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Plasma functionalization of polycarbonaturethane to improve endothelialization—Effect of shear stress as a critical factor for biocompatibility control

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Abstract

Medical devices made of polycarbonaturethane (PCU) combine excellent mechanical properties and little biological degradation, but restricted hemocompatibility. Modifications of PCU might reduce platelet adhesion and promote stable endothelialization. PCU was modified using gas plasma treatment, binding of hydrogels, and coupling of cell-active molecules (modified heparin, anti-thrombin III (ATIII), argatroban, fibronectin, laminin-nonapeptide, peptides with integrin-binding arginine-glycine-aspartic acid (RGD) motif). Biocompatibility was verified with static and dynamic cell culture techniques. Blinded analysis focused on improvement in endothelial cell (EC) adhesion/proliferation, anti-thrombogenicity, reproducible manufacturing process, and shear stress tolerance of ECs. EC adhesion and antithrombogenicity were achieved with 9/35 modifications. Additionally, 6/9 stimulated EC proliferation and 3/6 modification processes were highly reproducible for endothelialization. The latter modifications comprised immobilization of ATIII (A), polyethyleneglycole-diamine-hydrogel (E) and polyethylenimine-hydrogel connected with modified heparin (IH). Under sheer stress, only the IH modification improved EC adhesion within the graft. However, ECs did not arrange in flow direction and cell anchorage was restricted. Despite large variation in surface modification chemistry and improved EC adhesion under static culture conditions, additional introduction of shear stress foiled promising preliminary data. Therefore, biocompatibility testing required not only static tests but also usage of physiological conditions such as shear stress in the case of vascular grafts.

Keywords

Endothelial cell seeding, thrombogenicity, plasma treatment, hydrogel, polyurethane, sheer stress, biocompatibility

Introduction

Polycarbonateurethanes (PCUs) have been used for producing biostable implantable medical devices (e.g. artificial blood vessels, small-diameter vascular grafts) as they provide relatively beneficial thromboresistance, biocompatibility and mechanical properties similar to the polyether-based polyurethanes.[1–4] However, thrombus formation remained a critical risk factor.[5–7] An effective way to improve hemocompatibility of PCU is to optimize its material surface but not significantly change their intrinsic mechanical properties. Modification of the polyurethane surface chemistry

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combining reduced platelet adhesion/activation and increasing endothelial attachment would be an attractive task.

It is very attractive to activate polymer surfaces using gas plasma treatment.[8–13] In this context, it was shown that nitrogen (N_2) plasma treatment enriched the surface with nitrogen species,[9,10] and thereby reduced platelet adhesion, activation, and aggregation.[9,11] In addition, N_2 plasma activation promoted adhesion and proliferation of fibroblasts and osteoblasts onto chitosan membranes or biodegradable polybutylene succinate surfaces.[12,13] Furthermore, biocompatible hydrogels are generating growing interest in the community of tissue engineering because of their mechanical and chemical versatility and cell compatibility based on biomimicry of the extracellular matrix.[14,15] For example, polyethylene glycol (PEG) is one of the most widely investigated hydrogels,[16] that allowed adhesion of smooth muscle cells for improvement of cardiovascular tissue engineering applications.[17] Furthermore, PEG-diamines were used as branch molecules in the network which were linked to cell-active molecules.[18] Other interesting hydrogels in tissue engineering approaches are polyethyleneimine composites[19,20] and carboxymethyl dextran[21]. They are characterized by low cytotoxicity, simple processing, and variable applicability.[19] Additional immobilization of biomolecules on biomaterial surfaces has been also proven to be a very effective method for considerable improvement of blood compatibility or increasing cell attachment and proliferation.[22] Covalent binding of fibronectin[23] or RGD peptides[24,25] as well as a laminin-nonapeptide[26] improved integrin-mediated cell adhesion of endothelial cells or progenitor cells. Other strategies such as binding of anticoagulants like heparin,[27] phosphorylcholine,[28] or Argatroban[29] pursued the aim to reduce platelet adhesion, activation and aggregation.

Routinely, biocompatibility testing only used static culture methods according to international standard ISO-10993 requirements.[8,11,12,22–25,28] In cardiovascular tissue engineering, endothelial cell attachment to and retention on an appropriate scaffold is particularly important because of the high fluid shear forces applied to the graft lumen upon implantation within the arterial tree. Therefore, bioreactors and perfusion flow systems were used to endothelialize the graft lumen, to precondition the cells to blood flow and to mimic the physiological fluid-induced cyclical shear stress derived from blood flow through the vessels.[14,22,30] In the present study, static methods were introduced to analyze the bio- and hemocompatibiltiy of 35 different chemical modifications of PCU. The most promising modifications with low platelet adhesion and improved endothelial cell adhesion were analyzed in a bioreactor under pulsatile flow to evaluate the effect of shear stress on the integrity of the cell monolayer.

Materials and methods

Materials

Disks (area, 0.3 cm^2) and vascular grafts (length, 15 cm; diameter, 0.6 cm; wall thickness, 0.5 mm) made of PCU were purchased from DUALIS (Seefeld, Germany). The PCU-samples were cleaned (ethanol, p.a.) and air dried before processing.

N_2 and carbon dioxide (CO_2) were purchased from Linde (ultrapure grade, 99.998%; Ludwigshafen, Germany). Laminin-nonapeptide (CDPGYJGSR), short (GRGDSPK) and long (GCGYGRGDSPG) RGD-peptides were obtained from GenScript Corporation (Piscataway, NJ), Antithrombin III (ATIII, Kybernin®P) from CLS Behring (Marburg, Germany), Argatroban (Argatra®) from Mitsubishi Pharma (Düsseldorf, Germany), human fibronectin from BD Biosciences (Heidelberg, Germany), branched polyethyleneimine (PEI) and poly(ethylene glycol) bis(amine), (PEG-diamine, MW10,000) from Sigma-Aldrich (Munich, Germany). Unless otherwise indicated, chemicals were purchased from Sigma-Aldrich or Merck (Darmstadt, Germany). Water was deionized. Carbonate buffer (c-buffer) (0.1 M, pH 8.4) was prepared freshly. Carboxymethyl-dextran (CM-Dex) was prepared with 1 M bromoacetic acid as described earlier.[31] Heparin was modified by dissolving heparin (0.3%) in icy water, spiked with sodium nitrite (0.4 M), acidified with hydrogen chloride (pH 2.7), and stirred (2 h, 0°C). After adjustment to pH 7.0, the solution was filled with twice the volume of 2% sodium chloride (NaCl, in ethanol) and stored for 40 h (4°C). Crystals were harvested, dried in vacuum and stored at 4°C.

Surface modifications

Figure 1 presents all analyzed surface modifications using different manufacturing processes (Argatroban, Ar; ATIII, A; fibrinogen, F; Laminin-nonapeptide, L; short, Rs, and long, Rl, RGD-peptides). Due to blinded study design and block by block biological testing, not all molecules were immobilized onto each activated surface.

Procedure 1. All PCU-samples were plasma treated[32] using a Plasmabrush® (Reinhausen Plasma, Germany). The plasma chamber was thoroughly purged with a continuous flow of N_2 (20 NL/min; voltage, 4500 V) and CO_2 (1.5 NL/min). Before activation, disks were fixed on glass slides, and grafts were wrapped in grounded aluminum foil. Discs were treated for 7 s each. The lumen of grafts

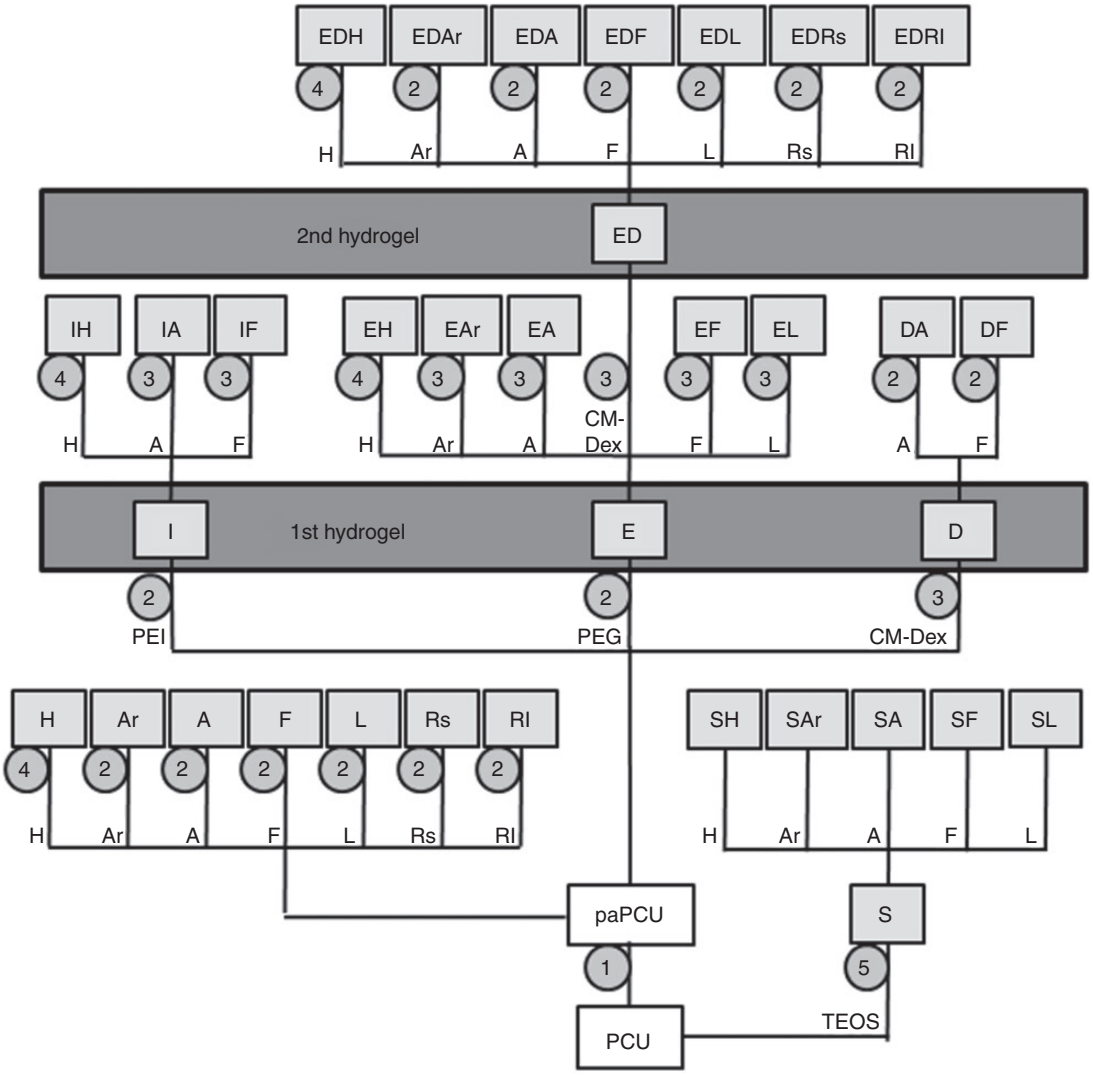

Figure 1. Manufacturing of different chemical modifications of PCU.
PCU was modified using different procedures (1–5, grey encircled) to introduce hydrogels (PEI, polyethyleneimine; PEG, poly(ethylene glycol) bis(amine)), cell-active molecules (Ar, argatroban; A, Antithrombin III; F, fibrinogen; L, laminin-nonapeptide; Rs, RI, short and long RGD-peptides; H, modified heparin), and carboxymethyl-dextran (CM-Dex). End products are framed at the end of the connecting lines. For details of the procedures and composition of the end products see "Materials and methods" section.

was treated with N_2-plasma from one end and CO_2 gas from the opposite site for 20 s once. The distance of the nozzle to the PCU was 0.5 cm. After 45 min on air, plasma-activated PCU-samples (paPCU) were incubated with 0.9% NaCl solution (2 h, 50°C), washed in water and immediately modified.

Procedure 2. NHS/EDC chemistry (N-hydroxysuccinimide/1-ethyl-3-(3-dimethylaminopropyl) carbodiimide)[33] was used to activate modified PCU-samples (paPCU, CM-Dex immobilized surfaces). Samples were incubated with NHS/EDC (0.1 M/0.1 M) in aqueous media (room temperature, RT, 20 min), rinsed with water and processed.

Procedure 3. A (4 IU/mL), Ar, L, and F (each, 0.4 mg/mL) and CM-Dex (4 mg/mL) in c-buffer were incubated in NHS/EDC (0.1 M/0.2 M) (20 min, RT), and filled up to the 4-fold volume of c-buffer. Modified PCU samples were incubated in A, Ar, L, and F solutions (19 h, 4°C) or CM-Dex solution (2 h, RT).

Procedure 4. Sodium cyanoborhydride ($NaBH_3CN$) was used to immobilize modified heparin (***H***) (see above). Modified heparin (1 mg/mL) was dissolved in $NaBH_3CN$ (3 mg/mL) in 0.15 M NaCl and incubated with modified PCU-samples (2 h, 50°C). After repeated short-time rinsing with water, the samples were dried and stored at 4°C until biological testing.

Procedure 5. Tetraethylorthosilicate (TEOS) was used as a precursor to polymerize on PCU-samples via sol-gel process.[34] TEOS gas was generated by heating up to 80°C and fed in with Argon gas carrier (10 NL/min; 4500 V) into the argon-plasma using the Plasmabrush® (***S***). Disks were treated (3×7 s). Immediately after polymerization of TEOS, disks were continuously moved in 10% 1,6-hexamethylenediisocyanate[35] in diethylether (72 h, RT) followed by diethylether washing (5 h) and repeated c-buffer washings. Thereafter, disks were incubated with A (1 IU/mL), modified heparin, Ar, L, and F (each, 0.1 mg/mL) (dissolved in c-buffer) over night at 4°C (***SA, SH, SAr, SL, SF***).

Hydrogels of PEI (***I***) and PEG (***E***) were produced by crosslinking PEI (3 mg/mL in c-buffer) (2 h, RT) and PEG (1 mg/mL, in 0.6 M potassium sulfate and c-buffer) (2 h, 50°C)[23] with activated PCU samples (*procedure 2*). For immobilization of CM-Dex, CM-Dex (4 mg/mL) was activated (*procedure 3*) and incubated with paPCU (***D***) and *E*-modified PCU (***ED***) (2 h, RT).

Covalent binding of cell-active molecules. The end product of chemical modification strategies consisted of covalent binding of argatroban (Ar), ATIII (A), fibrinogen (F), laminin-nonapeptide (L), short (Rs), and long (Rl) RGD-peptides. Molecules were either dissolved in c-buffer (A (1 IU/mL), Ar, L, Rs, Rl, F (each, 0.1 mg/mL)) and incubated with activated *paPCU*, activated *ED*-modified PCU, activated *D*-modified PCU (*procedure 2*) (19 h, 4°C) [***Ar, A, F, L, Rs, Rl, EDAr, EDA, EDF, EDL, EDRs, EDRl, DA, DF***], or activated using *procedure 3* and incubated (19 h, 4°C) with *E*- and *I*-modified PCU-samples [***EAr, EA, EF, EL, IA, IF***]. Heparin was immobilized onto *paPCU*, *S*-, *E*-, *ED*-, and *I*-modified PCU samples using *procedure 4* [***H, EH, EDH, IH***].

Each end product (framed within Figure 1) was thoroughly rinsed with water, dried, and stored at 4°C until blinding and biological testing. Blinded test samples were washed (ethanol, 70%; sterile PBS), and fixed into 96-well microplate (Nunc®, Wiesbaden, Germany) with sterile steel rings. Tissue-cultured polystyrene (TCP) (Nunc) was the reference for biological testing.[36]

Cytocompatibility

Human saphenous vein ECs (HSVEC) were cultured in growth medium with serum (GMS, Medium 199, 10% fetal calf serum, L-Glutamine (PAA Laboratories, Pasching, Austria), Supplement Pack (PromoCell, Heidelberg, Germany)).[36] Informed consent was obtained from cell donors. The protocol was approved by the local human ethics committee (no. 99/133). For adhesion (proliferation) tests, 66,000 (17,000) HSVECs/cm^2 were seeded onto non-coated test samples or fibronectin-coated (10 μg/mL) TCP for 24 h (3–7 days). Cells were fed with fresh GMS (days 3 and 5), harvested by addition of trypsin/EDTA (Promocell) on days 3, 5, and 7, and counted (CASY-TTC, Roche, Mannheim, Germany).

Hemocompatibility

Human citrated venous blood was drawn from male healthy volunteers with written consent as per institutional ethics guidelines (no. 10-101-0159), and centrifuged (300 *g*, 15 min, 37°C). Platelet-rich plasma (PRP) was transferred into a polypropylene syringe containing 10% aqueous citrate dextrose, and centrifuged again. Platelets were counted (Neubauer haemocytometer), double-stained (monoclonal anti-CD41-FITC/anti-CD62P-APC antibodies, BD Biosciences, Erembodegem, Belgium) (24 h, 4°C) and analyzed using flow cytometry. Only PRP preparations with <10% CD41+/CD62P+ platelets were used for platelet adhesion tests. PRPs (5×10^7 platelets/0.3 cm^2) were coincubated with test samples (30 min, 37°C). Non-adherent platelets were removed by careful washing with PBS. Adherent platelets were fixed (10% paraformaldehyde, RT, 10 min), permeabilized (0.5% TritonX-100 in PBS, RT, 5 min), washed with PBS, stained with rhodamine-phalloidin (Molecular Probes, ThermoFisher, Darmstadt, Germany) (100 nM, RT, 30 min), and visualized using fluorescence microscopy (Leica DMRBE, Biberach, Germany). Cell density was estimated using a platelet score (1, <20%; 2, 20–50%; 3, >50% surface coverage) (Figure 2(a) to (c)). Platelet score was evaluated in independent experiments using five different donors by three technicians in a blinded fashion. A mean platelet score <1.5 was defined as sufficient hemocompatible.

Effect of shear stress

Modifications with the best EC adhesion properties were used to analyze the effect of shear stress on cell adhesion in a bioreactor (DUALIS). Details for bioreactor processing see Riescher et al.[37] HSVECs ($2–6 \times 10^6$) were resuspended in 2 mL of GMS and added into the modified grafts. Fibronectin-coated grafts made of Elastollan®1180A (BASF, Ludwigshafen, Germany) were used as a positive control.[37] The grafts were perfused with a pulsatile flow for 72 h. Then, grafts were removed from the bioreactor, fixed with paraformaldehyde, and stained with 4′,6-Diamidin-2′-phenylindol-dihydrochloride (DAPI). The grafts were separated into pieces of 1 cm of length, opened longitudinally and fixed on a glass slide. The nuclei of the adherent cells were visualized with the fluorescence microscope (10× magnifications) and

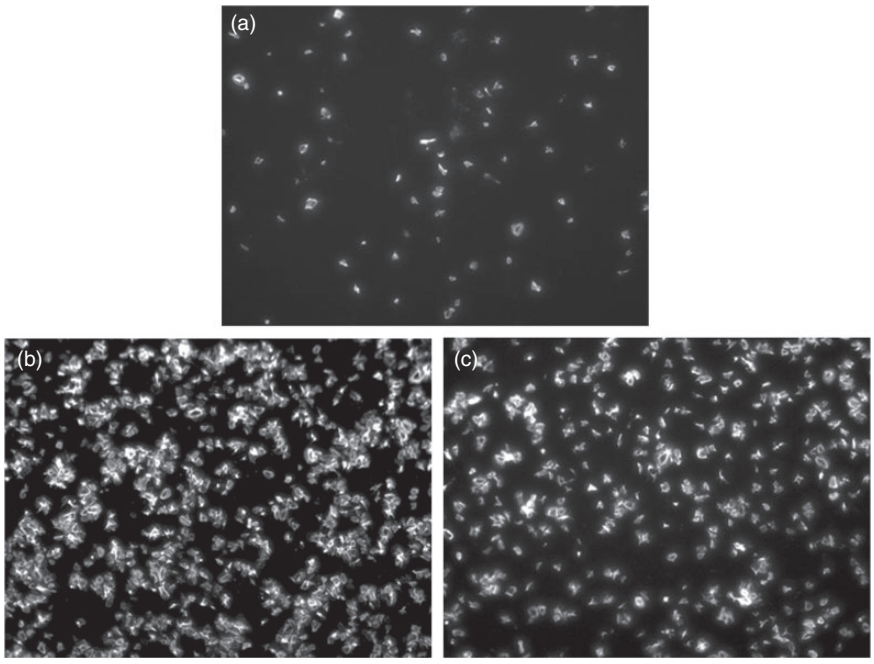

Figure 2. Estimation of the platelet score.
Different amounts of isolated platelets ((a) 6×10^6; (b) 2.5×10^7; (c) 5×10^7 per 0.3 cm^2) were seeded onto tissue-cultured polystyrene (TCP) for 30 min, fixed with formaldehyde, permeabilized, stained with rhodamine-phalloidin and visualized using fluorescence. Platelet score was defined as surface coverage of stained platelets: (a) platelet score 1, <20%; (b) platelet score 2, 20–50%; (c) platelet score 3, >50% surface coverage.

quantified using ImageJ software. Other sections of the grafts were stained with rhodamine-phalloidin (see above) and with fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-human CD31 antibody/ Ancell, Bayport, MN). Furthermore, a third part of the grafts was used for scanning electron microscopy (SEM) as described by Lehle et al.[38]

Statistics

Data were presented as mean ± standard deviation (SD). Due to non-normality of the data, the Wilcoxon-Signed-Rank-Test (Sigma-Stat, SPSS, Chicago, IL) was used after passing the Friedman-Test (Sigma-Stat, SPSS, Chicago, IL). p-Values ≤ 0.05 were considered significant. All analyses in the static cell culture were done with six EC and five platelet donors. Other details see text.

Results

Screening of different chemical modifications

Platelet adhesion (Figure 3(a)) was high for TCP and untreated PCU with a platelet score of 2.9 ± 0.3 and 2.2 ± 0.5, respectively. After chemical modification of PCU, 15 different strategies (43%) presented a significant reduction of the mean platelet score (1.3 ± 0.6, $p \leq 0.001$ vs. untreated PCU) (white bars, Figure 3(a)). As shown in Figure 3(b), TCP allowed high EC coverage (100%), while the cell density was significantly reduced for untreated PCU ($48 \pm 13\%$ of TCP, $p \leq 0.001$). Cell adhesion onto PCU was significantly improved in 19 modifications (54%) (white bars, Figure 3(b)) ($p \leq 0.05$ vs. untreated PCU). Nine modifications (26%) unified both anti-thrombogenic and EC-adhesive characteristics (shaded boxes, Figure 3(a) and (b)).

As shown in Figure 3(a) and (b), plasma activation of PCU (paPCU) did not reduce platelet adhesion (platelet score >2, $p > 0.05$) but significantly increased EC adhesion ($p = 0.024$). However, no additional treatment enabled a further increase in EC density compared to paPCU. Only the direct coupling of heparin (H) improved EC coverage; however, without statistical significance ($p = 0.063$, compared to paPCU). Surprisingly, binding of fibronectin to PEI hydrogels even reduced EC density (IF). Polymerization of TEOS and further modifications (S, SH, SAr, SA, SF, SL) did not affect EC-adhesive properties, but instead increased platelet score (>2). Therefore, this procedure was not pursued further. Further contemplation focused on anti-thrombogenic surfaces (platelet score <1.5, Figure 3(a)). PEG-based hydrogel coatings (E, ED) significantly reduced thrombogenicity (E, $p = 0.009$; ED, $p = 0.029$; compared to untreated PCU). Coupling of PEI (I) and CM-Dex (D) did not

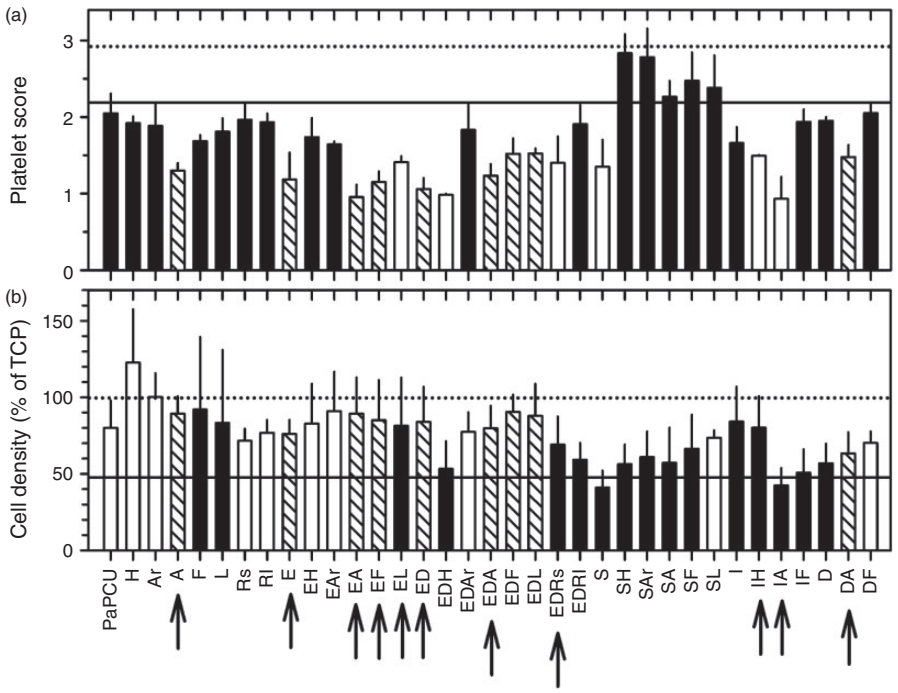

Figure 3. Biological screening of different chemical modifications.
Different chemical modifications (for abbreviations and manufacturing protocol see "Materials and methods" section) were tested for their (a) hemocompatibility and (b) cytocompatiblilty. Platelet adhesion was estimated via platelet score (5 donors). Endothelial cells (8 donors) were cultivated for 24 h and counted with CASY-TTC. Cell counts were specified in % of TCP (tissue-cultured polystyrene). Solid black line, untreated PCU; dotted black line, TCP; filled boxes, no significant alteration compared to untreated PCU; white boxes, significant reduction of platelet score (<1.5) or increase of EC density compared to untreated PCU; shaded boxes, concerning modifications combined both anti-thrombogenicity and endothelial cell adhesion; arrows, these modifications were used to demonstrate reproducibility of the manufacturing process. Data are presented as mean and standard deviation.

improve anti-thrombogenicity (platelet score > 1.5). Covalent binding of ATIII—A, EA, EDA, IA, DA—improved anti-thrombotic properties of untreated PCU (platelet score < 1.5) (each, $p \leq 0.001$ compared to untreated PCU). Comparing preconditioning treatment with respective ATIII modifications showed a significant reduction for A, IA, and DA (paPCU vs. A, $p = 0.004$; I vs. IA, $p = 0.010$; D vs. DA, $p = 0.002$). A benefit in anticoagulant function of Argatroban (Ar, EAr, EDAr) and modified heparin (H, EH, EDH, IH) failed. Additional binding of EC-adhesive proteins/peptides such as fibronectin did not affect the anti-thrombotic properties of respective pretreated PCUs (F, EF, EDF, IF, DF).

Reproduction of the manufacturing process

Eleven of thirty-five modifications were tested for reproducibility of the manufacturing process. Selection criteria included (a) anti-thrombogenicity (Figure 3(a)), (b) easy manufacturing, (c) preservation of EC-adhesiveness (Figure 3(b)). Manufacturing process was repeated three times in an independent fashion. As shown in Table 1, platelet adhesion was highly variable for different preparations of A, E, EF, ED, EH, and DA. Reproduction of low platelet scores was documented for EA, EL, EDA, EDRs, and IA (foregrounded in grey, Table 1). As shown in above, EC adhesiveness was only adequate for 7/11 selected modifications. EL, EDRs and IH showed high variability in EC coverage. IA prevented EC adhesion. These characteristics were reproducible for A, E, IH (maintenance of EC adhesiveness), and IA (prevention of EC adhesion) (foregrounded in grey, Table 1).

EC proliferation

Improved cytocompatibility of biomaterials was also defined by an increase in the proliferative activity of EC (Figure 4). Highest cell density was detected for TCP ($n = 8$, $p \leq 0.001$). Instead, cell density was by a factor of 2 significantly lower on untreated PCU ($p = 0.001$). Additional modifications either completely suppressed proliferation (EA, ED, EDA, EDRs, IA), or permitted an increase in cell count over 5 days but without a synergistic effect compared to untreated PCU (A, E, EF, EL, IH, DA).

Table 1. Reproduction of the manufacturing process of 11 modifications.

	EC adhesion (% of TCP)				Platelet adhesion (platelet score)			
Preparation	1st	2nd	3rd	4th	1st	2nd	3rd	4th
Modification								
Untreated	50 ± 10	48 ± 13	60 ± 19	61 ± 20	2.5 ± 0.5	2.2 ± 0.3	2.7 ± 0.1	2.2 ± 0.2
A	89 ± 11	55 ± 23	66 ± 23	67 ± 13	1.3 ± 0.6	1.7 ± 0.5	1.8 ± 0.7	1.8 ± 0.8
E	76 ± 9	56 ± 16	75 ± 18	56 ± 18	1.2 ± 0.5*	1.2 ± 0.6*	2.3 ± 0.8	1.2 ± 0.5*
EA	89 ± 24	29 ± 10	65 ± 9	53 ± 43	0.9 ± 0.6	1.1 ± 0.6	1.6 ± 0.7	1.2 ± 0.5
EF	85 ± 26	46 ± 13	80 ± 15	47 ± 21	1.2 ± 0.6	1.1 ± 0.6	1.9 ± 1.0	1.1 ± 0.7
EL	81 ± 31	49 ± 18	73 ± 11	56 ± 13	1.4 ± 0.5	1.2 ± 0.6	1.1 ± 0.6	1.2 ± 0.7
ED	84 ± 23&	39 ± 19	76 ± 22&	27 ± 17	1.1 ± 0.5*	1.1 ± 0.6*	1.8 ± 0.7	1.0 ± 0.7*
EDA	80 ± 15&	55 ± 21&	54 ± 12&	24 ± 10	1.2 ± 0.7	0.8 ± 0.3#	1.2 ± 0.4	1.0 ± 0.6
EDRs	69 ± 18	14 ± 4*&#	74 ± 14	31 ± 5*#	1.5 ± 0.7	0.6 ± 0.5#*	1.5 ± 1.1	1.0 ± 0.5
IH	80 ± 21	74 ± 18	70 ± 14	70 ± 15	1.5 ± 0.7	1.2 ± 0.5*	1.8 ± 0.8	1.1 ± 0.6*
IA	43 ± 11	31 ± 13	19 ± 10	27 ± 18	0.9 ± 0.5&	0.9 ± 0.5&	0.9 ± 0.5&	1.7 ± 0.6
DA	63 ± 14	27 ± 27*#	75 ± 21	42 ± 24	1.4 ± 1.0	1.6 ± 1.0	2.0 ± 1.0	1.5 ± 1.3

Data are mean ± standard deviation. Abbreviations of the modifications are described in the "Materials and methods" section and Figure 1. Each preparation (1st to 4th) was done independently in a triple approach.
EC: endothelial cell; TCP: tissue culture polystyrole (reference material).
Statistics: *significant vs. 3rd preparation; #significant vs. 1st preparation; &significant vs. 4th preparation.

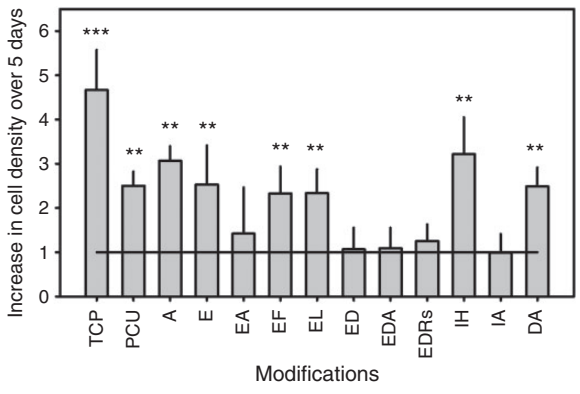

Figure 4. Proliferation of endothelial cells on the surface of selected modifications.
Endothelial cells (17,000 per cm^2) were seeded onto tissue-cultured polystyrene (TCP), untreated PCU and 11/35 modified test samples (for abbreviations and manufacturing protocol see "Materials and methods" section) for 5 days. Cells were counted and displayed relative to the cell count on the day of seeding (solid line). ***$p \leq 0.001$; **$p \leq 0.01$ compared to the cell count on the day of seeding. ##$p = 0.001$ comparing cell counts on day 5 after seeding on TCP and untreated PCU. Data are presented as mean and standard deviation from eight cultures.

Effect of shear stress

Modifications A, E, and IH combined benefits such as adequate EC adhesion and proliferation, and satisfactory reproducibility. These properties might enable cell seeding of vascular synthetic grafts in a bioreactor under pulsatile flow.[29] After 3 days under shear stress (maximum flow, 600–800 mL/min; laminar flow; shear force, 6 dyn/cm^2), cell density on fibronectin-coated Elastollan® grafts (positive control) was significantly higher than on untreated PCU grafts (Figure 5(a)). Nuclei from adherent cells on Elastollan® and untreated PCU showed an oval shape. The circularity (relative unit 1 represents a circle) of the nuclei described its roundness (Figure 5(b)). Adherent EC on the luminal surface of the Elastollan® graft formed a complete cell monolayer. The cells arranged in flow direction (Figure 5(c)). In contrast, pulsatile flow removed the integrity of adherent EC on untreated PCU grafts. As a result, cells detached and aggregated. Only small colonies remained adherent on the polymer surface.

In six independent experimental set-ups, modified PCU grafts (A, E, IH) were endothelialized and exposed to shear stress for 3 days. Immediately after induction of pulsatile flow EC completely detached from the surface of E-modified grafts. After 3 days, no EC was detected on these grafts. In contrast, EC remained attached on the surface of the other modified grafts (A, IH). As shown in Figure 6, EC formed a monolayer with its typical "cobblestone morphology". However, the cells differed in their size. Modification IH presented more cells with larger volume (cell swelling) (Figure 6(a)). EC on A-modified grafts were irregularly arranged (Figure 6(b)). In any case, cell arrangement in flow direction failed. In addition, the integrity of the monolayer was disrupted as shown in

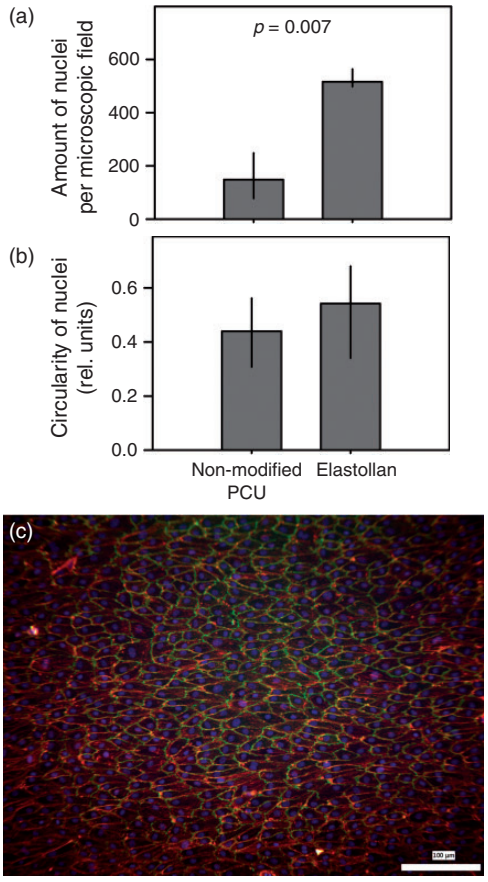

Figure 5. Effect of shear stress on the cell adhesion into grafts made of Elastollan® and untreated PCU. Details for usage of the bioreactor see reference #29 and text. After 3 days under pulsatile flow, cell density on fibronectin-coated Elastollan® grafts (positive control) was significantly higher than on untreated PCU grafts (a). Nuclei from adherent cells were round (circularity; 1 represents a circle) (b). Endothelial cells formed a confluent monolayer. Cells arranged in flow direction (c). Immunofluorescent staining of fixed cells: blue, DAPI, red, rhodamine-phalloidin; green CD31-FITC.

representative REM photographs in Figure 6(c). The median cell density was quantified by computation of DAPI-stained nuclei per microscopic field (Figure 6(d)). Median cell density on modifications A and IH was about half of the positive control (high variation of cell counts). In detail, only one of six EC cultures showed good cell attachment on A-grafts, and three of six EC cultures formed a monolayer on IH-grafts. There was no difference in the circularity of the nuclei from cells on Elastollan®, A-graft, and IH-graft. The shape of the nuclei of remaining cells on E-grafts was deformed—a sign of absent viability (Figure 6(e)).

Discussion

In this study, a multitude of chemical modifications was produced to reduce thrombogenicity and to improve cytocompatibility of PCU. Three of 35 modifications (A, E, IH) fulfilled biological improvements including reduced platelet adhesion, stable EC adhesion and proliferation, and a reproducible manufacturing process. However, under shear stress, the benefit of good EC adhesion/proliferation and satisfactory reproducibility disappeared.

Modification of the PCU surface chemistry combining reduced platelet adhesion/activation and increasing endothelial attachment whilst preserving the mechanical properties would be an elusive goal. Here, 9/35 modifications met both biological requirements. A promising approach was the usage of PEG-based hydrogel coatings alone or conjugated with ATIII and ECM proteins/peptides. PEG is biocompatible with blood and tissue, nontoxic to cellular systems, nonimmunogenic, an excellent conjugate for polymer graft materials, and has been approved by the FDA.[39,40] Therefore, PEG hydrogels have been widely used as a supporting matrix in almost every field of tissue engineering (nerve, cartilage, liver, pancreas, bladder, skin, heart). The reduced platelet binding capacity resulted from its inert surface and low protein adsorption.[41,42] The additional binding of anticoagulants had no synergistic anti-thrombogenic effect. Furthermore, the PEG-based hydrogels allowed EC adhesion. Single coating with PEG-diamine hydrogels even allowed EC proliferation. Furthermore, the manufacturing process of PEG-diamine hydrogel coating to plasma-activated PCU was highly reproducible. Similar results were presented for smooth muscle cell attachment to the surface of the PEG-genipin hydrogels[17] and cultivation of neural cells.[43–45] Additional conjugation with ECM peptides/proteins (e.g. RGD-peptides, fibronectin, laminin) had no synergistic effect on EC adhesion. This was in contrast to other studies using functionalized PEG hydrogels. Binding of RGD increased migration/adhesion of smooth muscle cells and allowed the construction of artificial blood vessels.[46,47] We supposed that the heterogeneous data from functionalized PEG-hydrogels resulted from different preparation strategies of PEG-based hydrogels, various conjugation protocols and the pretreatment and type of the underlying polymer. Nevertheless, the potential of EC adhesion might further aggravate bacterial contamination to reduce risk of infection of such blood contacting surfaces.[48]

Of special interest for improved antithrombogenicity of cardiovascular devices was the immobilization of anticoagulants (e.g. heparin). In contrast to other studies, our binding strategies of heparin and Argatroban suppressed their anticoagulant function. Independent of pretreatment process (except silanization), conjugation with ATIII resulted in a reduced platelet adhesion. The manufacturing process that ensures active ATIII function was highly reproducible for PEG-based and

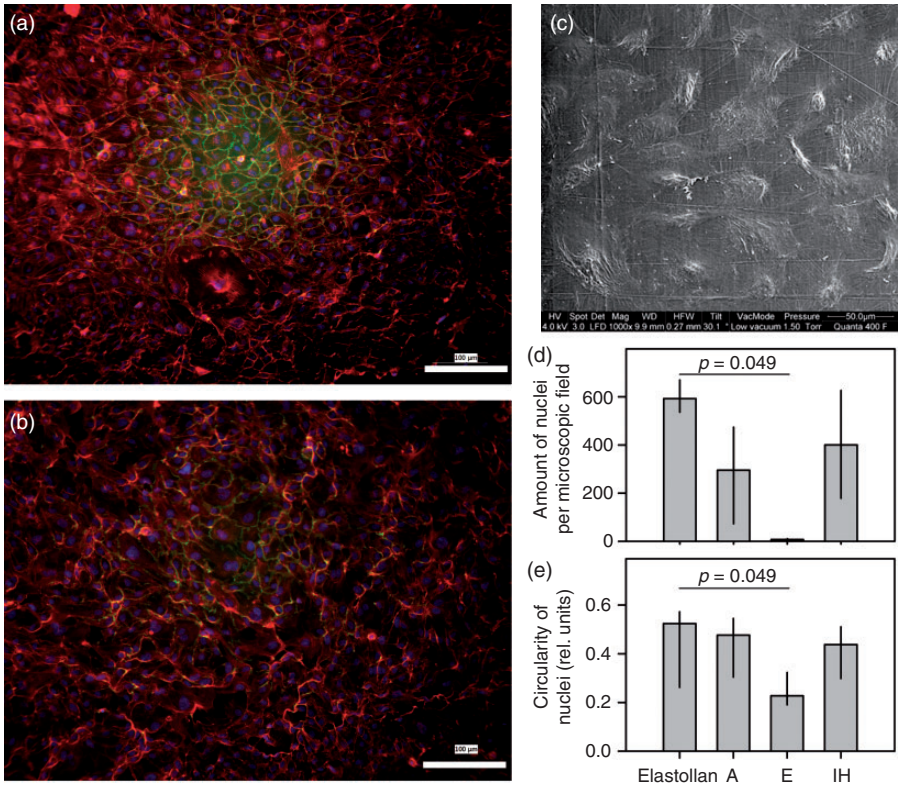

Figure 6. Effect of shear stress on endothelial cell coverage on modified PCU.
For details of protocol and abbreviations see "Materials and methods" section. After 3 days, endothelial cells formed a cobblestone-like monolayer on the luminal surface of IH- (a) and A-grafts (b). However, arrangement of the cells was different to Elastollan® grafts (Figure 4(c)). Staining was described in legend of Figure 4. Integrity of the monolayer was disrupted as shown in a representative SEM photograph from IH-grafts (c). Cell density (verified as described in legend of Figure 5) (d) and circularity of the nuclei (e) differed to data from Elastollan® grafts.

PEI hydrogels. The primary goal of this study was the creation of surfaces that prevent platelet adhesion and allow EC adherence as well. Therefore, EC-adhesive properties of 4/5 ATIII binding strategies were analyzed. Unfortunately, ATIII binding strategy to PEG, PEG-CM-Dex, and CM-Dex hydrogels resulted in high variability of EC coverage. Furthermore, the PEI hydrogels conjugated with ATIII showed cytotoxic effects for EC. This was surprising because in this study PEI alone was not cytotoxic. Nevertheless, under in vitro conditions PEIs induced molecular weight dependent cytotoxic effects.[49] There was no convincing explanation for cytotoxic effects of ATIII-modified PEIs. Finally, only direct coating of ATIII to plasma-activated PCU was a reproducible method for EC adhesion, but critical for reproducible low platelet adhesion (as discussed above).

The last interesting surface modification in this context comprised PEI hydrogel in combination with heparin binding. EC not only adhered and proliferated on PEI-heparin surfaces but also the manufacturing process was highly reproducible regarding EC adhesion. Reproducibility of low platelet adhesion was acceptable. PEI-heparin nanogels were already used as gene delivery system in ovarian cancer cells[50] that availed its excellent blood compatibility and low cytotoxicity.[51]

Only three PCU modifications (A, E, IH) fulfilled biological improvements including reduced platelet adhesion, stable EC adhesion and proliferation, and a reproducible manufacturing process. In cardiovascular tissue engineering applications, biocompatibility control also required analysis of shear forces on the retention of the cells on the luminal surface of the bio-engineered vessel. Placement of endothelialized grafts into the arterial tree resulted in the removal of seeded cells because of high fluid shear forces applied to the graft lumen. Retention of a complete and stable EC layer on the lumen of vascular grafts in the presence of shear stress is an important prerequisite for long-term patency and prevention of thrombus formation.[52] Therefore, a bioreactor was used to evaluate the strength of EC adhesion onto modified PCU surfaces within a vascular graft under pulsatile flow. In a previous study, it was already shown that a stable

endothelialization can be achieved at pulsatile flow within the fibronectin-coated Elastollan® graft.[37] As shown now, EC seeded onto this surface (reference material) not only formed a confluent monolayer but also rearranged to align themselves to the direction of flow, thus establishing a characteristic polarization that is observable both in vivo and in vitro.[53–55] However, the Elastollan® material did not fulfill the mechanical properties required for usage in arterial grafts or a pump chambers in ventricular assist devices (data not shown). Instead, the PCU from the present study was more compatible as graft material, but after seeding of EC onto untreated and modified PCU grafts, shear stress caused loss of cell-cell-interactions of ECs which resulted in partial or complete removal of the cells. Similar results were shown by Inoguchi et al.[30] Human umbilical vein ECs seeded onto compliant small-diameter grafts made of poly(L-lactide-co-epsilon-caprolactone) fiber meshes got lost after exposure to high shear forces (>9 dyne/cm^2). The shear forces used in the present study (6 dyn/cm^2) did not cause cellular damage because the EC monolayer on reference material remained stable and exhibited polygonal cobblestone morphology with aligned cells in flow direction. Therefore, it could be speculated, that the anchorage of the EC onto the selected modified PCU was too weak and the cells got lost under shear stress. Only PCU grafts with the IH-modification remained attached after 3 days under pulsatile flow. Nevertheless, the monolayer was incomplete and the cells were not arranged in flow direction. The denuded areas may lead to thrombus formation when implanted in the vascular network.[52,56] Restricted polarization seems to be an indicator for missing shear force resistance.[55] Attempts to seed ECs on current vascular prosthesis materials remains problematic. Despite different strategies to modify scaffold materials such as binding of cell-adhesive proteins such as fibronectin, laminin, and collagen VIII, the exposure to shear stress in vivo resulted in detachment of EC and clot formation.[57,58] Promising results included titanium-coating[37] and usage of nanocomposite biomaterial-based vascular grafts.[59] Used PCU grafts were modified with RGD-peptides and demonstrated under static and dynamic conditions a relatively rapid endothelialization from endothelial progenitor cells. In addition to a compatible surface treatment for stable endothelialization, control of shear forces is inalienable in biocompatibility analysis of bioengineered grafts and vascular assist devices.[55] Future studies must include not only static tests but also standardized bioreactor systems that produced physiological flow streams before implantation into animals or humans.[60]

The present study has some limitations. We used primary human endothelial cells instead of blood-derived progenitor cells[59] that might be an attractive cell type to demonstrate in vivo endothelialization of implantable cardiovascular grafts.[61] Furthermore, our study focused on the biological characteristics of the surface modifications. Due to the fact, that none of the introduced modifications fulfilled our requirements, physicochemical properties were not analyzed.

Conclusion

Bio- and hemocompatibility testing of plasma-treated and chemically modified biomedical-grade PCU exhibited promising surface modification that permitted platelet adhesion and improved endothelialization. However, improvement of EC adhesion as detected under static culture conditions failed after introduction of shear stress. This study suggests the importance of including mechanical forces in terms of endothelial retention and ability to resist circulating blood elements under physiologic conditions. Functionalization of PCU to promote stable endothelialization for bioengineered vascular grafts or ventricular pump chambers required further scientific input with special consideration of shear stress.

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