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Sulfated Hyaluronan coating of polyurethane-based implant materials

Abstract: Thermoplastic polycarbonate urethane elastomers (TPCU) are potential implant materials for treating degenerative joint diseases thanks to their adjustable rubber-like properties, their toughness, and their durability. We developed a water-containing high-molecular-weight sulfated hyaluronic acid-coating to improve the interaction of TPCU with the synovial fluid. It is suggested that trapped synovial fluid can act as a lubricant that reduces the friction forces and thus provides an enhanced abrasion resistance of TPCU implants. Aims of this work were (i) the development of a coating method for novel soft TPCU with high-molecular-weight sulfated hyaluronic acid to increase the biocompatibility and (ii) the *in vitro* validation of the functionalized TPCUs in cell culture experiments.

Keywords: PURs, surface modification, sulfated hyaluronic acid, implant coating

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1 Introduction

Recent therapeutic approaches for osteoarthritis focus on the deceleration of the disease progression by using minimally invasive treatment methods before replacing the knee joint by endoprostheses as last therapeutic resort [1,2]. Polymers are potentially interesting materials for knee cushion implants supporting physiological issue regeneration or allowing improved implant performance under high mechanical load. Polyurethanes with systematically variable soft and hard segments are commonly used for load-bearing orthopedic applications like chondral implants. Due to their long-term

biostability and physical properties such as strength and flexibility as well as their tunable mechanical properties they can maintain their function in well-designed cyclic loaded implants for years. However, unmodified TPCUs often show non-specific protein adsorption onto the surface leading to inflammatory responses. Furthermore, insufficient tribological properties of TPCUs may result in the formation of wear debris particles, inducing harmful reactions or impair the biological environment [2]. To avoid such disadvantages, various surface modification techniques have been proposed to alter surface properties without affecting the bulk properties [3,4]. For example, functionalization of joint replacement material surfaces (UHMWPE, TPCU) with native biomolecules such as hyaluronic acid (HA) can improve the bio-compatibility and mimic the natural synovial joint ensuring abrasion resistance of chondral implants [5]. However, it is well known that native HA is susceptible to fast enzymatic degradation, especially in the inflammatory environment of arthritic cartilage joints [3]. To achieve a hydrogel-coated TPCU surface resistance against enzymatic degradation we use high-molecular-weight sulfated hyaluronic acid for the surface modification of soft TPCU (Fig.1). We performed *in vitro* biological tests to investigate the responses of chondrocytes on the modified TPCU, suggesting an improved biological activity.

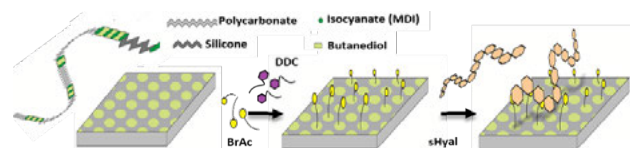


Figure 1: Schematic representation of the functionalization with sulfated hyaluronic acid (sHyal).

2 Materials and Methods

2.1 Materials

Sodium hyaluronate (HA; Mw 700 kDa) was purchased from Lifecore. Dimethylacetamide (DMAc), Tetrahydrofuran

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(THF), Methanol (MeOH), Acetone, were purchased from ABCR. N,N'-Dicyclohexylcarbodiimide (DCC), bromoacetic acid, Sulfur trioxide N, N -dimethylformamide complex (SO₃-DMF), Tetrabutylammonium hydroxide (TBA), phosphate buffer saline (PBS), Click-iT-EdU Alexa Fluor 594 imaging kit and the ion exchanger Dowex 50W-X8 were purchased from Thermo Scientific.

2.2 Activation of Polyurethane samples

Within the scope of this study, a newly developed thermoplastic silicone-polycarbonate-urethane (TPCU) surrounded by long polydimethylsiloxane chains was used as a base material described in previously [7,8]. For modification with sulfated hyaluronic acid (sHyal), the TPCU-elastomers were functionalized with bromoacetic acid (BrAc) by the procedure of Magnani et al. [3,4]. Briefly, in a solution of TPCU in DMAc (10% w/w), combined with 0.5M DCC, bromoacetic solution (40% w/v in DMAc) was added dropwise. After filtration, the activated TPCU (TPCU-BrAc) was precipitated in methanol and dried under vacuum. To obtain thin polymer films for further experiments, a TPCU solution (10% w/v in THF) poured into a Teflon petri dish and the solvent evaporated under vacuum (500 mbar). For surface modification, specimens were cut into cylinders with 3,5 mm diameter and cleaned by 2-Propanol to remove chemical residues.

2.3 Sulfation of Hyaluronic acid and immobilization on TPCU surface

For the synthesis of high-sulfated hyaluronic acid (sHyal), the SO₃/DMF-complex was used as a sulfation agent. To dissolve HA in DMF, the native, water-soluble sodium hyaluronate first needed to be converted into the organic soluble tetrabutylammonium hyaluronate (HA-TBA) salt. Therefore, a sodium hyaluronate solution (0.5 % w/v in deionized water) was mixed with the ion-exchange resin Dowex (25% w/v), which was previously activated with a 40% TBA solution. After stirring overnight at room temperature, the HA-TBA solution was filtered and lyophilized.

To obtain sHyal with a high degree of sulfation, 4 g of SO₃/DMF-complex was added to 100 ml of an HA-TBA solution (0.5% w/v in DMF) and stirred under nitrogen purge at room temperature overnight. After reaction quenching, by adding 100 deionized water the pH of the solution was adjusted with 0.1 M NaOH (pH 9) for obtaining the corresponding sodium salt of sulfated hyaluronic the acid.

sHyal was then precipitated in acetone and after filtrating and washing, the product was dried at 40 C under vacuum. Afterwards, sHyal was incorporated on the TPCU-BrAc surface. The polymer films were immersed in a solution of 10 mg/ml sHyal in PBS (pH 7.4) at 50 °C to reach the formation of ester bonds between the COO⁻ groups of sHyal and the reactive bromomethyl groups on the polymer surface. After 24 h the sHyal solution was removed and the samples were rinsed thoroughly with PBS. These samples are referred to as TPCU-BrAc-sHyal. Before cell experiments, the cast polymer substrates were placed in a multiwell plate and sterilized with 70% ethanol.

2.4 Cell culture and proliferation assay

In vitro tests of sHyal-coated samples were performed with chondrocytes, isolated from porcine cartilage. Chondrocytes (passage 2) were cultured in a mixture of DMEM/Ham's F-12 (v/v 2:3) growth medium, supplemented with 0.15 mM Ascorbic acid 2-phosphate sesquimagnesium salt, 10% FCS and 1% PEN/Strep. To determine the proliferation rate, cells were seeded on the sample surfaces (20.000 cells/cm²) and cultivated for 24 hours under standard cell culture conditions. EdU staining was performed using the Click-iT-EdU Alexa Fluor 594 imaging kit according to the manufacturer's instructions. Images were acquired by a A-Plan 10×, 0.25 Ph1 objective and counting of nuclei was carried out using the Zen Blue image analysis software (Zeiss Axio Observer).

2.5 Characterization methods

FTIR: ATR-FTIR spectroscopy (Perkin Elmer, Spectrum one) was used to characterize the synthesis of sulfated hyaluronan. Measurements were carried out between 4000 and 700 cm⁻¹ with a resolution of 4 cm⁻¹.

Contact angle: Measurements were performed at room temperature using a DSA 10 MK2 goniometer (Krüss). Prior to examination samples were dried, 3 µl deionized water droplets were placed on the surfaces. Each sample was measured with 10 drops placed at different positions. Young La Place equation was used for sessile drop fitting.

Water uptake: The uptake of water was determined by measuring the amount of absorbed water relative to the dry weight of the films after 7 and 14 days of storage in PBS at 37°C.

SEM: The surface morphology of the samples was observed using scanning electron microscopy (Zeiss DSM 962). Prior to examination the sHyal polymer films were freeze-dried and sputter-coated with a thin gold layer.

3 Results

Before surface modification of the TPCU samples, the sulfation of HA was investigated by FTIR analysis. Fig. 2 shows the spectra of unsubstituted hyaluronic acid, tetrabutylammonium hyaluronate, and sulfated derivative. After ion exchange, the CH₃ and CH₂ valence and deformation vibrations of the TBA are visible in the ranges 3000-2800 cm⁻¹ and 1487-1464 cm⁻¹. The peaks at 1438 cm⁻¹, 1387 cm⁻¹ and 990-850 cm⁻¹ are assigned to the R-O-SO₃⁻ group in sHyal. The decrease in the free primary hydroxyl group band (ν OH) at 3300 cm⁻¹ indicates a high degree of substitution.

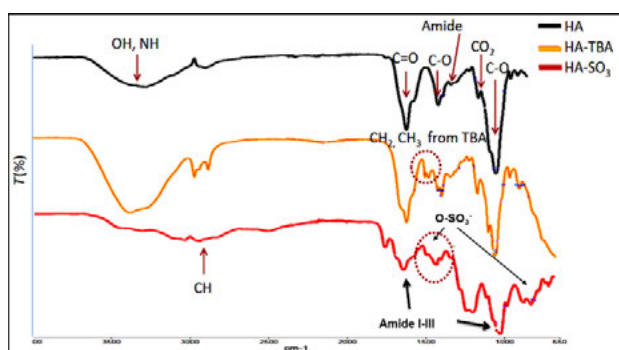


Figure 2: ATR-FTIR spectra of HA (black), HA-TBA (orange) and HA-SO₃ (red).

SEM pictures (Fig. 3 A-C) reveal structural changes of the surfaces after each individual modification step. The images in Fig. 3D-I show significant topography differences after

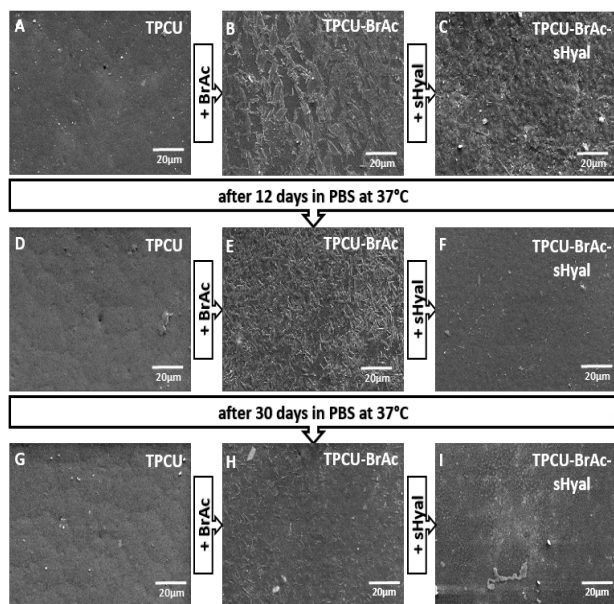


Figure 3: SEM images of the TPCU, TPCU-BrAc and TPCU-BrAc-sHyal films directly after functionalization (top), after 12 d (middle) and 30 d (bottom) storage at 37° in physiological buffer.

storage in a simulated body fluid (12d and 30d in buffer solution at 37°C). The TPCU-BrAc-coated surfaces (Fig. 3 B, E, H) exhibited a rough texture compared to the untreated samples (Fig. 3 A, D, G) due to the aggregation of bromomethyl groups after functionalization. The surfaces showed a smoothed topography after coating with sHyal. As shown in Fig. 3, surface restructuring of the TPCU has occurred and the differences in surface topography become less obvious after two weeks of storage in physiological solution. The untreated hydrophobic TPCU surfaces exhibited a contact angle of around 105°.

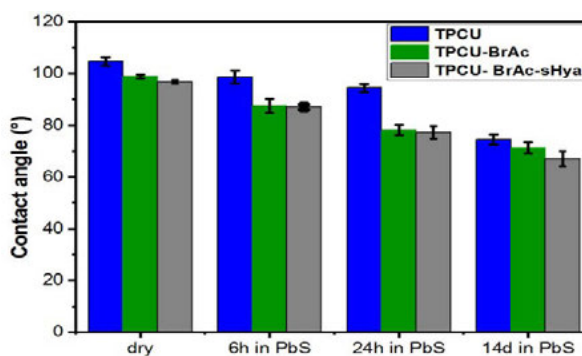


Figure 4: Average contact angles of TPCU (blue), TPCU-BrAc (green) and TPCU-BrAc-sHyal (grey) depend to storage conditions

After modification with BrAc or sHyal, only a slight decrease of contact angle to 95° was observed, implying only a minor hydrophilization and wettability effect of TPCU surfaces. This result may suggest that storage of samples in a physiological buffer solution at 37°C leads to a reorganization of the TPCU microdomain structures. The resulting stabilization of the surface modification can be observed in a decreasing the contact angles of 80° for TPCU and 70° for TPCU-BrAc and 65° TPCU-BrAc-sHyal if stored in PBS immediately after modifying the samples (Fig. 4). Compared to the non-modified material (TPCU), the modified polymers show also a significantly higher water uptake nearly saturation after approximately 7 days (Tab. 1). We suggest that in the aqueous environment, the hydrophilic segments of the bulk polymer (aliphatic polycarbonate, urethane) and the functional groups of the sHyal coating increasingly migrate to the surface, which also increases the surface energy and wettability in an aqueous environment.

Table 1: Water uptake of the TPCU films after 7, 14 days of storage in PBS at 37 ° C related to the dry weight at day 0.

Sample	7d (%)	14d (%)
TPCU	0.73±0.03	0.74±0.04
TPCU-BrAc	1.52±0.12	1.80±0.11
TPCU-BrAc-sHyal	2.22±0.09	2.23±0.10

To assess cellular responses to the coatings porcine chondrocytes were cultured in direct contact with the modified TPCUs for 24h. Cells adhere and proliferate to varying degrees on the surfaces, as shown by the microscope images in Fig. 5. Cells cultured on TPCU-BrAc surfaces (Fig. 5B) show morphological alterations as well as reduced cell adhesion and proliferation. To quantify the cell division rate of the chondrocytes on the three different samples a proliferation assay was performed (Fig. 6). The microscopic images of the fluorescence-labeled nuclei are shown in Fig. 5. Red labeled are only cell nuclei with EDU intercalations (DNA duplication), while all cell nuclei on the sample are marked blue. Cells on the TPCU-BrAc-sHyal substrates show a significantly increased cell proliferation rate compared to unmodified or BrAc-treated TPCU as shown in Fig. 6. The adhesion of the chondrocytes to the sHyal-coated samples is also evident according to the phase contrast microscope image in Fig. 5.

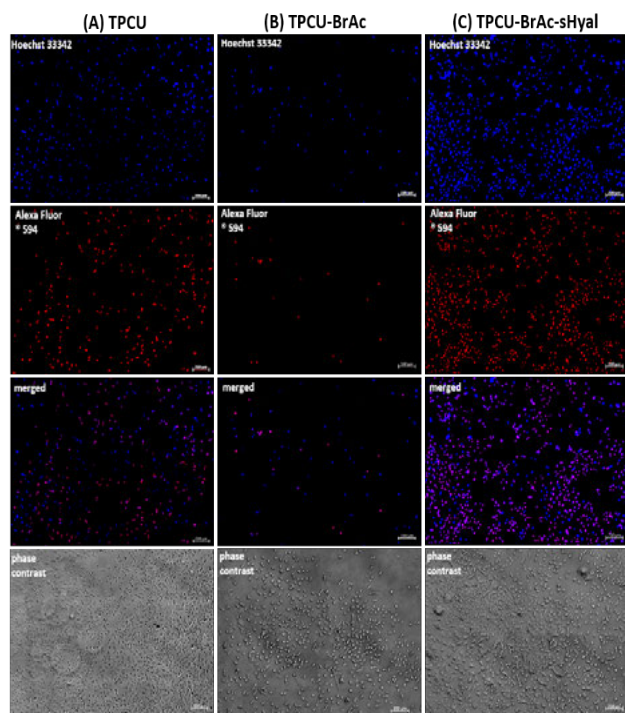


Figure 5: Proliferation assay with chondrocytes after 24 h. (A) TPCU, (B) TPCU-BrAc (C) TPCU-BrAc-sHyal. The cells show an increased proliferation rate on the sHyal coated surface. Red are cell nuclei of the proliferating cells with EDU storage, blue marked all cell nuclei. The scale corresponds to 200 μ m.

In conclusion, we successfully achieved a chemical grafting of sHyal onto the surface of the novel bio-stable thermoplastic polycarbonate-urethane by a straight forward strategy. The TPCU surface was first functionalized with bromoacetic acid, a spacer, bearing bromomethyl groups as anchorage sites. Afterwards, sHyal grafted directly on the TPCU-BrAc surface. In vitro experiments show an enhanced cell proliferation of chondrocytes on the TPCU-BrAc-sHyal surfaces.

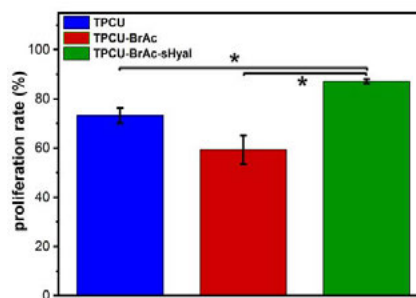


Figure 6: Cell proliferation determined with Click-it EdU Alexa Fluor 594 assay. Error indicators represent the mean and standard deviation of 10 samples with * $p < 0.05$.

Author Statement

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