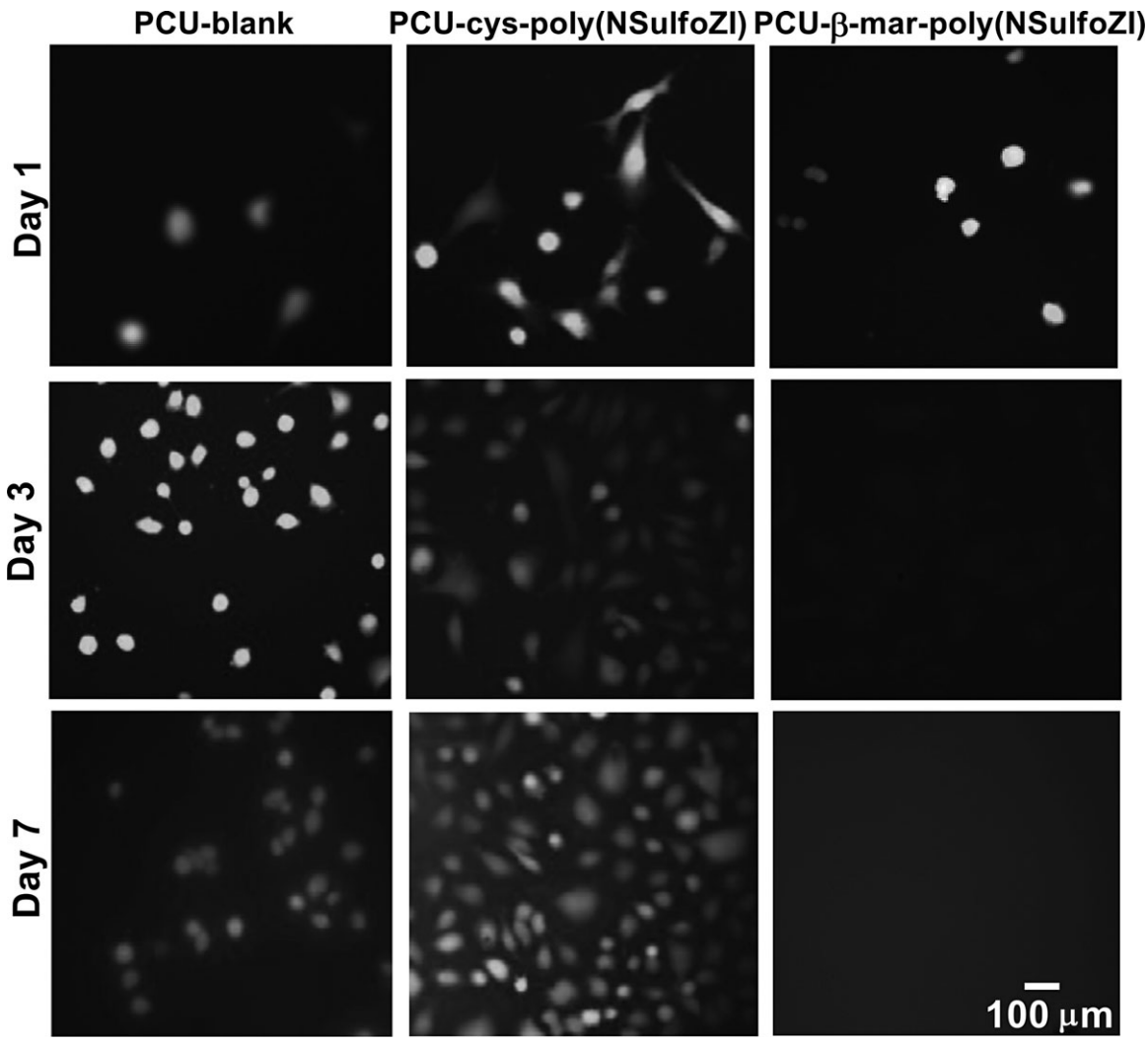


Figure 6. Fluorescence micrographs of EA.hy926 cells, showing adhesion and proliferation on the modified PCU film surfaces at 1, 3, and 7 d culture time.

PCU surface, and then covalently conjugated with zwitterionic poly(NSulfoZI) polymers to obtain poly(NSulfoZI)-modified PCU surface (Scheme 1). The surface hydrophilicity was enhanced after zwitterionic poly(NSulfoZI) grafting, for example, PCU-cys-poly(NSulfoZI) surface showed very low water contact angle value of $57 \pm 2^{\circ}$. The thiol-ene *click-reaction* provided an effective approach to improve the hydrophilicity of PCU surface.

The MTT assay clarified that the cytocompatibility was significantly improved after zwitterion grafting onto the PCU surface via l-cysteine. More importantly, PCU-cys-poly(NSulfoZI) surface showed highest cell viability after 7^{th} day culture. Therefore, PCU-cys-poly(NSulfoZI) surface is beneficial from l-cysteine as a linker. On the other hand, PCU-β-mar-poly(NSulfoZI) surface, which was prepared by using β-marcaptoethanol as a thiol agent, exhibited relative high cytotoxicity, and therefore is inappropriate for tissue engineering application. Because β-marcaptoethanol is high toxic reagent, the remained β-marcaptoethanol in the modified surface may cause the high cytotoxic effect of PCU-β-mar-poly(NSulfoZI) surface. Thus, β-marcaptoethanol method is not suitable for preparation of modified surface.

The quantification of cell growth and proliferation indicated that the zwitterion polynorbornene-modified surface using l-cysteine as linker (PCU-cys-poly(NSulfoZI)) could provide a suitable candidate for a future biomedical application.

5. Conclusion

The elastomeric polycarbonate urethane surface was modified with zwitterionic poly(NSulfoZI) via a convenient

route of thiol-ene *click-chemistry* to create a biomimetic platform. The sulfhydryl groups were first introduced onto a pre-treated NCO-terminated PCU surface using β-marcaptoethanol/l-cysteine. Subsequently, poly(NSulfoZI) was grafted via thiol-ene *click-reaction*, which provided high surface hydrophilicity. These designed surfaces were evaluated for cytocompatibility against model endothelial cells (EA.hy926) for one week period, which showed enhanced cell adhesion and proliferation in comparison to PCU-blank surface. Therefore, the zwitterion-modified surface, i.e., PCU-cys-poly(NSulfoZI), provided a suitable platform for tissue-engineering application.

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