Kiriaki Athanasopulu[1], Larysa Kutuzova[1], Joana Thiel[1], Günter Lorenz[1] and Ralf Kemkemer[1, 2*]

Enhancing the biocompatibility of silicone-polycarbonate urethane based implant materials

Abstract: Polyurethane-bases block copolymers (TPCUs) are block-copolymers with systematically varied soft and hard segments. They have been suggested to serve as material for chondral implants in joint regeneration. Such applications may require the adhesion of chondrocytes to the implant surface, facilitating cell growth while keeping their phenotype. Thus, aims of this work were (i) to modify the surface of soft biostable polyurethane-based model implants (TPCU and TSiPCU) with high-molecular weight hyaluronic acid (HA) using an optimized multistep strategy of immobilization, and (ii) to evaluate bioactivity of the modified TPCUs in vitro. Our results show no cytotoxic potential of the TPCUs. HA-bioactive molecules (Mw =700kDa) were immobilized onto the polyurethane surface via polyethylenimine (PEI) spacers, and modifications were confirmed by several characterization methods. Tests with porcine chondrocytes indicated the potential of the TPCU-HA for inducing enhanced cell proliferation.

Keywords: PURs, surface modification, hyaluronic acid.
https://doi.org/10.1515/cdbme-2019-0114

1 Introduction

The development of biocompatible polymer materials is one of the key challenges in biomaterial engineering. One general strategy is to modify the polymer surface with biocompatible molecules, such as extra-cellular matrix.
Such approaches, realized often via combination of grafting and coating techniques, allow the creation of polymer surfaces with improved biocompatibility, and application-appropriate mechanics and friction characteristics [1; 2]. One promising class of polymers are polyurethane-based block copolymers (TPCUs) with systematically variable soft and hard segments, thus tunable mechanical properties. These polymers have been suggested to be interesting biomaterials. Several reports show also their principle usability for chondral implants in joint regeneration. [2; 3]
In such applications, the improvement of cell adhesion and proliferation, for instance of chondrocytes, on the surface of the polymer, may be an attractive option for creating functional implants. Therefore, functionalization of segmented thermoplastic polyurethanes (TPU) was reported using bioactive heparine and hyaluronic acid (HA) [4; 5]. Highly functionalized TPU-HA materials with an immobilization density of 2.3 µg/cm^3 had a notably bioactivity [4]. Despite previous work on the functionalization of TPUs, different effects on the functionalization efficiency are still not fully understood due to structural complexity and high sensitivity towards even small changes in the synthesis conditions.
Therefore, we systematically modified the surface of novel soft biostable polyurethane-based implants (TPCU and TSiPCU) with high-molecular weight HA using an optimized multistep strategy of immobilization, evaluated the cytotoxicity of the materials, and investigated the responses of chondrocytes on the modified TPCU. Notably, the surface modification by PEI and HA suggests that the surfaces may facilitate low bacterial and platelet adhesion as well as enzymatic degradation. [5; 6; 7]

2 Materials and Methods

2.1 Polyurethane Samples

The core material is based on a novel soft polycarbonate urethane surrounded with long polydimethylsiloxane chains (PC-PDMS-MDI-BD block copolymer) which was developed by G. Lorenz *et al.* [8]. Two different thermoplastic polycarbonate polyurethanes (TSiPCU and TPCU) with and without polydimethylsiloxane (PDMS) in the backbone were used. The samples had a disk geometry of 40 mm in diameter

***Corresponding author: Ralf Kemkemer[1,2*]**
1. Applied Chemistry, Reutlingen University, Reutlingen, Germany
2. Max Planck Institute for Medical Research, Heidelberg, Germany, Ralf.Kemkemer@Reutlingen-University.de
Kiriaki Athanasopulu[1], Larysa Kutuzova[1], Joana Thiel[1], Günter Lorenz[1]; Kiriaki.Athanasopulu@Reutlingen-University.de.

and 6 mm thickness. For surface modification specimens were cut into equal pieces (length: 20 mm; width: 2mm) or cylinders with 2mm diameter and cleaned by 2-Propanol for 5 min to remove chemical residues. After washing, the samples were dried to a constant weight at 40°C under vacuum for 24 hours.

2.2 TPCUs surface modification

The optimal reaction conditions for wet-chemical surface modification with methylene diphenyl isocyanate (MDI, Sigma Aldrich) were established by determining a suitable solvent mixture for the PURs. The swelling behavior of PUR in heptane (Sigma Aldrich) compared to toluene (Sigma Aldrich) is low, resulting only in the desired surface swelling. Because of the low solubility of MDI in heptane, the optimum condition was determined by adding a minimum volume of toluene, until the MDI was dissolved. The PURs were pretreated in various toluene/heptane mixtures. The dry (24h at 40 °C, under vacuum) and weighed samples (cylinder with Ø=2mm) were stored in the different solutions for 24 h at 50°C to simulate reaction conditions during MDI activation. After the excess surface solution was removed with filter paper, the swollen samples were weighed again, and the swelling ratio was calculated as following (Eq. 1):

$$\Delta m = \frac{m - m_0}{m_0} \times 100\% \quad (1)$$

where Δm is the swelling ratio, m and m_0 are the weight of the swollen sample, respectively the dry sample.

Then, the PU surface was activated by chemically grafting of MDI to introduce free-NCO-groups. Samples were immersed into a solution consisting of 1% MDI, 1% Triethylamine as catalyst in a toluene/heptane mixture (2:3, v/v) under nitrogen purge at 50° C for 30 min.. After NCO-activation, samples were washed 3 times in a toluene/heptane solution to remove unreacted MDI. The samples were dried at 40°C under vacuum for 24 hours.

Afterwards, PEI was incorporated on the TPCU-NCO surface. Samples were immersed at 50° C for 24 h under nitrogen purge, in a solution of 2-Propanol/ Triethylamine (1:1, v/v) containing 6 % w/v branched PEI (Mw: 800 g/mol; Sigma Aldrich) to reach the saturated surface density of primary amino groups. Samples were washed with 2-Propanol and dried to constant weight at 40°C under vacuum. These products are referred as TPCU-PEI and TSiPCU-PEI.

Hyaluronic acid was eventually grafted on the PUR-PEI surface. HA (Mw: 700K, Lifecore), was mixed in a solution of 10 mg/ml HA in 0.05 M MES buffer (2-(N-morpholino)ethanesulfonic acid; pH 5.4; Thermo Scientific), combined with 200 mM EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, Thermo Scientific) and 50 mM NHS esters (N-hydroxysuccinimide esters; Thermo Scientific) and was used for the reaction with the amino- functionalized samples. After 24 h the HA solution was removed and the samples were rinsed thoroughly with water.

2.3 Cell culture and cytotoxicity assay

The biocompatibility of the selected bulk materials tested according to DIN EN ISO 10993-5. Cell-medium extracts of the sterilized samples (extraction ratio: 0.1 g/ml) were tested using L929 mouse fibroblasts cell line (ACC 2, DSMZ). High density polyethylene (HDPE) was used as negative control, while Pellethane 80A served as reference for biocompatibility. Extract medium (DMEM with 10% FBS and 1% PEN/Strep) containing 1% sodium dodecyl sulfate (Sigma Aldrich) was used as positive control. The extractions were carried out under standardized conditions for 72 h at 37 °C, 5% CO_2 and gyratory shaking. Cells were seeded in a 96-well plate ($1x10^4$ cells/well) in a humidified incubator (Heraeus, Thermo Fisher Scientific Inc., Germany) for 24 h at 37 °C and 5% CO_2. Subsequently, medium was replaced by the extracts and controls. Cells were allowed to grow for overnight. Cell proliferation was measured by an MTS assay (CellTiter 96® Aqueous One Solution, Promega). Experiments were repeated three times with three replicates for each extract. The Proliferation was normalized corresponding to HDPE samples. *In vitro* tests of HA-coated samples were performed with chondrocytes, isolated from porcine cartilage. DMEM / Ham's F-12 in a volume ratio of 2:3, supplemented with 0.15 mM Ascorbic acid 2-phosphate sesquimagnesium salt, 10% FCS and 1% PEN/Strep was used as cell growth medium. Cells were cultured in a humidified incubator for 24h at 37 °C and 5% CO_2. Phase contrast images were acquired by an A-Plan 10×, 0.25 Ph1 objective (Zeiss).

2.4 Characterisation methods

Young's modulus was determined in accordance to DIN EN ISO 527-2. The mechanical properties of individual modification steps were performed using a Zwick tensile testing machine with a 10 kN load cell. Microtensile test bars of type 5A (test length 20 mm) were used, which were punched out of injection-molded discs. Test speed was set as 1 mm/min. Young's modulus was the slope of a linear regression of the stress-strain curve. Tensile strength and elongation at break were determined at a test speed of 100 mm/min.

Contact angle measurements were performed using a Drop shape analysis system DSA 10 MK2 (Krüss). 3 µl deionised water droplets were placed on the surfaces. Young La Place

equation was used for sessile drop fitting. Measurements were carried out at room temperature and repeated 6 times for each sample.

Surface density quantification: The surface density quantification of immobilized HA was determined by a modified HA sandwich Elisa Assay (Echelon Bioscience). The cylindrical HA-functionalized samples were used instead of the assay detection plate. Further enzymatic reaction were performed according to the manufacturer's recommendations.

3 Results

Biocompatibility tests of the bulk polymer samples used for further modifications show that cell growth with TCPU extracts is comparable to reference and control samples suggesting no cytotoxic potential of the selected polyurethane samples (Fig. 1).

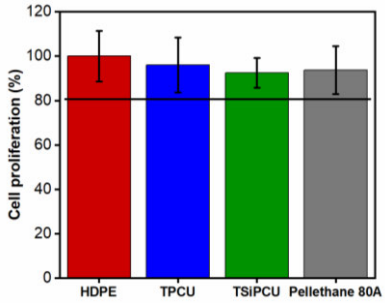

Figure 1. Relative cell proliferation according to MTS assay.

The swelling ratios of PURs in the tested solvents are summarized in Table 1. The swelling of PUR in heptane compared to toluene is significantly reduced, resulting only in the desired surface swelling. To obtain a homogeneous 1% MDI solution the optimum mixture of toluene/heptane was 2:3 (v/v). Moreover, due to adding of heptane, the swelling of the polymer is inhibited in comparison to using the pure toluene solvent.

Table 1: Swelling ratios of PURs in different solvent mixtures

Solvent	TPCU (%)	TSiPCU (%)
Toluene	47.1 ± 0.3	51.0 ± 2.3
Heptane	6.3 ± 0.4	3.4 ± 1.1
Toluene/heptane (2:3)	8.9± 0.3	10.8± 0.4

The mechanic property of PUR-NCO after MDI modification was determined. Dynamic tensile tests show that the modified polymers have similar mechanical properties to the polymeric base material (Fig. 2). The stress-strain curves overlap within the 100% strain (Fig. 2A). As shown in Fig. 2B, the elastic modulus is constant around 13 MPa. The tensile strength is slightly reduced by 3 MPa of the modified sample.

Next, PEI was bound to the MDI-activated surface. Contact angles were measured for both PEI surface modified PURs.

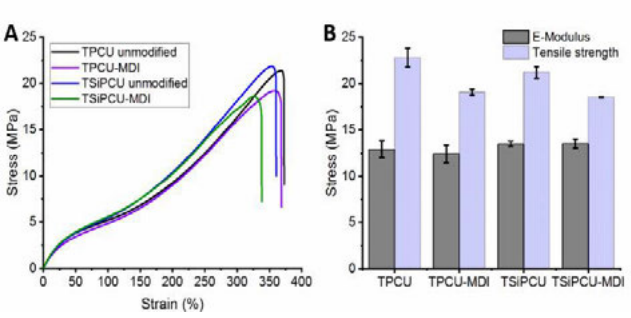

Figure 2. Tensile tests of unmodified and NCO-activated PURs. A. represents the exemplary stress-strain curves of TPCU or TSi-PCU before and after MDI modification. B shows the slopes of the stress-strain curves after linear fitting within the first 30% strain in grey. Moreover, the tensile strength at break is shown in purple.

The images in Fig.3A-C show a significant reduction of the contact angle for the modified samples. Contact angles of the untreated hydrophobic polymers are on average about 100°, of PEI modification leads to a decrease of contact angle of 70° for TPCU and 76° for TSiPCU (Fig. 3D).

Immobilization of high molecular HA on the polymer surfaces led to further increase of the sample wettability in an aqueous environment. The contact angle after coating with HA was 46 ° for both TPCU and TSiPCU (Fig. 3D).

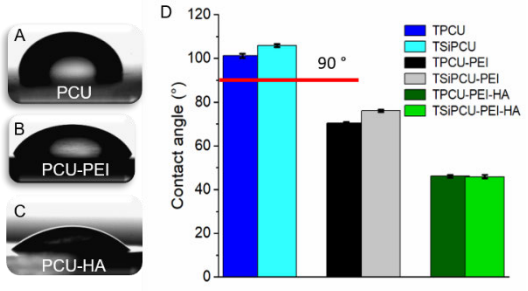

Figure 3. Contact angle for the two samples before and after PEI and HA-modification. A-C are exemplary contact angle images of pure PCU and PEI and HA modified PCU. D summarizes the contact angles of TPCU or TSiPCU before and after modifications.

The polymers were characterized by measuring the swelling ratio in water. After 48 h storage, the equilibrium swelling occurs, average maximum swelling was then 0.7% (TSiPCU), 1.51% (TSiPCU-PEI), and 0.99% (TPCU), and 1.53% (TPCU- PEI) respectively. The swelling ratio increased after PEI surface modification for both TPCU and TSiPCU. Generally, the increase of swelling in TSiPCU block copolymers is lower compared to the pure PUR due to the hydrophobic PDMS soft segment.

The surface density of the immobilized HA was approximated using a modified sandwich Elisa assay. A study of Chuan et al. [3] showed that increasing solution concentration and molecular weight of HA result for in an increase of hyaluronic acid on the activated PUR surfaces. In our tests, the immobilization of HA with the molecular weight (700 kDa) and the solution concentration (10 mg/ml) averaged a surface density of 130 ng/cm^2. For both polymer modifications, statistically similar HA densities were detected on the PUR surfaces.

Our cell culture assays show that chondrocytes isolated from porcine cartilage adhere on the TPCU-PEI-HA substrates and proliferate over 7 days (Fig.4). Cells cultured on untreated TPCU and TPCU-PEI show reduced cell adhesion and proliferation. Quantitatively, this observed cell behaviour was supported by the results of the cell viability experiments. There, cells show a significantly increased cell viability on the PUR-PEI-HA substrates after 4 and 7 days compared to the non-modified or PEI-modified surfaces of polymers (Fig. 5).

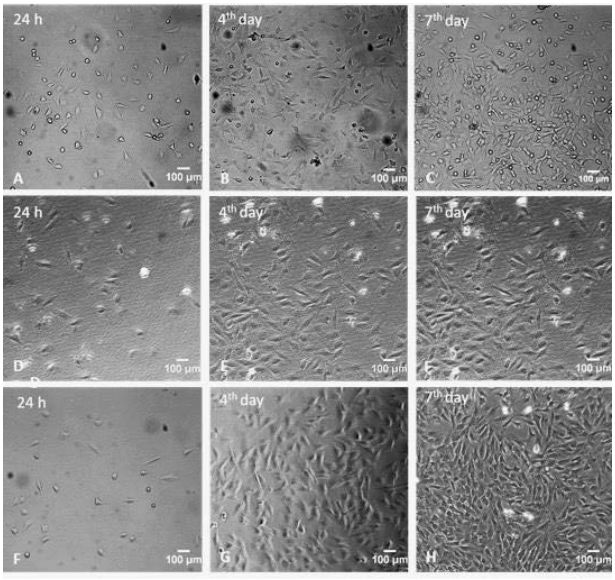

Figure 4. Exemplary microscopy images of primary porcine chondrocytes cultured on TPCU, TPCU-PEI and TPCU-PEI-HA at 24h, day 4 and day 7 after cell seeding.

In summary, in order to improve the biocompatibility of novel silicone-polycarbonate urethane based implant materials, we systematically optimized a strategy for immobilizing HA on the polymer surface. First cell experiments show an enhanced growth of chondrocytes on the HA-modified PUR surfaces.

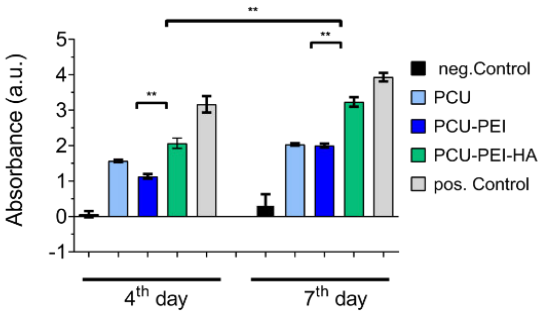

Figure 5 Resazurin assay results of the metabolic activity of primary porcine chondrocytes cultured on the differently modified samples represented as multiples of day 0. The measurements were performed on days 0, 4 and 7. (n=3; p<;0. 05)

Author Statement

Research funding: Financial support by BMBF under grand number 01EC1406C (project TOKMIS) is gratefully acknowledged. Conflict of interest: Authors state no conflict of interest. Informed consent: not applicable. Ethical approval: The conducted research is not related to either human or animal use.

References

[1] Ruiz A, Rathnam KR, Masters KS.; Effect of hyaluronic acid incorporation method on the stability and biological properties of polyurethane–hyaluronic acid biomaterials; J. Mater Sci. Mater Med. 2014; 25:487–498

[2] Andrzej B. Jozwiak, Cay M.Kielty, Richard A. Black; Surface functionalization of polyurethane for the immobilization of bioactive moieties on tissue scaffolds; J. Mater. Chem., 2008, 18: 2240-2248

[3] Imran Khan, Nigel Smith, Eric Jones, Dudley S Finch, Ruth Elizabeth Cameron; Analysis and evaluation of a biomedical polycarbonate urethane tested in an in vitro study and an ovine arthroplasty model. Biomaterials 2005; 26: 633-643

[4] Tzu-Wen Chuang, Kristyn S. Masters; Regulation of polyurethane hemocompatibility and endothelialization by tethered hyaluronic acid oligosaccharides; Biomaterials 2009; 29: 5341–5351

[5] Tan, M., Feng, Y., Wang, H. et al., Immobilized bioactive agents onto polyurethane surface with heparin and phosphorylcholine group, Macromol. Res. 2013; 21: 541

[6] Merve Gultekinoglu, Yeliz Tunc Sarisozen, et al.; Designing of dynamic polyethyleneimine (PEI) brushes on polyurethane (PU) ureteral stents to prevent infections; Acta Biomaterialia 2015; 21: 44-54

[7] Filiz Kara, E. Ayse Aksoy, Zehranur Yuksekdag, Serpil Aksoy, Nesrin Hasirci; Enhancement of antibacterial properties of polyurethanes by chitosan and heparin immobilization; Applied Surface Science; 2015; 357: 1692-1702

[8] L. Kutuzova, Y. Shu, R. Koslik, C. Zhang, G. Lorenz. Tailoring the mechanical properties of implantable polyurethanes by variation in the chain structures, BioNanoMat. 2016,17:149-15