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Protein adsorption steers blood contact activation on engineered cobalt chromium alloy oxide layers

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ABSTRACT

Biomaterials upon implantation are immediately covered by blood proteins which direct the subsequent blood activation. These early events determine the following cascade of biological reactions and consequently the long-term success of implants. The ability to modulate surface properties of biomaterials is therefore of considerable clinical significance.

Goal of this study was an in-depth understanding of the biological response to cobalt chromium stent alloys with engineered surface oxide layers, which showed altered body reactions *in vivo*. We analyzed *in vitro* the biological events following initial blood contact on engineered cobalt chromium surfaces featuring said oxide layers. Surface-specific blood reactions were confirmed by scanning electron microscopy and the adsorbed protein layers were characterized by mass spectrometry. This powerful proteomics tool allowed the identification and quantification of over hundred surface-adhering proteins. Proteins associated with the coagulation cascade, platelet adhesion and neutrophil function correlated with the various blood surface activations observed. Furthermore, results of pre-coated surfaces with defined fibrinogen–albumin mixtures suggest that neutrophil adhesion was controlled by fibrinogen orientation and conformation rather than quantity. This study highlights the importance of controlling the biological response in the complex protein–implant surface interactions and the potential of the surface modifications to improve the clinical performance of medical implants.

Statement of Significance

The blood contact activation of CoCr alloys is determined by their surface oxide layer properties. Modifications of the oxide layer affected the total amount of adsorbed proteins and the composition of the adsorbed protein layer. Additionally fibrinogen coatings mediated the surface-dependent neutrophil adhesion in a concentration-independent manner, indicating the influence of conformation and/or orientation of the adsorbed protein. Despite the complexity of protein–implant interactions, this study highlights the importance of understanding and controlling mechanisms of protein adhesion in order to improve and steer the performance of medical implants. It shows that modification of the surface oxide layer is a very attractive strategy to directly functionalize metallic implant surfaces and optimize their blood interaction for the desired orthopedic or cardiovascular applications.

1. Introduction

The success of biomedical devices depends on the biological reactions occurring at their surfaces. The first biological interactions occur during surgery, when blood components – first proteins then cells – contact and react to the implant surfaces [1].

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While a strong thrombogenic response to osseous implant surfaces correlates with better osseointegration and is therefore often desired [2,3], the opposite holds true for cardiovascular stents for which thrombosis and in-stent restenosis (re-narrowing) lead to implant failure [4].

Studying the underlying mechanisms of blood contact activation, it was shown that the biological processes are driven by an instantaneous blood protein adsorption [1]. Barbosa and colleagues elegantly showed that neutrophils specifically adsorb to defined model surfaces *in vivo*. These surface specific cell adhesions were observed also *in vitro* when the surfaces where precoated with specific blood proteins [5,6] emphasizing the importance of protein adsorption on biomaterial surfaces for blood activation. This has lead to an increasing interest in studying protein adsorption on surfaces with defined properties.

In particular, a lot of effort has been put into exploring protein adsorption and blood reaction on highly defined model surfaces. Especially self-assembled monolayers (SAMs) functionalized with controlled moieties (i.e. –OH, –COOH, $–CH_3$ or combinations), allowed the systematic study of surface blood interactions [6–9]. These studies showed that after whole blood incubation, surfaces functionalized with $–CH_3$ groups lead to increased platelet adhesion, while on –OH terminated SAMs only leukocytes adhere. In contrast neither platelets nor leukocytes (including neutrophils) adhere on –COOH presenting surfaces. By carefully investigating changes in plasma protein adsorption onto these surfaces, three aspects of protein adsorption were identified as crucial for blood activation processes: protein quantity [10,11], protein layer composition [12,13] and protein conformation [7,14,15]. Increased platelet adhesion did not correlate with the amount of adsorbed fibrinogen, but with the conformational change of fibrinogen [7,14].

While SAM functionalized surfaces with defined moieties are very useful for studying the consequences of steered protein adsorption on surfaces, their implementation on metallic biomedical devices is difficult due to their poor stability on metallic surfaces [16]. Therefore, other surface functionalization strategies are currently being investigated for use in implantology. Chemical surface modifications were shown to be very effective in altering blood reactions and implant performance *in vivo* [2,17]. One such chemical surface modification would be the controlled modification of the surface oxide layer composition, as it has the great advantage of good stability and unmodified bulk material composition. In a recent study, we showed that modifying the cobalt chromium (CoCr) stent oxide layer composition resulted in a treatment-specific alteration of blood activation [18]. Additionally, when analogously-treated stents were implanted in coronary arteries of pigs, biological responses were altered leading to significant reduction of in-stent restenosis [17].

The alterations of blood activation processes and resulting modulation of the performance of implanted stents are likely to occur on the modified oxide layers due to differential protein adsorption. In this work, we investigated the role of protein adsorption for blood contact activation triggered by CoCr surfaces with 3 defined oxide layer compositions (described in [18]). We first confirmed differences in blood activation in terms of platelet adhesion, fibrin clot formation and neutrophil adhesions on the surfaces. We then analyzed the quantity and identity of blood plasma proteins adsorbed on the differently treated surfaces using mass-spectrometry. The role of plasma proteins in controlling neutrophil adhesion on specific surfaces was verified and possible correlations between changes in oxide layer composition and protein adsorption as well as their implication on blood components adhesion and activation are discussed. Finally, the role of fibrinogen in controlling the neutrophil adhesion was investigated by coating surfaces using defined protein mixtures.

Taken together, we describe how surface oxide modification of CoCr alloys altered the composition and quality of surface-adhering blood proteins further underlying differences in biological performance of these surfaces previously observed *in vitro* (blood contact activation) [18] as well as *in vivo* (porcine coronary restenosis model) [17].

2. Materials and methods

2.1. Preparation of cobalt-chromium alloy test surfaces

In order to generate test surfaces comparable to those of coronary stents, 15 mm disks of cobalt-chromium alloy MP35N (ASTM F562) were prepared, as described in detail in [18]. The disks were cleaned using an ultrasound bath series (HF-power 180 W, frequency 35 kHz) of 15 min in 3% Deconex PA 12 (Borer Chemie AG, Switzerland), 5 min in tap water, 5 min in distilled water, and 5 min in WFI (water for injection, Laboratorium Bichsel AG, Switzerland) and dried. These disks, stored for 2 weeks in 12-well cell culture plates made of polystyrene (TPP AG, Switzerland), were used as control surfaces and are referred as untreated throughout the manuscript. Modifications of the surface oxide layer to produce TREATED N and TREATED A surfaces were performed by Qvanteq AG (Switzerland) as described in [18]. Briefly, TREATED N samples were additionally treated with oxygen plasma (PDC-002, Harrick Plasma, USA, using a pressure of 8×10^{-3} mbar and oxygen 99.995%). Instead, TREATED A disks were immersed for at least 2 weeks in alkaline aqueous solution with a pH of 12.4, obtained by adding 1.0 g/L of NaOH pellets (Merck, Germany) to NaCl sterile irrigation solution 0.9% (Laboratorium Dr. G. Bichsel AG, Switzerland). Before incubation with blood, plasma or protein solutions, the disks were immersed in NaCl sterile irrigation solution 0.9% (Laboratorium Dr. G. Bichsel AG, Switzerland). The treatments did not affect surface roughness, but differences in oxide layer compositions were associated with changes in surface charge and wettability [18].

2.2. Protein adsorption onto modified cobalt-chromium alloy test surfaces

All test surfaces were briefly rinsed with 0.9% NaCl solution, immediately transferred into the blood plasma samples (platelet-depleted plasma, 3 donors, obtained from the blood bank Zurich, Switzerland), and incubated for 30 min at 37 °C under static conditions. After incubation with blood plasma, cobalt-chromium alloy test surfaces were rinsed 3 times with PBS and used for either total protein adsorption assays or for identification of the adsorbed proteins by mass spectrometry (after digesting and elution).

2.2.1. Quantification of plasma protein adsorption

For quantification of protein adsorption, an adapted protocol of microBCA (Sigma–Aldrich, Germany) was used as described by Ye et al. [19]. Briefly, 150 µL of micro BCA working reagent was added to the protein-coated CoCr surfaces covered with 150 µL of PBS. Samples were shaken for 30 s and left in the dark at room temperature for 1 h. After incubation, 100 µL of the supernatant of each sample was transferred into a well of a 96 well-plate and optical density at 540 nm was measured with a precision microplate reader (Molecular Devices, Switzerland). Triplicates of 3 independent samples were measured for each condition.

2.2.2. Protein digestion and peptide elution from CoCr surfaces

The surfaces were incubated with 400 µL 1 M urea in 0.1 M tris buffer (pH 8.5). Dithiothreitol (DTT) was added to reach a final concentration of 5 mM and shaken for 1 min on an orbital shaker and

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incubated 30 min at room temperature (RT). Afterwards, Iodoacetamide was added (to achieve 15 mM final concentration), and the samples were left in the dark at RT for 20 min. More DTT was added to achieve a final concentration of 10 mM and the samples were further incubated for 15 min at RT in the dark. Proteins were then digested overnight at 37 °C with trypsin (Promega, Madison, WI), in a ratio of 1:10 trypsin to protein. To stop trypsin activity, the solution was acidified with 2 μL of 10% formic acid (FA).

Eluted peptides were collected in fresh eppendorf tubes and a fraction (10 μL) was further used to quantify the total amount of eluted peptide by Qubit (Qubit® Fluorometric Quantitation, Invitrogen, Germany) according to the manufacturer's protocol.

Peptide solutions were then desalted using Ziptip (Millipore, Switzerland) protocol; briefly, the ZipTips were equilibrated by aspirating and dispensing 10 μL of 60% acetonitrile (ACN), 0.1% trifluoroacetic acid (TFA) twice, followed by 2 rinsing steps with 10 μL of double distilled water (ddH_2O). The samples were then loaded onto the ZipTip by repeated aspiration and dispensing for a total of 10 cycles. The ZipTips with bound samples were then washed by aspirating 10 μL of ddH_2O and dispensing the wash material into a waste reservoir two times. Samples were then eluted from the ZipTips by aspirating elution buffer (60% ACN, 0.1% TFA) and dispensed in a fresh eppendorf tube. Peptide solution was then dried in vacuum concentrator (30 °C for 30 min), and peptides were then dissolved in 12 μL of 3% ACN and 0.1% FA for 15 min RT.

2.2.3. Mass spectrometry

The samples were analyzed with an LTQ Orbitrap or an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Germany) coupled to an Eksigent Nano HPLC system (Eksigent Technologies, USA). Samples were dissolved in 3% ACN and 0.1% FA. Peptides were loaded onto a self-made tip column (75 μm × 80 mm) packed with reverse phase C18 material (AQ, particle size 3 μm, 200 Å, Bischoff GmbH, Germany) and eluted at a flow rate of 200 nL per min. Solvent composition of buffer A was 0.2% FA and 1% ACN in ddH_2O, and buffer B contained 0.2% FA and 99.8% ACN. The following LC gradient was applied: 0 min: 5% buffer B, 56 min: 40% B, 60 min: 47% B, 64 min: 97% B, 71 min: 97% B.

Mass spectra were acquired in the m/z range 300–2,000 in the Orbitrap mass analyzer at a resolution of 60,000. MS/MS spectra were acquired in a data dependent manner from the five most intense signals in the ion trap, using a normalized collision energy of 28%. Charge state screening was enabled, and singly charged precursor ions and ions with undefined charge states were not considered. Precursor masses already selected for MS/MS acquisition were excluded from further selection for 120 s, and the exclusion window was set to 20 ppm.

2.2.4. Protein identification and protein quantification

The raw files from the mass spectrometer were loaded into Progenesis LC–MS (v.4.0.4265). Before the automatic aligning the loading and the wash phase of the gradient were cut, the aligning reference was chosen where most features visually were observed.

From each Progenesis feature (default sensitivity for feature detection) a maximum of the top four tandem mass spectra were exported using charge deconvolution and deisotoping option, and a maximum number of 200 peaks per MS/MS was permitted. The Mascot generic file (.mgf) was searched with Mascot Server v.2.4.1 (www.matrixscience.com) using the parameters 10 ppm for precursor ion mass tolerance and 0.8 Da for fragment ion tolerance. Trypsin was used as the protein-cleaving enzyme and two missed cleavages were allowed during identification. Carbamidomethylation of cysteine was specified as a fixed modification, and oxidation of methionine, pyroglutamate formation from glutamine and N-terminal acetylation of proteins were selected as variable modifications.

We searched a forward and reversed human database (downloaded in December 2012) concatenated to 259 known mass spectrometry contaminants in order to evaluate the false discovery rate using the target-decoy strategy [20]. The mascot results were exported as XML file and an in-house java script cleaned the XML file to only keep rank one peptide-spectrum-matches while all other ranks were deleted.

This cleaned XML file was re-imported into Progenesis LC–MS. The experimental design was set according to the three different conditions to be compared. Normalization across the different measurements was default as suggested by the manufacturer.

2.2.5. Fibrinogen adsorption onto CoCr surfaces from model protein mixtures

A stock solution of fluorescent fibrinogen was prepared mixing 1 portion of Oregon-green Fibrinogen (Molecular Probes, The Netherlands) with 19 portions of an untagged fibrinogen (Fib III, Enzyme laboratories, UK).

Protein solutions were prepared in PBS. The final concentration of albumin (Sigma, Switzerland) was set at 50 mg/mL, and the final concentration of fibrinogen was set at 3 mg/mL, 0.3 mg/mL, 0.03 mg/mL and 0 mg/mL.

Proteins mixtures were then incubated with the test samples (triplicates) for 30 min at 37 °C and rinsed 3 times in PBS. Fluorescence was measured with a Synergy HT microplate reader (BioTek), exciting the samples using a filter at 485 nm with a bandwidth of 20 nm, and reading the emitted signal using a filter at 535 nm with a bandwidth of 25 nm. The signal measured on surfaces without fibrinogen was used as baseline and subtracted from the sample values.

2.3. Bioactivity of modified cobalt-chromium alloy test surfaces

2.3.1. Blood exposure to CoCr surfaces

The use of human blood and neutrophils for this study was approved by the Cantonal Ethical Commission of Zurich (KEK-ZH-Nr. 2012-0302). Whole blood obtained from healthy volunteer donors who had not used any aspirin in the previous two weeks was drawn into 5 mL vacutainer tubes (Vacutainer™ No Additive (Z) Plus Tubes, BD, Switzerland) and supplemented with 100 μL of 150 IU/mL heparin (B. Braun AG, Switzerland) to reach a final concentration of 3 IU heparin per mL blood. 500 μL fresh blood (not older than 2 h) was distributed into wells of a 24-well plate. Test surfaces were briefly rinsed with 0.9% NaCl solution, immediately transferred into the blood samples, and incubated for 2 h at 37 °C under static or dynamic (on a rotating plate with 100 rpm) conditions. After incubation with blood, cobalt-chromium alloy test surfaces were rinsed 3 times with PBS followed by fixation with 4% paraformaldehyde (PFA).

2.3.2. Neutrophil adhesion to protein-exposed CoCr surfaces

Neutrophils were isolated from heparinized whole blood using the PolymorphPrep™ system (Axis-Shield, USA). Neutrophils were resuspended in HBSS (without Ca/Mg) at a final concentration of 2×10^6 cells/mL. Five hundred μL of the neutrophil solution was added to treated CoCr surfaces, which had been previously incubated with either blood plasma (obtained from the same donor as the isolated neutrophils), albumin/fibrinogen mixtures or PBS for 30 min. After 2 h incubation at 37 °C under static conditions, test surfaces were rinsed 3 times with PBS followed by fixation with 4% PFA.

2.3.3. Neutrophil quantification

After fixation, blood or neutrophil-exposed surfaces were stained with a 1:1000 Hoechst 33342 solution in PBS for 15 min (Molecular Probes, The Netherlands). The samples were analyzed

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by fluorescent microscopy (DM550B, Leica Microsystems, Germany). For quantification five images per sample were acquired, three technical replicates (different surfaces) of biological replicates (different donor) were used. Nuclei were counted using ImageJ software.

2.3.4. Scanning electron microscopy inspection of adherent human blood components

After blood incubation cobalt-chromium alloy test surfaces were rinsed with PBS and fixed for 30 min in 3% glutaraldehyde in PBS. Samples were then dehydrated in a graded series of ethanol (from 25% to 100%) followed by drying over the critical point of CO_2 (T_k = 31 °C, P_k = 73.8 bar) using a critical-point dryer (CPD 030 Critical Point Dryer, Bal-Tec AG, Liechtenstein). The samples were sputter-coated with 10 nm platinum and the images were recorded with a Leo 1530 scanning electron microscope (Zeiss, Germany) using a secondary electron detector and 5 kV acceleration voltage.

2.4. Statistical analysis

All mean values were compared by two-way ANOVA analysis using Matlab 7.9 (the MathWorks Inc, USA). Statistical significance was accepted for $p < 0.05$ after comparing the mean values by Bonferroni *post hoc* test and was designated by an asterisk. For the statistical analysis which is testing differential individual protein amounts in the three groups (Fig. 4), the Progenesis LC–MS internal test was used. This is an ANOVA test based on the transformed normalized protein abundances in the hyperbolic arcsine space. Throughout the manuscript the data is presented as a mean ± standard deviations.

3. Results

3.1. Blood cell adhesion to variously treated cobalt-chromium alloy surfaces

After incubation of Cobalt-chromium alloy disks with engineered oxide layers (for surface properties refer to [18]) with freshly drawn human whole blood under static conditions platelets adhered to the untreated samples (Fig. 1a), while many leukocytes (neutrophils) adhered to TREATED N samples (Fig. 1b) and few fibers (presumably fibrin) where formed at the TREATED A surface (Fig. 1c) as seen by SEM micrographs. Under dynamic conditions, the differential activation of blood components on oxidized versus untreated surfaces was represented by extensive platelet coverage of the untreated surfaces (Fig. 1d), only few neutrophils were present on the TREATED N samples (Fig. 1e), and a dense network of fibrin-fibers has been observed on the TREATED A sample surfaces (Fig. 1f).

3.2. Blood responses are protein mediated

It is generally thought that alterations of blood activation are mediated through changes in the adsorbed plasma-protein layer. To investigate the role of plasma proteins, we focused on neutrophil adhesion. Indeed, one of the most striking differences between the variously treated surfaces was the presence of great numbers of neutrophils on the TREATED N surfaces (Fig. 2b), especially under static conditions, while they were almost absent on the untreated and TREATED A surfaces (Fig. 2a and c, respectively). To understand whether surface properties directly triggered this behavior or whether it was mediated by surface adsorbed proteins, neutrophils were seeded onto treated surfaces in absence of proteins or onto surfaces previously coated with blood plasma proteins.

When isolated neutrophils were seeded in absence of any proteins (resuspended in HBSS), they adhered well to all surfaces (Fig. 2d–f). The numbers for all samples reached values comparable to TREATED N surfaces incubated with whole blood (Fig. 2b). When surfaces were pre-incubated with plasma proteins for 30 min, neutrophils of the same donor adhered similarly as in the whole blood case: many neutrophils adhered on the TREATED N surface with plasma proteins (Fig. 2h), while only very few neutrophils adhered on the untreated (Fig. 2g) and TREATED A (Fig. 2i) surfaces. Quantification of cell nuclei confirmed the clear trend already observed qualitatively, with strongly reduced adhesion on untreated and TREATED A surfaces in presence of proteins (whole blood or pre-coated) but no significant effect of the presence of proteins on TREATED N surfaces, which showed always large numbers of neutrophils (Fig. 2j).

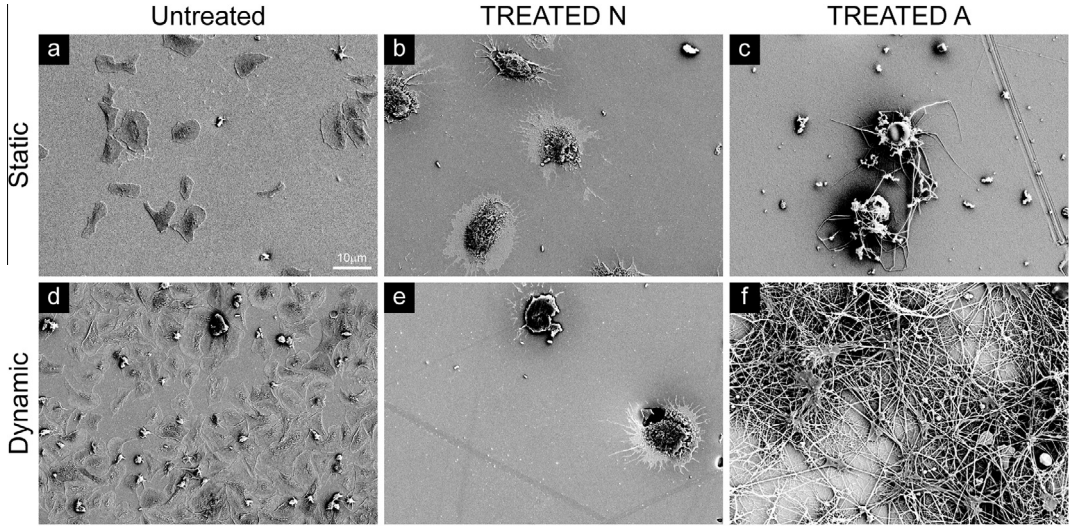

Fig. 1. Scanning electron micrograph of untreated (a and d), TREATED N (b and e) and TREATED A (c and f) CoCr surfaces after incubation with low heparinized whole human blood under static or dynamic conditions (2 h incubation time at 37 °C). While untreated CoCr surface are covered with platelets, only neutrophils adhered on the TREATED N surfaces and a network of fibrin-fibers was observed on the TREATED A surfaces. Dynamic conditions enhanced platelet adhesion (untreated surface), reduced neutrophil adhesion (TREATED N surfaces) and enhanced fibrin fiber formation (TREATED A).

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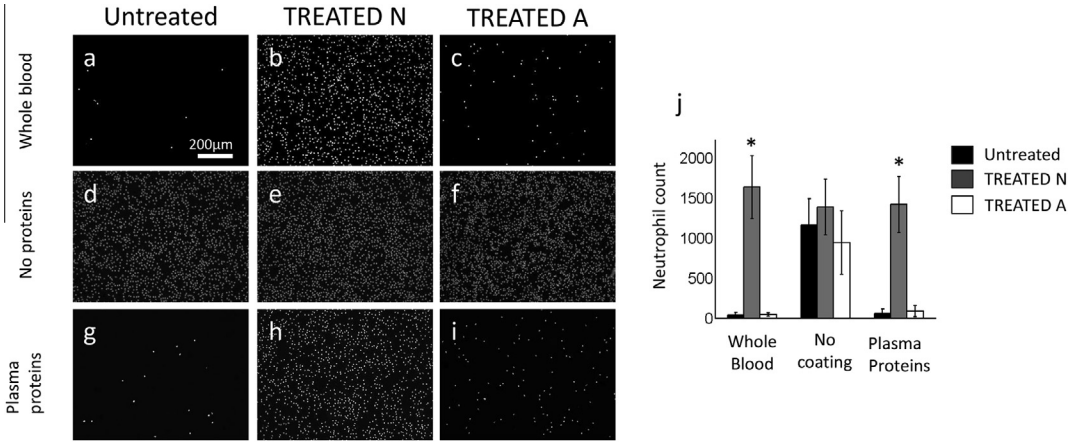

Fig. 2. Fluorescent images of untreated (a, d and g), TREATED N (b, e and h) and TREATED A (c, f and i) surfaces, showing the nuclei of adherent neutrophils (white dots). The samples had been incubated for 2 h with one of the following media: whole human blood (a–c); freshly isolated neutrophils (without proteins) (d–f); freshly isolated neutrophils, seeded on surfaces previously incubated for 30 min with plasma proteins (g–i). In absence of proteins, neutrophils adhered in large numbers to all surfaces. Instead, in presence of plasma proteins or whole blood they only adhered to TREATED N surfaces. (j) Quantification of the nuclei counts. The plot shows the means and standard deviations of 3 donors in triplicates.

3.3. Surface properties alter protein adsorption

The importance of plasma proteins for altered neutrophil adhesion on the various surfaces emphasized the central role of plasma proteins in blood reactions to the surface. To gain more information about proteins adsorbed on the differently treated surfaces (untreated, TREATED N and TREATED A), the samples were incubated with blood plasma proteins for 30 min, and the quantity and identity of proteins adsorbed on the various surfaces was assessed.

Micro-BCA measurements performed directly on the surfaces showed a significantly lower protein adsorption on TREATED N surface compared to both untreated and TREATED A surfaces (Fig. 3a). In order to objectively identify and quantify individual surface-adsorbed proteins, proteins were proteolytically digested on the surface and their peptides were eluted from the surfaces followed by mass-spectrometry analysis. The total amount of eluted peptides measured by Qubit was in agreement with Micro-BCA measurements: i.e. significantly lower amounts were eluted from TREATED N surfaces compared to untreated and TREATED A surfaces (Fig. 3b).

The database search approach yielded a list of identified peptides with a Mascot ion score of at least 25 or more. These peptides were compared with protein sequences in a database and where found to belong to 144 proteins.

For quantification, peptides found in more than one protein (shared peptides) were excluded, additionally only proteins for which at least two peptide ions had been identified were included. After applying these filters, the resulting list of quantified proteins included 105 proteins (Supplementary Table S1) into which no decoy protein were found, which suggests a very high confidence (protein false discovery rate close to zero, estimated as in [20]).

A representative composition of eluted proteins with relatively high abundance on the surface (Suppl. Fig. S1) and described in other studies to play a role in blood activation on biomaterial surfaces are shown and compared in Fig. 4. While levels of some proteins were found to change just slightly on the differently treated surfaces (albumin, complement C3, fibronectin, vitronectin and IgG), others were found to be significantly increased (fibrinogen) or decreased (apolipoprotein E, coagulation factor XI, kininogen) on the untreated surface compared to the treated ones. In a similar manner α2-macroglobulin and apolipoprotein A levels were significantly reduced and plasminogen significantly increased on TREATED N compared to the other two surfaces. More peptides belonging to prothrombin and to the coagulation factors X and IX were identified on TREATED A samples compared to untreated and TREATED N samples.

3.4. Role of fibrinogen in altered neutrophil adhesion

Fibrinogen (FGN) has been described to be actively involved in neutrophil adhesion [22]. In order to test its role in neutrophil adhesion on oxidized CoCr implants, untreated and TREATED N surfaces were incubated with physiological concentration of FGN

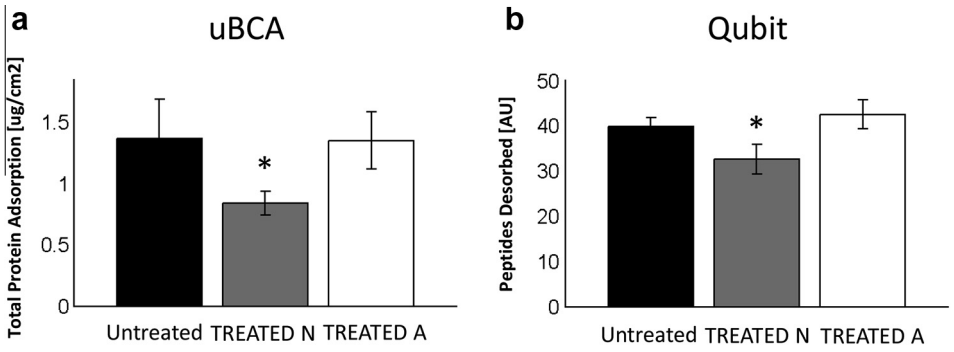

Fig. 3. (a) Total protein adsorption on the differently treated surfaces assessed by micro-BCA after 30 min incubation with blood plasma. (b) Total amount of eluted peptides from the different surfaces after protein digestion and elution.

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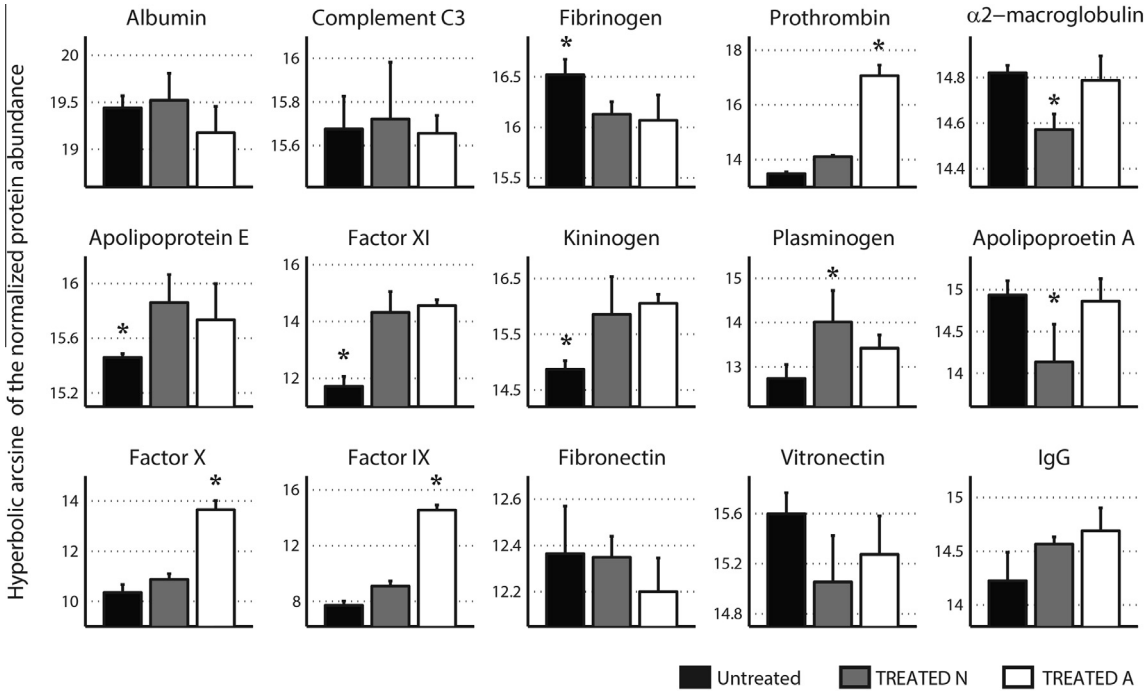

Fig. 4. Comparison of proteins of interest identified by mass spectrometry in the eluates from the differently treated surfaces. The proteins of interest include: albumin, complement C3, fibrinogen, prothrombin, α2-macroglobulin, apolipoprotein E and A, coagulation factor IX, X and XI (factor IX, factor X and factor XI), kininogen, plasminogen, fibronectin, vitronectin and immunoglobulin G (IgG). Other detected proteins not primarily discussed in this manuscript are reported in Table S1.

(3 mg/mL corresponding to 100%) and various fractions thereof (10%, 1% and none) mixed with physiological concentration of albumin (the most abundant blood protein, 50 mg/mL). On TREATED N surfaces the amounts of adhered FGN (at 3 and 0.3 mg/mL) were significantly reduced compared to untreated surfaces (Fig. 5a). On both untreated and TREATED N surfaces the amount of adsorbed FGN decreased with the decreasing concentration of fibrinogen in the incubation medium (faster decrease for untreated surfaces, such that at diluted concentrations the amounts of FGN on both surfaces are similar).

When surfaces were pre-incubated with physiological mixtures of FGN and albumin, neutrophils adhered in large numbers to TREATED N surfaces, comparably to blood plasma coated samples, whereas almost no adhesion was observed to untreated surfaces (Fig. 5c and d). When the concentrations of FGN were reduced 10 or 100-fold in the FGN/albumin protein mixture, the number of adhering neutrophils did not change on the untreated (Fig. 5e and g) and TREATED N surfaces (Fig. 5f and h) compared to physiological concentrations of FGN in albumin (Fig. 5c and d). However, if no FGN at all was present in the protein mixture (only albumin), a large, similar number of neutrophils was observed on both untreated and TREATED N surfaces (Fig. 5i and j).

4. Discussion

In this study, we characterized which proteins adhered to biomaterials surfaces and evaluated their role in initial blood contact activation, and potentially subsequent biological events. We used differently oxidized CoCr alloys (untreated, TREATED N and TREATED A) featuring various physico-chemical surface properties to perform our studies [18]. These surfaces are of particular relevance since they were shown to modulate treatment-specific *in vivo* response (e.g. in-stent restenosis) [17] additionally to different levels of *in vitro* blood activation [18].

Blood exposure of the various surfaces resulted in different biological reactions. While in the TREATED A samples, only scarce fibrin fibers deposition was observed after static exposure to blood, dense fibrin networks where formed on the same surface exposed to blood under dynamic conditions. This observation is supported by previous work showing increased activation of coagulation under flow conditions [21]. Also in accordance with previous work [22,23], platelet adhesion was altered by shear stress. Indeed, a strong increase in platelet adhesion was observed on untreated samples and sometimes single platelets adhered even on treated surfaces. The increase in platelet adhesion correlated to higher flow rates [22]. Also consistent with the literature [24,25], the opposite trend was observed for leukocytes (neutrophils) which tended to have reduced adhesion to substrates under dynamic conditions (Fig. 1b and e).

Both surface treatments TREATED N and TREATED A resulted in strongly reduced platelet adhesion compared to untreated samples (Fig. 1), which may be at least partially explained by the highly hydrophilic surfaces compared to the moderately hydrophobic untreated surface [18]. Indeed, similar observations of preferential platelet adhesion on hydrophobic surfaces have been made also on other materials [26–29].

Nevertheless, in this study, changes in surface wettability are not the sole explanation for alterations of blood activation. Indeed, major differences in neutrophil adhesion were observed on surfaces with equivalent hydrophilicity (Fig. 2b and c). The strong adhesion observed on TREATED N as compared to TREATED A surfaces must be the result of differences in surface charge (negative versus neutral-slight positive charge) and/or oxide layer composition (Cr-rich versus Ni and Co-rich oxide layer).

Moreover, to understand whether surface properties affect blood cell adhesion in a direct or protein-mediated way we investigated the role of plasma proteins in neutrophil adhesion. Neutrophils seeded in a salt solution without any proteins adhered to all substrates with equal efficiency. Instead, if samples were incubated with plasma proteins prior to incubating with neutrophils, neutrophil adhesion varied strongly depending on the surface treatment, analogously to the whole blood case. These results indicate that plasma proteins passivate selected surfaces

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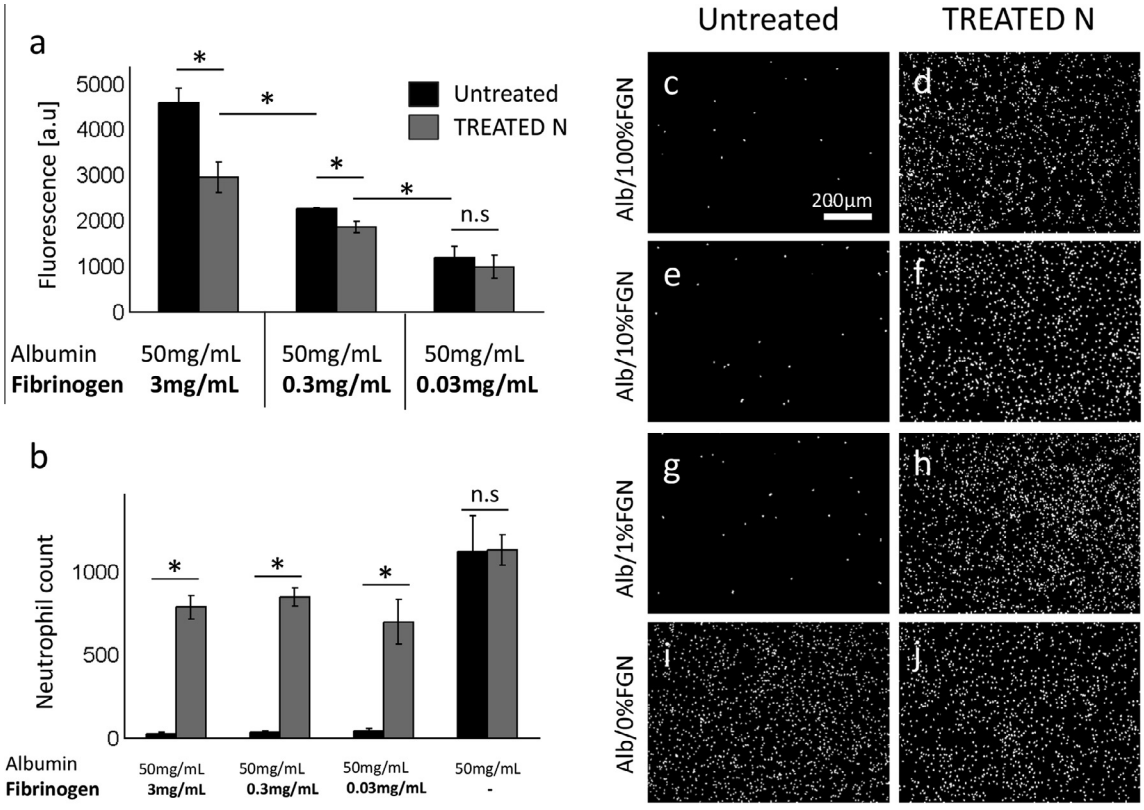

Fig. 5. Effect of fibrinogen concentration on neutrophil adhesion. (a) Fluorescent signal of untreated or TREATED N surfaces incubated with various albumin/Alexa 488-tagged fibrinogen mixtures. 100% corresponds to the typical blood concentrations: 50 mg/mL albumin, 3 mg/mL fibrinogen. (b) Quantification of the nuclei on untreated and TREATED N surfaces pre-incubated with various concentration ranges of albumin/fibrinogen. (c–j) Fluorescent images of untreated (c, e, g and i) and TREATED N (d, f, h and j) surfaces, pre-coated with various albumin/fibrinogen ratios for 30 min, 2 h after incubation with freshly isolated neutrophils. The nuclei of adherent neutrophils appear as white dots.

(untreated, TREATED A) modulating negatively the adhesion of neutrophils (Fig. 2). This is not surprising since, during blood contact, protein adhesion is much faster than cell adhesion. This model therefore separates the two adhesion steps which take place in the same order as during whole blood incubation and underlined the crucial role of proteins in controlling neutrophil adhesion. This observation is also in accordance with previous work showing that the presence or absence of plasma proteins is crucial for the modulation of platelet – [30] and neutrophil–adhesion [6]. We here performed a 30 min pre-incubation with plasma proteins, as it was shown that this pre-incubation time triggers biological responses representative of the whole blood case [5,6].

Protein adsorption can vary in various ways: total amount of protein, composition of the adsorbed protein layer and orientation/conformation of the adsorbed proteins. To find the key parameter of the protein layer controlling the cell adhesion we first compared the total amount of adsorbed proteins with two different techniques (micro BSA, measuring directly on the surfaces or by Qubit, measuring after proteolytic digestion and elution). Both analyses indicated that similar amounts of protein adsorbed on untreated and TREATED A surfaces, whereas less proteins were adsorbed on TREATED N surfaces (Fig. 3). While it is uncertain if the total amount of adsorbed protein quantity correlates with blood response, with contradictory observations in literature [7,31–33], these results support that (i) protein adsorption is altered by the surface treatment and (ii) after elution the total amount of proteins (peptides) is still treatment-specific. In order to identify the adsorbed proteins and investigate possible changes in protein layer composition, which may explain the different cellular adhesion, two main strategies are commonly used: analysis of proteins directly on the biomaterial surface, or analysis of proteins eluted from the surface [10,34,35]. The second approach was chosen here, since it is more suitable for screening an unknown protein mixture as it does not require the use of specific antibodies or labeling of selected proteins [34].

As a first screening step to identify as many proteins as possible and to look for potential interesting candidates, proteins were proteolytically digested, eluted and analysed by mass spectroscopy, analogously to procedures described in the literature [35,36]. Not surprisingly, abundant blood proteins such as albumin, fibrinogen and complement C3 were found in large quantities among the proteins desorbed from all surfaces (Fig. S1 and Table S1). However, the protein layer composition varied as a function of the surface characteristics, indicating that selected proteins adhered with unequal efficiency on the differently treated surfaces (Fig. 4). On TREATED A surfaces much larger amounts of proteins involved in the coagulation cascade [37] (i.e. prothrombin, factor X and factor IX) were observed compared to both TREATED N and untreated surfaces. This suggests that TREATED A surfaces may have an increased tendency to induce blood coagulation and could be the reason for the fibrin network formation observed by SEM only on these surfaces, especially under dynamic conditions (Fig. 1). While this effect would not be desirable for cardiovascular implants, where thrombus formation would potentially have catastrophic consequences, TREATED A surfaces may show superior properties for orthopedic implants, where higher thrombogenicity improves osseointegration. On untreated surfaces, instead, differences in the protein layer composition which may correlate with the increased platelet adhesion were found: kininogen, which is known to have regions inhibiting platelet adhesion and aggregation [38], indeed adhered in lower amounts on the untreated surfaces compared to both treated surfaces. Fibrinogen,

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known to have an impact on cell adhesion [32,44], was instead observed in increased amount on untreated surfaces.

Moreover, in proteins eluted from TREATED N surfaces – onto which many neutrophils adhered during blood exposure – highest quantities of plasminogen were found, while significantly lower amounts of apolipoprotein A and α2-macroglobulin were detected. Previous findings suggested that α2-macroglobulin inhibits leukocyte adhesion to biomaterials surfaces [39], and apolipoprotein A reduces neutrophil recruitment and function [40–42], while plasminogen recruits neutrophils [43]. We conclude that the TREATED N surfaces were therefore covered with less neutrophil inhibitors and more neutrophil recruiters, matching the increased amounts of neutrophils on these surfaces compared to TREATED A and untreated surfaces. However, it should be noted that current evaluations of protein quantities were restricted to only a few identified, surface-bound proteins that could play a role in blood activation. Therefore it cannot be excluded that the efficiency of adsorption of other, less abundant proteins could also play a role in the blood-surface response.

Given all the data available in the literature indicating an important, though not fully understood role of fibrinogen in cell adhesion which shows significant changes in adsorbed amount as a function of the surface treatment, fibrinogen adsorption and its effect on neutrophil adhesion was investigated in more detail (Fig. 5). The equally efficient adhesion of neutrophils on albumin-coated TREATED N and untreated surfaces (Fig. 5b, datapoints without fibrinogen) indicates that the surface-dependent (anti-)adhesion was not influenced by albumin. In contrast, when TREATED N and untreated surfaces were pre-coated with albumin-fibrinogen mixtures at physiological concentrations, the neutrophil adhesion behavior as observed in plasma or whole blood was reproduced, indicating a fibrinogen-dependent mechanism of the inhibition of neutrophil adhesion (Fig. 5b, datapoint with 3 mg/mL fibrinogen). However, since at physiological fibrinogen and albumin concentrations more fibrinogen adhered onto the untreated surfaces compared to the TREATED N surfaces, a concentration dependent effect could not be excluded. For this reason the amount of adsorbed fibrinogen was reduced on selected samples by decreasing its concentration in the incubation protein medium (Fig. 5a), exploiting a correlation (though not linear) between adsorbed amount and concentration in the incubation medium described in the literature [44,45]. Reducing the amount of fibrinogen adsorbed on the untreated surfaces to as low as 1% of the physiological concentration did not compromise the surface anti-adhesive properties for neutrophils. Thus our data, in contrast to a study were increasing levels of adsorbed fibrinogen were shown to inhibit neutrophil adhesion [32], strongly suggests that fibrinogen quantity is not responsible for inhibiting neutrophil adhesion. This is further underlined by the fact that on TREATED N and TREATED A surfaces comparable amounts of fibrinogen were measured, but neutrophil adhesion was very different. Fibrinogen has been described to affect neutrophils adhesion in a conformation-dependent manner [46], suggesting that on TREATED N surfaces fibrinogen is probably in a different conformation and/or in a different orientation as compared to untreated surfaces. Since fibrinogen conformational changes are usually specific to the biomaterial surface properties, and are even more pronounced for dilute protein solutions [7], we assume that the herein described inhibition of neutrophil adhesion is mediated by the fibrinogen conformation or orientation.

A limitation of this analysis is that no direct information concerning protein conformation is available. Such an analysis is not possible in our case due to the non-transparent metallic substrates used. Additionally, some studies report that experiments with individual proteins show effects on platelets/neutrophils which may not always represent what happens in presence of plasma, when the role of the single proteins might be different or masked by other proteins. This is the reason why the experiments were performed with mixtures of albumin (the most abundant blood protein) and fibrinogen and not only with pure fibrinogen alone. Despite these convincing results with fibrinogen, the significant differences in other protein adsorption and possibly conformation should not be overlooked and should be further studied. Most likely the surface-dependent neutrophil adhesion is the result of a combination of ratio and conformation of several proteins at the same time.

5. Conclusions

The blood contact activation of CoCr alloys is determined by the properties of their surface oxide layer in a protein-mediated manner. Indeed, modifications of the oxide layer affected the total amount of adsorbed proteins, but more importantly they influenced the composition of the adsorbed protein layer. Using mass-spectrometry we identified over hundred adsorbed proteins, and the analysis of the most abundant ones correlated well with the observed blood activation. For instance, surfaces showing enhanced fibrin clot formation showed also an adsorbed protein layer containing increased concentrations of proteins involved in the coagulation cascade. Analogously, higher platelet adhesion was associated with decreased amounts of adsorbed kininogen and enhanced neutrophil adhesion was observed on surfaces exhibiting increased plasminogen amounts combined with reduced apolipoprotein A and α2-macroglobulin. Moreover, pre-incubation assays with fibrinogen largely replicated the surface-dependent neutrophil adhesion observed with whole blood or plasma. This effect was concentration-independent, suggesting that cell adhesion and activation on implant surfaces is also controlled by the conformation and/or orientation of the adsorbed proteins.

Despite the complexity of protein–implant interactions, this study highlights the importance of understanding and controlling these mechanisms to improve and steer the performance of medical implants. These results show that modification of the surface oxide layer is a very attractive strategy to directly functionalize metallic implant surfaces and optimize their blood interaction for the desired application, for instance by increasing its thrombogenicity for orthopedics or by reducing the thrombogenicity and inhibiting platelet adhesion for cardiovascular applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2015.06.020.

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