Design of anticoagulant surfaces based on cellulose nanocrystals

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Heike M. A. Ehmann[a*]**, Tamilselvan Mohan**[b]**, Maria Koshanskaya**[b]**, Sylvia Scheicher**[c]**, Doris Breitwieser**[b]**, Volker Ribitsch**[b]**, Karin Stana-Kleinschek**[d]**, Stefan Spirk**[a,d]*****

The anticoagulant activity of surfaces decorated with cellulose nanocrystals (CNCs) prepared via sulfuric acid hydrolysis, is explored. Such surfaces bear a high amount of negatively charged sulfate groups, which mimic the naturally 10 **occurring anticoagulant heparin in terms of charge density. It is demonstrated that CNC decorated surfaces significantly enhance the coagulation times of blood plasma and whole blood as proven by QCM-D and simple clotting tests.**

15 Cellulose nanocrystals (CNCs) are a fascinating class of cellulose based materials due to their variety of unusual properties and resulting applications.[1-6] The quest for new materials based on CNCs started in 1992 when the liquid crystalline nature of interacting colloidal cellulose suspensions was observed for the 20 first time.[7] At low concentrations, CNCs form an isotropic phase, where the rod like nanocrystals are distributed in the suspension in a random fashion. By increasing the concentration, an alignment of the CNC takes place, which is accompanied by the formation of nematic phases. A variety of interesting phenomena 25 has been observed in these phases such as strong iridescence, optical activity and selective reflection of circularly polarized light.[1, 8] These properties could be relevant in the development of security features in banknotes, as polarization rotators in lasers (e.g. laser surgery), or as optical storage devices for instance. An 30 alignment of the CNCs can also be achieved by applying strong magnetic fields since cellulose exhibits a negative magnetic susceptibility.[9, 10] This negative magnetic susceptibility could be exploited in the controlled therapy of tumors for instance since a magnetic field causes the nanocrystals to heat up, finally 35 destroying pathogenic tissues. Besides these ensemble-based phenomena, the extremely high Young's modulus and stiffness of CNCs (comparable to steel or even Kevlar) have entered into the focus of material scientists with the aim to reinforce organic polymers. Lately, such composites have been shaped into 40 different forms such as thin films, electrospun fibers and foams to mention only a few.[6] In many of these composites, CNCs act as stabilizers providing mechanical strength and stiffness. Since cellulose (and also CNC)[11] is highly biocompatible and biodegradable, the potential of cellulose nanocrystals to be used 45 in medical materials has attracted very recently significant interest (e.g. as injectable hydrogels[12], drug carriers[13], for bioimaging[14]). A very important feature of CNCs in this respect is the wide range of surface functionalities that can be easily introduced into the material. The most widely used functional

50 groups are sulfates, which are created in the course of the preparation of CNC, where sulfuric acid is added to microcrystalline cellulose at low temperatures.[1] This treatment does not only remove amorphous domains of cellulose but also esterifies the surface of the remaining crystallites. When 55 comparing the obtained sulfated CNCs with other naturally occurring polysaccharides, the similarity to heparin is obvious. Heparin is a key player in the body to prevent coagulation of blood and thrombosis via several mechanisms.[15] One important point in this respect is the very high negative charge density of 60 heparin due to the presence of sulfate groups. Particularly for the design of materials that come into contact with blood, *e.g.* artificial blood vessels, dialysis tubings, or catheters, anticoagulant activity and hemocompatibility of the surfaces are crucial issues. Usually, the base materials such as PET (Dacron[R]) 65 or PTFE are subjected to surface treatments (grafting, plasma etching etc.) in order to achieve anticoagulant activity and protein repellency. Protein repellency is a main prerequisite for cathethers or artificial blood vessels since protein adsorption onto the materials would cause clotting and consequently a thrombus 70 leading to severe health problems (*e.g.* thrombosis, infarction).
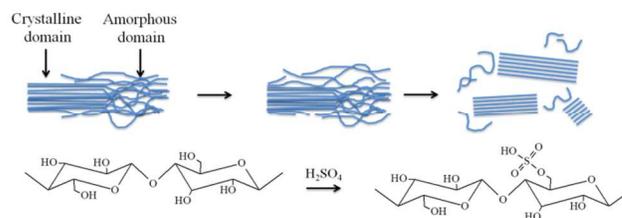
Figure 1. Schematic representation of the preparation of CNC starting from microcrystalline cellulose. The surface esterification reaction to introduce sulfate groups is shown schematically as well (only at C6 due to 75 clarity reasons).

In this contribution, we employ sulfated CNCs to create anticoagulant surface coatings, which could serve as components in medical materials such dialysis tubings. For testing of anticoagulant properties either whole blood or blood plasma, 80 (easier to handle, commercially available) are employed. Sulfated CNCs are prepared using well known literature protocols starting from microcrystalline cellulose and sulfuric acid. The successful introduction of sulfate groups can be easily followed by determinations of the zeta potential of these suspensions.[16] As 85 expected, the CNC suspensions exhibit a highly negative zeta potential over a wide pH range. In order to test the suitability of

CNCs to serve as anticoagulant surface layer, a suitable immobilization method has to be established. As it turns out, the adsorption of sulfated CNCs onto positively charged polyelectrolytes such as branched polyethyleneimine (PEI) is the
5 best option, since these layers can be easily attached onto other surfaces as well.

Firstly, adsorption experiments are performed on glass slides in order to determine the surface charges and the isoelectric points of the so-prepared surfaces by the streaming potential method. As
10 expected, surfaces exhibit a positive charge ($\zeta \sim$ +2 mV at pH 7) after PEI coating (0.5 M KCl) and the isoelectric point for piranha cleaned glass (4.5) is shifted to ca 8.0 in case of the PEI coated surfaces. Adsorption of CNCs (c = 1 mg/ml) on the PEI coated glass slides results in a charge inversion of the surface at
15 neutral pH and shifts the IEP to 2.4 which corresponds to the pK_a of a sulfate group. The ζ of the CNC rendered surfaces is -37 mV (Fig. 2, left), which is in the same range as the anticoagulant heparin features in solution (-39 mV[17]). In addition, the streaming potential measurements also provides information about the
20 stability of the attached layers under oscillating flow conditions, similar to those present in the human body (average time of single blood cell to pass heart ~45 seconds). As expected, the layers are stable on glass and a change of the zeta potential over a time period of 2 hours was not observed on the slides (compare
25 dialysis treatment: 4-5 hours).
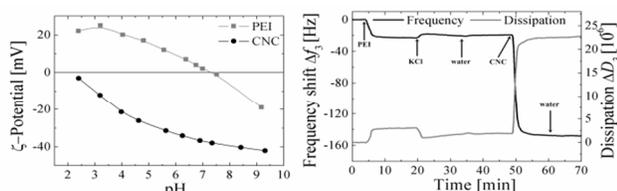
Figure 2- Left: Zeta potential obtained by streaming potential measurements of the modified surfaces. Right QCM-D experiment for the immobilization of PEI/CNC on a QCM-D sensor.

30 On these CNC rendered glass slides a simple coagulation test using whole blood is performed. Fresh blood from a female healthy volunteer (M.K., 40 µl) is placed on a CNC coated slide and is covered with another slide having the same coating. PEI coated slides have been used as control. The coagulation time is
35 then assessed using an optical microscope, where the movements of the erythrocytes are followed. In case of PEI, an immediate clotting occurs (within 20 seconds), which is related to the formation of aggregates of the negatively charged erythrocytes (ζ ca -17 mV[18]) initiated by the positively charged PEI (Fig. 3). In
40 contrast, the negatively charged CNC surfaces exhibit much longer coagulation times of up to seven minutes. It should be noted here that standardized tests concerning the testing of anticoagulant surfaces do not exist so far. A short movie (time span 15 minutes, one picture each 3 seconds, film speed, 3
45 pictures/seconds) showing the coagulation of the erythrocytes is given in the Supporting Material.

Based on these results, a QCM-D adsorption experiment was set up, which allows the determination of the adsorbed mass of material by employing the Sauerbrey equation (rigid films) or by
50 viscoelastic modelling (Voigt-model; soft films). This experiment comprises following steps: (i) adsorption of PEI onto the QCM sensor, (ii) rinsing (KCl and water) of the sensors, (iii) adsorption of an aqueous CNC suspension (c = 0.5 mg/ml) followed by (iv) final rinsing steps (KCl and water) to remove loosely bound
55 material. As depicted in Figure 2, PEI adsorbs as a monolayer (Δf_3 ca. -20 Hz) onto the QCM sensors concomitant with partial swelling that can be observed in the dissipation channel (ΔD_3: ca 3 x 10^{-6}). On these positively charged surfaces, a relatively large amount of negatively charged CNCs is adsorbed at native pH
60 causing a frequency shift Δf_3 of ca -120 Hz (30.4 ±2.2 mg m^{-2}). In contrast to the PEI, the adsorbed layer is highly hydrated and contains a large amount of water which can be clearly seen in the dissipation channel (ΔD_3 ca. 20 x10^{-6}). It is known that the highly crystalline domains of CNC are not accessible to water; therefore
65 it is obvious that the coupled water is coordinated by surface functional groups (e.g. sulfate, hydroxy) of the CNCs.

Besides the determination of adsorbed masses on surfaces, QCM-D can also be used for the determination of coagulation times of blood plasma as demonstrated recently.[19] Using this approach
70 two phenomena can be tracked during the coagulation, *i.e.* adsorption of plasma proteins resulting in a frequency shift as well as the increase in the rigidity of the layer during blood coagulation (*i.e.* conversion of fibrinogen to fibrin) resulting in a dissipation decrease. From these experiments, information of the
75 last two phases in the coagulation cascade, namely the thrombin formation time (TT) and the fibrin clotting time (FT) can be extracted with high reproducibility and the sum of TT and FT is commonly referred to as coagulation time. The formation rate of fibrin can be evaluated as well by analyzing the first deviation at
80 the inflection point of the frequency domain ('fibrin deposition rate', df/dt). In contrast to the adsorption experiments, the test is performed in specially designed open QCM chambers which are kept sterile at 37°C. Via the opening bores, citrated blood plasma is deposited onto the coated surfaces and a solution containing
85 $CaCl_2$ is added to trigger the coagulation cascade. In the initial phase, thrombin is formed (TT) and after reaching a plateau a decay of the frequency takes place, which mainly corresponds to the amount of formed fibrin on the surfaces. A variety of surfaces has been tested using similar setups such as synthetic polymers
90 poly(methyl methacrylate) PMMA, polydimethylsiloxane (PDMS), polyethylene terephtalate, (PET), and biopolymer based coatings such as carboxymethyl cellulose and 6-O-chitosan sulfate (S-Chi).[19-22] The main difference between synthetic polymers and polysaccharides is unspecific protein adsorption
95 onto their surfaces, which is rather low for polysaccharides in general (compare Δf_3 values in Hz: -30 (S-Chi), -100 (CMC), -550 (PMMA), -750 (PDMS), -1400 (PET)). However, the values for synthetic polymers cannot be compared directly since a slightly different setup (lower temperature, way how coagulation
100 was initiated). Referring to these values, the CNC decorated surfaces perform well with Δf_3 of -30±1 Hz indicating low protein adsorption. The fibrin formation rate is very low with a value of -0.9±0.1 Hz/min, which is much lower than those reported for CMC surfaces decorated with S-Chi (average: -6 Hz/min)[22] or
105 heparin@PET (-2.7±0.3 Hz/min).[19] Although the coagulation times can be assessed in the frequency domain, the clotting behavior on surfaces can be alternatively followed in the dissipation channel, which measures the rigidity of the system. In some cases, the dissipation is a better indicator for coagulation
110 times since it is less sensitive towards external influences. The

QCM-D curves obtained for CNC gave a slightly negative ΔD_3 dissipation shift, which means that the layer is more rigid than the film prior to coagulation. For all the other materials tested by QCM-D always (slightly) positive ΔD_3 values have been
[5] reported.[19-22] In terms of blood plasma coagulation time, the CNC rendered surfaces described here indicate coagulation after 9 ± 1 minutes in both domains, which is much longer than for CMC coatings evaluated under the same conditions. The FT is determined to be 6 ± 1 minutes while TT is considerably low (ca
[10] 3 ± 1 minutes). Although reproducibility is rather good, there are variations in the frequency domain compared to other systems (e.g. S-Chi@Ag), which we investigated recently.[22] Probably, this phenomenon is based on the rather high roughness of the substrates, which of course has a certain influence on the amount
[15] of adsorbed proteins onto the surfaces causing variations in Δf_3. However, the total coagulation times are not affected to a large extent by these factors and evaluation of coagulation times by the dissipation channel always leads to very similar results.
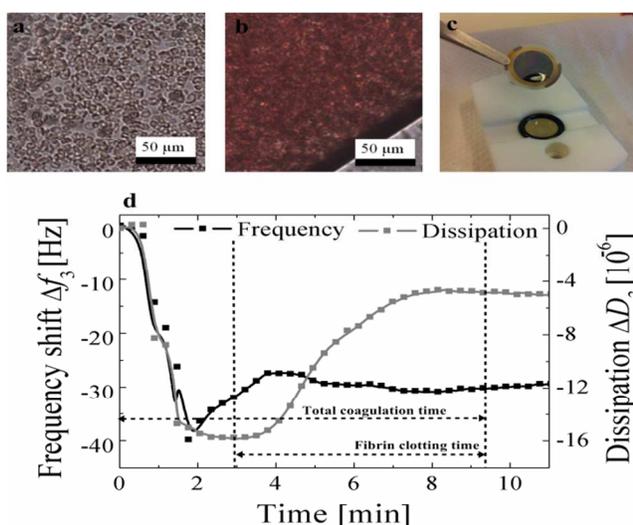
[20]   Figure 3. Optical microscopy images from whole blood contacted with PEI (a) and CNC (b) after coagulation of whole blood as well as a photograph of the QCM-D sensor (c) after blood plasma coagulation. The lower row (d) shows the corresponding QCM-D data.

In contrast, as already shown on glass slides, the positive charge
[25] of the PEI immediately leads to the formation of a clot (data not shown) followed by immediate (within seconds) detachment of the whole films from the QCM-sensor from the surface after contacted with blood plasma.

In summary, sulfated CNCs have been used as coating for the
[30] built-up of anticoagulant surfaces. The sulfate groups on the CNCs are highly negatively charged, which mimics one way of action of heparin, the most commonly used anticoagulant. However, while heparin remains the gold standard in anticoagulant therapy, our results show that sulfated CNCs may
[35] have potential to be used as component in the design of anticoagulant surfaces. A feasible approach would be either to immobilize CNCs onto the surface of polymers (e.g. by electrostatic attraction) or to incorporate CNCs during the shaping/processing of the polymers. Since both areas are well
[40] developed, the possibilities to use such sulfated, biocompatible and readily available CNCs in (bio)medical materials are tremendous and still remain to be explored.

Notes and references

[a] Graz University of Technology, Institute for Chemistry and Technology
[50] of Materials, Stremayrgasse 9, 8010 Graz, Austria. E-Mail: stefan.spirk@tugraz.at, heike.ehmann@tugraz.at. Tel.: +43-316-873-32284.
[b] Institute for Chemistry, University of Graz, Heinrichstrasse 24, 8010 Graz, Austria.
[55] [c] Joanneum Research Materials, Institute for Surface Technologies and Photonics, Franz-Pichlerstrasse 30, 8160 Weiz, Austria
[d] University of Maribor, Institute for the Engineering and Design of Materials, Smetanova Ulica 17, 2000 Maribor, Slovenia.

[60] †ESI available [detailed experimental procedures, atomic force microscopy, viscoelastic properties of the films]

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