Sulfated cellulose thin films with antithrombin affinity

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Abstract. Cellulose thin films were chemically modified by *in situ* sulfation to produce surfaces with anticoagulant characteristics. Two celluloses differing in their degree of polymerization (DP): CEL I (DP 215–240) and CEL II (DP 1300–1400) were tethered to maleic anhydride copolymer (MA) layers and subsequently exposed to SO₃·NMe₃ solutions at elevated temperature. The impact of the resulting sulfation on the physicochemical properties of the cellulose films was investigated with respect to film thickness, atomic composition, wettability and roughness. The sulfation was optimized to gain a maximal surface concentration of sulfate groups. The scavenging of antithrombin (AT) by the surfaces was determined to conclude on their potential anticoagulant properties.

Keywords: biocompatible polymers, cellulose, sulfation, maleic anhydride copolymer

1. Introduction

Cellulose is one of the most widely used materials of natural origin [1]. The biopolymer was also one of the first biomaterials used [2] and cellulosic membranes still play an important role in extracorporeal life support [3]. However, the blood compatibility of cellulosic materials is limited [4, 5]. Among the related processes thrombosis is a major problem [6]. Thrombus formation is a life-threatening event resulting from a series of enzymatic hydrolysis steps known as the blood coagulation cascade. The serpin antithrombin (AT) is the most important regulator of the coagulation enzymes under physiological conditions. Its activity is drastically enhanced after complexation with polyanionic polysaccharides such as heparin [7]. Although chemically bound heparin shows a reduced biological activity compared to free heparin [8], the blood

compatibility of cellulosic membranes was found to be improved upon heparinization [9]. Beyond that, partially sulfated cellulose was considered promising for biomaterials coatings [10]. Due to their action on AT, such coatings were demonstrated to reduce the coagulation processes at the blood/material interface [11–13]. Such coatings could be useful for the surface-selective modification of catheters and several other devices temporarily applied in direct blood contact. However, neither the degree of polymerization (DP) of the cellulose chain nor the degree of sulfation (DS) were so far systematically studied with respect to the bioactivity of the obtained coatings. Since these characteristics influence the structure and AT affinity of the cellulose layers knowledge about the relevance of these parameters appeared important for the intended application of this approach in the sur-

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face modification of biomaterials we performed a series of experiments to explore the impact of these features.

Maleic anhydride copolymer (MA) thin films were shown to be a versatile platform for the biomolecular functionalization of solid supports [14]. Previous studies included the covalent attachment of cellulose on MA pre-coatings via an esterification reaction [15]. This approach was applied in the present work to gain thin and stable cellulose coatings using two types of cellulose CEL I (DP 215–240) and CEL II (DP 1300–1400). Based on that, we report a straightforward modification of cellulose layers by in situ sulfation of the cellulosic hydroxyl groups.

A schematic representation of the derivatized poly(ethylene-alt-maleic anhydride) copolymer (PEMA) thin films is displayed in Figure 1. The shown three different types of investigated surfaces – Group A: PEMA-HYDRO, hydrolyzed PEMA; Group B: PEMA-CEL I, II, cellulosic PEMA; Group C: PEMA-CEL I, II-sulf, sulfated cellulosic PEMA ($R=OH$, SO_3^-) – shall present a systematic approach towards heparinoid interfaces. Several reaction parameters (concentration of the sulfation reagent $SO_3 \cdot NMe_3$, reaction temperature, and reaction time) were varied to investigate their influences on the sulfation process and the resulting surface properties. Changes in film thickness, elemental composition, wettability, and morphology were thoroughly investigated. In particular, the

degree of sulfation was assessed from XPS (X-ray photoelectron spectroscopy) data. As a measure of the bioactivity of the films, the AT adsorption onto the sulfated cellulose layers was determined and compared.

2. Experimental section

2.1. Materials

All solvents and reagents were used without further purification. Aqueous ammonia solution (Acros Organics, Geel, Belgium) and hydrogen peroxide solution (Merck, Darmstadt, Germany) were employed for the pre-cleaning of the silicon wafers and glass coverslips.

Poly(ethylene-alt-maleic anhydride) copolymer (PEMA, $M_w = 125\,000$ g/mol, Sigma-Aldrich, Munich, Germany) was used for solid support coating. The immobilized native cellulose types were CEL I (DP 215–240, Aldrich-Fluka Chemicals) and CEL II (DP 1300–1400, kindly provided by Membrana GmbH, Wuppertal, Germany). The solvents dimethyl sulfoxide (DMSO), 4-methylmorpholine-4-oxide monohydrate (NMMO), and dimethylformamide (DMF) as well as the sulfur trioxide-trimethylamine complex $SO_3 \cdot NMe_3$ were purchased from Aldrich-Fluka Chemicals. Human antithrombin (AT) was supplied by Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Normal donkey serum (NDS, Jackson ImmunoResearch Laboratories Inc., USA) was used as blocking

Figure 1. Schematic representation of the surfaces investigated in this study. The modified poly (ethylene-alt-maleic anhydride) copolymer (PEMA) thin films are divided into three groups. Group A: PEMA-HYDRO, hydrolyzed PEMA; Group B: PEMA-CEL I, II, cellulosic PEMA; Group C: PEMA-CEL I, II-sulf, sulfated cellulosic PEMA ($R=OH$, SO_3^-). The comonomer units (ethylene) as well as the linkage to the solid support are not drawn.

buffer. Fluorescein isothiocyanate (FITC) conjugated sheep anti-human AT was purchased from Cedarlane Laboratories LTD (Hornby, Canada). The substances used during the AT adsorption experiments were dissolved in Phosphate buffered saline buffer (PBS, Sigma, Schnellendorf, Germany).

2.2. Surface preparation and modification

Stable copolymer thin films were prepared by spin-coating (RC5, Suess Microtec, Garching) of a 0.15 wt% solution of PEMA in acetone/THF (1/2) onto previously cleaned and amino-silanized silicon wafers (10×20 mm) or glass coverslips (22×22 mm) following a procedure described elsewhere [14]. The two celluloses (CEL I, CEL II) were subsequently immobilized onto the PEMA films. A 0.5 wt% solution of cellulose (CEL I or CEL II) in dimethyl sulfoxide/4-methylmorpholine-4-oxide monohydrate (solution of 40 wt% DMSO and 60 wt% NMMO) was used for immobilization following a published procedure [15]. The polymer-coated (Si wafer or glass) carriers were tempered at 120°C for 2 h just prior to spin coating to regenerate the anhydride form of the copolymers. Subsequently, the carriers (preheated at 45–50°C) were spin coated with the hot cellulose solutions (60 s, 3000 rpm, 1500 rpm/s). The samples were prepared as series at the same temperature guaranteeing equal preparation conditions. After spin coating, the samples were immersed into MilliQ water for precipitation of the cellulose layers. Air drying overnight followed by vacuum-drying for 2 h at 120°C. Solvent residues were removed by immersion of the samples in MilliQ water (twice for 1 h). The samples were dried then under a N₂ stream. Prior to the introduction of the sulfate groups, the cellulose-PEMA samples were dried at 70°C for 1 h in a vacuum oven. The samples were then immersed into a SO₃-NMe₃ solution in DMF and heated up. Three cellulosic samples were treated per different reaction condition. After cooling down to ambient temperature, the samples were washed thoroughly with DMF and immersed into a 0.01 N NaOH solution for 10–15 min. Afterwards, the sulfated films were kept in deionized water overnight, dried under a N₂ stream, and placed at 120°C for 2 h.

2.3. Methods

The layer thickness values were determined by ellipsometry on polymer-coated Si wafers in the dry state. Investigations were carried out on a variable angle spectroscopic ellipsometer M-2000VI (371–1680 nm, Woollam Inc., Lincoln, USA) in a polarizer compensator sample analyzer (PCSA) configuration. Two samples were measured and at least three measurements on each wafer were performed. The two Stokes parameters Δ (phase shift) and Ψ (amplitude ratio) were calculated using an optical 4 phases model (Si/SiO₂/polymer/ambient) [16]. The optical constants of Si and SiO₂ were taken from the literature [17, 18]. The best fit results (without roughness correction) gave the effective refractive indices and thickness values for the overall polymer layer system on the wafer surface.

X-ray photoelectron spectroscopy (XPS) measurements were performed to determine the elemental composition of the polymer films. XPS spectra of cellulose bearing surfaces were recorded on an Axis Ultra spectrometer (Kratos Analytical, Manchester, United Kingdom). Monochromatic AlK α X-rays of 300 W at 20 mA were applied. The kinetic energy of photoelectrons was measured using a hemispherical analyzer (take-off angle of 0°) having a constant energy pass of 160 eV for survey spectra and 20 eV for high-resolution spectra. All spectra were referenced to 285.0 eV (C_{1s} binding energy). Quantitative elemental compositions were determined from the peak areas of the survey spectra using experimentally determined sensitivity factors and the spectrometer transmission function. The high-resolution spectra were deconvoluted by means of Kratos Analytical software. The following parameters were fitted: binding energy, peak height, full width at half-maximum, and Gaussian-Lorentzian ratio of the component peaks.

The surface densities [mol/cm²] of anhydride groups (Γ_{PEMA}) and of cellulose repeating units AGU (Γ_{CEL}) were estimated using a model developed by Pompe *et al.* [19]. Estimations from the elemental composition determined by XPS were executed based on a linear regression analysis assuming a density of 2.85 g/cm³ of the detected SiO₂ from the silicon wafer substrate and a mean

attenuation length of the XPS signal in the substrate and the surface layer of about 3 nm [20, 21]. Within this routine the elemental compositions (C_xH_y , C–OH, COOH, N, Si) of the layer components (SiO₂, PEMA, PEMA-CEL I or PEMA-CEL II) were fitted to the XPS data in a least square sense. The grafting yields (Φ) were defined as the ratio of the surface densities of cellulose units (AGU) and the PEMA repeating units ($\Gamma_{CEL}/\Gamma_{PEMA}$). The degree of sulfation (DS) was calculated from XPS data (C1s and S 2p), Φ and the carbon number of a modified MA unit.

Dynamic contact angle measurements were carried out on an optical contact angle device OCA 30 (Dataphysics, Filderstadt, Germany). Droplets of deionized water were placed onto freshly annealed samples. At least three measurements on each sample were performed. The data presented as mean value and standard deviation.

The surface topography was investigated on one sample using a Dimension 3100 Nano-Scope IV AFM (Veeco, USA). An area of 2×2 μm was measured in tapping mode. The cantilever (Si-SPM-cantilever, BudgetSensors, Bulgaria) had a spring constant of ca. 40 N/m and a resonance frequency of ca. 300 kHz. The tip radius was lower than 10 nm. The mean surface roughness values were calculated using the Nanoscope software.

2.4. Antithrombin binding studies

Antithrombin (AT) adsorbed onto the modified surfaces was detected by immunostaining-fluorescence microscopy. Glass slides bearing sulfated coatings were placed into home-developed screening chambers having a test-material area of 2 cm². Two samples were incubated with a 0.1 U/ml AT solution in PBS buffer for 30 min at room temperature. As reference a third sample was incubated in PBS buffer for 30 min at room temperature. After incubation, the three samples were rinsed twice with PBS, thereafter incubated with 60 mg/ml normal donkey serum solution for 30 min and subsequently washed twice using a 0.1% PBS-donkey serum buffer (PBS-NDS). The surfaces were then incubated with a 7 $\mu\text{g}/\text{ml}$ solution of FITC-conjugated sheep anti-human AT in 0.1% PBS-NDS for 1 h. Finally, the samples were washed twice with 0.1% PBS-NDS, then mounted on microscopy slides, and imaged using a confocal laser scanning

microscope (TCS SP, Leica, Bensheim, Germany) having a 40× oil immersion objective. The microscope settings were kept constant to allow a direct comparison of the fluorescence intensities measured on the different surfaces. The images were quantified using Image J software (freeware, <http://rsb.info.nih.gov/ij/>). The experiments were performed in duplicate and the relative fluorescence intensities are reported as average of at least 5 measurements on each slide. The results are shown as mean value and standard deviation.

3. Results and discussion

3.1. Cellulose-grafted PEMA surfaces

Prior to cellulose attachment, the PEMA pre-coatings had a film thickness of 6.3 ± 1.1 nm. The cellulose immobilization resulted in thin films of 14.9 ± 1.3 nm (PEMA-CEL I) and 19.0 ± 1.5 nm (PEMA-CEL II). The refractive indices (measured at a wavelength of $\lambda = 630$ nm) of PEMA-CEL I (1.56 ± 0.02) and PEMA-CEL II (1.52 ± 0.02) were slightly different.

The atomic composition of PEMA-CEL I and PEMA-CEL II was determined by XPS (see Table 1). Based on detailed XPS investigations previously reported [15], 4 types of carbon atoms with different chemical environment were defined within the cellulosic layers and are listed in Table 2. The related high-resolution C1s spectrum of a cellulose film on a PEMA support is given in Figure 2a. The C1s spectrum deviates from the typical shape expected for cellulosic materials, however similar spectra were previously obtained for

Table 1. Atomic concentrations (at %) of the survey XPS spectra of PEMA-CEL I and PEMA-CEL II (for preparation condition of cellulosic surface see chapter 2.2); binding energies: C1s: 286.7 eV, O1s: 533.0 eV, Si2p : 103.4 eV, N1s: 401.2 eV

	C1s	O1s	Si 2p	N1s
at %PEMA-CEL I	57.8±1.4	38.6±1.1	2.4±0.6	1.2±0.1
at %PEMA-CEL II	57.0±0.2	42.1±0.2	0.4±0.0	0.8±0.4

Table 2. Components of the C1s peak of the cellulosic PEMA thin films, R = organic rest

Components	Binding energy [eV]	Chemical group
A	285.0	aliphatic carbons
B	286.6	–CH(H,R)OH
C	288.1	–O–CHR–O–, CHO
D	289.1	–COOR, COOH, –CO–O–CO–

Figure 2. High-resolution C1s (left) and S 2p (right) XPS spectra of a cellulose film immobilized with PEMA on a support before (a) and after its sulfation (b); the sulfation conditions were $\text{CSO}_3\cdot\text{NMe}_3 = 5 \text{ wt}\%$, 90°C , 3 h

similar sample preparations [15]. Component peak A results from saturated hydrocarbons of the PEMA plus adsorbed hydrocarbon contaminations, which are commonly observed on surfaces. The reacted (ester bonds between cellulose and PEMA) and remaining anhydride groups of PEMA were assigned to component peak D. The two component peaks B and C mainly reflect the $\text{C}-\text{OH}$ and $\text{C}-\text{O}-\text{C}$ (B) and acetal groups of cellulose (C). Autocatalytic degradation of cellulose in NMMO solutions is described in the literature [22]; oxidized cellulose species, such as keto groups are produced and contribute to the component peaks C as well. The corresponding S 2p spectrum does not show any traces of sulfur. The atomic percentages obtained by deconvolution of the C1s signal for PEMA-CEL I and PEMA-CEL II are given in Table 3. The ratio of $\text{C}-\text{OH}/-\text{O}-\text{CHR}-\text{O}$ (component B/component C) is about 4/1 for both types of cellulose. This value differs from the theoretical value (5/1) of pure cellulose and may indicate the occurrence of cellulosic oxidation products which contribute to component C. The cellulose surface

Table 3. Atomic concentrations [%] of the components of the C1s peak in PEMA-CEL I and PEMA-CEL II

Surfaces	A	B	C	D	B/C
	atomic %				
PEMA-CEL I	38.9	42.1	10.7	8.3	3.9
PEMA-CEL II	21.4	59.0	14.5	5.6	4.1

densities obtained from the XPS data are summarized in Table 4. The anhydride density Γ_{PEMA} was calculated to be $1.43 \text{ nmol}/\text{cm}^2$. Surface densities of $\Gamma_{\text{CEL I}} = 0.83 \text{ nmol}/\text{cm}^2$ and $\Gamma_{\text{CEL II}} = 1.88 \text{ nmol}/\text{cm}^2$ were estimated for the PEMA-CEL I and PEMA-CEL II films, respectively. For PEMA-CEL I a grafting yield of 0.58 AGU:PEMA units was calculated whereas a value of 1.30 AGU:PEMA units was determined for PEMA-CEL II. The differences in grafting yield are attributed to the different molecular weight of the cellulose samples with CEL II being approx. 5 times bigger than CEL I. The calculated grafting yield might be considered quite low, however, its normalization to the number of PEMA units is the origin of the low value as only a few PEMA units of the PEMA layer are available for the grafting of the cellulose molecules. Hence, still a good coverage of the surface by CEL I and CEL II can be expected.

The wettability characteristics of the cellulosic films are reported in Table 5. The hydrophilicity of the layered substrates increased upon cellulose attachment: compared to the plain PEMA (65°), cellulosic layers displayed advancing contact angle of 51 and 33° for PEMA-CEL I and PEMA-CEL II, respectively. In comparison, water contact angles of 25 – 30° were previously reported for pure cellulose samples and cellulosic membranes used for hemodialysis were characterized with values of about 33° [23, 24]. The wettability of PEMA-CEL II, close to values of the pure cellulose, seems to indicate an almost complete coverage of PEMA by the high DP polysaccharide. The hysteresis values of 28 and 16° , found for PEMA-CEL I and PEMA-CEL II films respectively, point to a more homogenous surface coverage in the case of

Table 4. Surface densities of disaccharide units Γ_{CEL} , calculated from XPS data [19], S 2p/C1s ratio from XPS measurement (take-off angles $\Theta = 0^\circ$), degree of sulfation (DS) of PEMA-CEL I-sulf, PEMA-CEL II-sulf, and the corresponding sulfate densities of the modified surfaces ($\text{CSO}_3\cdot\text{NMe}_3 = 5 \text{ wt}\%$, 90°C , 3 h)

Surfaces	$\Gamma_{\text{CEL}} [\text{nmol}/\text{cm}^2]$	S 2p/C1s ratio	DS	$\rho [\text{pmol}/\text{cm}^2]$
PEMA-CEL I-sulf	0.83	0.05	0.56	460
PEMA-CEL II-sulf	1.88	0.03	0.41	770

Table 5. Advancing/receding water contact angles and hysteresis values of PEMA-CEL I-sulf and PEMA-CEL II-sulf after 3 and 24 h ($\text{CSO}_3\cdot\text{NMe}_3 = 5 \text{ wt\%}$, 90°C)

Surfaces	Adv. contact angle $\pm$ SD [$^\circ$]	Rec. contact angle $\pm$ SD [$^\circ$]	Contact angle hysteresis [$^\circ$]
PEMA-CEL I	50.7 ± 0.2	22.7 ± 0.1	28.0
PEMA-CEL I- sulf (3 h)	46.4 ± 0.2	19.3 ± 0.3	27.0
PEMA-CEL I- sulf (24 h)	38.4 ± 0.3	17.4 ± 0.2	21.0
PEMA-CEL II	33.3 ± 0.3	17.8 ± 0.2	15.5
PEMA-CEL II- sulf (3 h)	52.9 ± 0.2	15.8 ± 0.3	37.2
PEMA-CEL II- sulf (24 h)	51.0 ± 0.2	17.1 ± 0.1	33.9

PEMA-CEL II. The surface roughness determined from AFM measurements were 2.9 and 2.3 nm for PEMA-CEL I and PEMA-CEL II, respectively. These values are in good agreement with data on cellulose films immobilized from solutions with higher cellulose concentration ($> 0.5 \text{ wt\%}$) [15].

3.2. Sulfation of the cellulosic PEMA surfaces

The cellulose coatings were sulfated using a sulfur trioxide complex with DMF as the solvent. This experimental condition was chosen based on previous results reported for the sulfation of glycosides performed in the liquid phase [25, 26].

The high-resolution C1s and S 2p spectra of a cellulose film recorded after sulfation are reported in Figure 2b. The shape of the C1s spectrum (Figure 2b left) differs only slightly from the spectrum of the non-sulfated sample. Compared to component peak *B* the intensities of the two component peaks *A* and *D* seem to be slightly decreased. The S 2p spectrum clearly shows the presence of sulfur (Figure 2b right). The S 2p peak is composed of the S 2p_{1/2} and S 2p_{3/2} peaks. Their binding energy difference and the intensity ratio indicate that only one sulfur species is present on the sample surface. The binding energy of the S 2p_{3/2} peak, 168.7 eV, is typical for sulfate groups.

The sulfation conditions were optimized on PEMA-CEL I films to achieve a maximal decoration of the surface with the sulfate groups. The concentration of the sulfation reagent $\text{SO}_3\cdot\text{NMe}_3$ in DMF ($\text{CSO}_3\cdot\text{NMe}_3 = 1, 2.5, 5 \text{ wt\%}$), the reaction temperature (60, 90°C) as well as the reaction time (3, 24 h) were varied. No relevant film thickness alterations were detected by ellipsometry even under the most drastic reaction conditions (5 wt% $\text{SO}_3\cdot\text{NMe}_3$ at 90°C during 24 h).

The sulfur/carbon (S/C) ratios of PEMA-CEL I-sulf films calculated from XPS data are displayed

in Figure 3. Variation of the $\text{SO}_3\cdot\text{NMe}_3$ concentration at 60°C resulted only in minor S/C ratio changes (data not shown). The elevation of the reaction temperature had a stronger impact on the sulfation process. For a reaction time of 3 h using 5 wt% $\text{SO}_3\cdot\text{NMe}_3$, an increase of the S/C ratio was found for 90°C (0.046) compared to 60°C (< 0.01). As the solubility of the cellulose in DMF increased with the temperature, a better penetration of the sulfation reagent into the films can be assumed. At the same reaction temperature (90°C), the total S/C value was not dependent on the reaction time: no significant changes could be observed after 24 hours compared to 3 hours. However, angle dependent XPS measurements (take-off angles $\Theta = 0, 60, 75^\circ$) carried out for reactions times of 3 and 24 hours (Figure 4) revealed the chemical composition of the film at different depths. For the shorter reaction time (3 h), a gradient of the S/C could be observed. The number of introduced sulfate groups was declining towards the interior of the film. For a 24 hours reaction time the S/C gradient vanished: surface and interior of the PEMA-CEL I were sulfated in a similar way. We assume that the solubility of the celluloses in DMF is kinetically controlled.

Figure 3. Impact of reaction temperature and reaction time on the sulfur/carbon ratio (XPS measurements) using $\text{CSO}_3\cdot\text{NMe}_3 = 5 \text{ wt\%}$

Figure 4. PEMA-CEL I-sulf ($\text{CSO}_3\cdot\text{NMe}_3=5$ wt%, 90°C): dependency of the S2p/C1s on reaction time (3 and 24 h) at 3 different film depths

As a maximal S/C ratio of PEMA-CEL I-sulf was obtained using a 5 wt% $\text{SO}_3\cdot\text{NMe}_3$, the same concentration was utilized to produce PEMA-CEL II-sulf. As the DP of CEL II was about 6 times the DP of CEL I, a decrease of the S/C value due to decreased solubility in DMF was expected. However, at 60°C , the S/C value of 0.033 (see Figure 3) was about 5 times higher than PEMA-CEL I-sulf which suggests a higher OH abundance at the solid-liquid interface. A marginal increase of the S/C ratio was observed for PEMA-CEL II-sulf when the temperature was increased to 90°C . This observation suggests that an almost complete sulfation of the surface hydroxyl groups was already reached at 60°C . At longer reaction times (24 hours) OH groups within the bulk volume of the film became accessible to the $\text{SO}_3\cdot\text{NMe}_3$ complex resulting in a further increase of the S/C ratio (0.041).

From the atomic concentrations of C1s and S2p (detected by XPS) the degree of sulfation (DS) was estimated for CEL I and CEL II surface obtained using 5 wt% $\text{SO}_3\cdot\text{NMe}_3$ at 90°C for 3 h (Table 4). Lower S/C ratios were obtained for PEMA-CEL II (0.033) when compared to PEMA-CEL I (0.046). These ratios correspond to calculated DS values of 0.41 and 0.56, respectively. Both values remain far below the DS of heparin (2.7) and correspond to about one sulfate group per disaccharide unit (which is similar to the sulfation grade of heparan sulfate molecules) [27]. Based on the DS values, the surface concentrations of the sulfate groups were calculated to be 460 pmol/cm^2 for PEMA-CEL I-sulf and 770 pmol/cm^2 in the case of PEMA-CEL II-sulf.

Low concentrations of $\text{SO}_3\cdot\text{NMe}_3$ (1 and 2.5 wt%) did not influence the advancing contact angle values (CA) of PEMA-CEL-sulf (data not shown). As listed in Table 5 the treatment of PEMA-CEL I with a 5 wt% sulfation solution at 60°C during 3 hours led to a slight increase of the surface hydrophilicity (from 51 to 47°). A comparable value was obtained for the experimental conditions: 5 wt%, 90°C , 3 h. Significant changes were observed after increasing the reaction time from 3 to 24 h. After 24 h, decreased advancing contact angle (38°) and hysteresis (20°) were observed for PEMA-CEL I-sulf. PEMA-CEL II-sulf (5 wt%, 90°C , 3 h) displayed more hydrophobic (53°) and inhomogeneous (hysteresis = 40°) surface properties. After 24 h sulfation, the advancing contact angle decreased but remained above the values determined before sulfation i.e. the introduction of sulfate groups on the surface did not lead, as expected, to an enhanced surface wettability. Since the surface roughness did not change upon sulfation (see below) we assume rearrangements of the amorphous-crystalline structure of the cellulose layers to cause this unexpected behavior.

The AFM height images and the cross-section profiles of PEMA-CEL I-sulf and PEMA-CEL II-sulf (5 wt% $\text{SO}_3\cdot\text{NMe}_3$, 90°C , 24 h) are given in Figure 5. Similar surfaces topographies were obtained for both surfaces. The surface roughness values did not significantly change compared to samples before sulfation.

3.3. Surface binding of antithrombin

In this study, the hydrolyzed polymer layers PEMA-HYDRO were taken as a negative reference (no sulfate groups, no polysaccharidic backbone). The total hydrolysis of the anhydride moieties was achieved by autoclaving at 120°C , under saturated water vapor during 20 min. The PEMA CEL I-sulf and CEL II-sulf were prepared under the sulfation conditions: 5 wt% $\text{SO}_3\cdot\text{NMe}_3$ in DMF, 90°C , 3 h.

The amount of antithrombin (AT) bound to the hydrolyzed and derivatized MA substrates was estimated by immunostaining-fluorescence microscopy. The fluorescence intensities Λ measured by cLSM are shown in Figure 6. The reference values (black bars) were obtained after incubation with a fluorescent labeled antibody anti-antithrombin (anti-AT) and showed a weak non-specific adsorption of the

Figure 5. AFM height images and cross-section profiles of PEMA-CEL I-sulf (A) and PEMA-CEL II-sulf (B) obtained after treatment using 5 wt% $\text{SO}_3\cdot\text{NMe}_3$ at 90°C for 24 h

Figure 6. Adsorbed antithrombin (AT) on modified and hydrolyzed PEMA surfaces as determined by laser scanning microscopy (sulfation conditions: 5 wt% $\text{SO}_3\cdot\text{NMe}_3$ in DMF, 90°C, 3 h)

antibody on all the surfaces. Surface-scavenged AT was observed on all films. Hydrolyzed MA (PEMA-HYDRO) providing a highly negatively charged surface without glucose moieties or sulfate groups had the lowest AT binding capacity (18 a.u.). A significantly higher AT anchorage has been observed on a PEMA-CEL I-sulf surface (95 a.u.) while the less sulfated PEMA-CEL II-sulf

scavenged comparatively almost 50% (44 a.u.) less AT.

The comparison of the results obtained with sulfated CEL I and CEL II samples clearly demonstrates the importance of the DS for the AT binding: the slightly lower DS of CEL II correlates with a lower AT binding. The total sulfate density of the surfaces does not correlate with the AT binding indicating that only sulfate groups on the top-most part of the surface seem to contribute.

4. Conclusions

Systematic studies on structure-bioactivity correlations of potentially anticoagulant surfaces are required for the rational design of hemocompatible materials. In this work, two celluloses were attached onto poly(ethylene-alt-maleic anhydride) pre-coatings. The grafting of the cellulose depended on the degree of polymerization (DP) of the polysaccharide: CEL II (DP~1350) > CEL I (DP~230). The reactivity of the hydroxyl groups of the surface confined cellulose films with $\text{SO}_3\cdot\text{NMe}_3$ complex was found to be a function of reaction time, reac-

tion temperature, and concentration of the sulfation agent. For the applied sulfation conditions (5 wt% $\text{SO}_3\cdot\text{NMe}_3$ at 90°C for 3 h) the calculated degree of sulfation (DS) of the films was lower for layers consisting of cellulose with higher DP. The cellulose films showed an enhanced antithrombin binding (compared to the highly negatively charged PEMA surface) pointing at the importance of the molecular structure for the affinity. Antithrombin binding was furthermore found to correlate well with the DS of the cellulose sample, but not with the total sulfate content of the layers. The latter observation indicates that only sulfate groups on the topmost surface of the films influence the interaction with the serpin.

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