Analysis of rat plasma proteins desorbed from gold and methyl- and hydroxyl-terminated alkane thiols on gold surfaces

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It is believed that adsorbed blood or plasma components, such as water, peptides, carbohydrates and proteins, determine key events in the concomitant inflammatory tissue response close to implants. The aim of the present study was to develop a procedure for the collection and analysis of minor amounts of proteins bound to solid metal implant surfaces. The combination of a sodium dodecyl sulfate washing method coupled with a polyacylamide gel electrophoretic protein separation technique (SDS–PAGE), Western blot and image analysis enabled the desorption, identification and semiquantification of specific proteins. The analyzed proteins were albumin, immunoglobulin G, fibrinogen and fibronectin. Concentration procedures of proteins were not required with this method despite the small area of the test surfaces. The plasma proteins were adsorbed to pure gold and hydroxylated and methylated gold surfaces, which elicit different tissue responses *in vivo* and plasma protein adsorption patterns *in vitro*. The image analysis revealed that the pure gold surfaces adsorbed the largest amount of total and specific proteins. This is in accordance with previous ellipsometry/antibody experiments *in vitro*. Further, the principles described for the protein analysis can be applied on implant surfaces *ex vivo*.

1. Introduction

The mechanisms whereby certain biomaterials become rejected, encapsulated with fibrous tissue or integrated into tissues are not fully understood. It is believed, that the ''biofilm'' formed immediately after contact with blood is composed of polypeptides, carbohydrates and proteins. This layer may determine the key events in the early inflammatory tissue response but also later reparative processes [1–5], and some of the mechanisms in the intricate interplay between proteins and cells are known. Different types of materials induce different cell responses *in vitro* and *in vivo* [3, 6–11] and certain surface topographies, i.e. micropatterning can influence cell differentiation and orientation *in vitro* [12–16]. Surface wettability is shown to alter various protein/cell and tissue reactions [17–23]. Further, preincubation with specific proteins alters the cellular response *in vitro* [24–26].

A large number of differently chemically modified implant materials are suitable for a systematic evaluation of the role of surface properties for tissue/cellular reactions in *in vivo* and in *in vitro* model systems. In previous studies, surfaces have been covered with different functional groups and used in order to study protein and cell behavior on the surfaces [21, 27–29]. Alkane thiolates with functional groups ($—OH, —CH_3, —F, —Cl$) on pure gold have been used to modulate the chemical characteristics in a systematic way, thus providing well defined surfaces [30]. These surfaces have varying chemistries and wettabilities, which in turn result in different protein compositions after incubation in human sera [31–33]. Such intended surface modifications also affect inflammatory cell recruitment and distribution *in vivo* (rat model) and during *in vitro* culturing conditions [17]. However, for technical and practical reasons the studies on protein adsorption to implant materials have been performed in various model systems *in vitro*. Ellipsometry/antibody *in vitro* techniques are suitable for studying proteins adsorbed to smooth reflecting surfaces [34]. Specific proteins can be detected by the binding of antibodies to the already adsorbed organic layer, which enables

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estimation of biological access *per se* of the adsorbed proteins. Multilayered proteins or proteins sterically hidden in the organic layer cannot, however, be detected [31]. Another method of studying protein interactions with implant surfaces *in vitro* is to study the adsorption and desorption behavior of ^{125}I-labeled proteins from single or oligo protein solutions [9, 25, 32, 35, 36]. A method based on direct immunodetection where the proteins are analyzed on the surface without disturbing their original arrangement has been described [37]. The ellipsometry/antibody techniques and this method, however, suffer from the lack of accessibility of all antibodies to their antigens. Elution of adsorbed proteins with sodium dodecyl sulfate (SDS) and subsequent concentration prior to analysis has been employed, but the method is uncertain due to losses of proteins during the concentration steps.

The aim of this study was to develop a simple method to identify and semiquantify specific proteins incorporated in the initial biofilm on implant surfaces. We used the polyacrylamide gel electrophoretic (PAGE) protein separation technique, supplemented with SDS [38]. The SDS–PAGE technique has been used previously to study proteins on implant surfaces [39–43]. The method applied in the present study is a modification of a technique used for the desorption and analysis of salivary proteins in biofilms formed on dental materials [44]. The model surfaces used were pure gold and hydroxyl- and methyl-terminated alkane thiols on gold. The adsorbed proteins were dissolved using SDS together with a standardized mechanical rubbing procedure. Then the extracted proteins were electroblotted to a nitro-cellulose membrane and identified by antibody/enzyme color visualization techniques [45, 46]. Some proteins were quantified by densitometric image analysis of the staining obtained on the membranes using purified rat plasma proteins as standards. The surfaces induced different total amounts and adsorption patterns of plasma proteins. Therefore the described method can be used for *in vitro* screening of proteins adsorbed from plasma to various-surfaces. Further, the technique can be applied in the analysis of small amounts of proteins on explanted surfaces without concentration steps.

2. Materials and methods

2.1. The surfaces

Some 200 nm of pure gold was evaporated on clean, circular shaped, machined (pure) titanium discs (ϕ10 mm, thickness 1 mm) by physical vapor deposition (PVD) methods in ultra-high vacuum (UHV, $P \approx 10^{-7}$ torr). The surface roughness (Rq) of the gold coated discs was $< 0.2\,\mu$m (Nanoscope III, Digital Instruments, Santa Barbara, CA). The discs were incubated overnight in 2 mmol 16-thiol-hexadecanol $(HS-(CH_2)_{16}OH)$ or n-hexadecanethiol $(HS-(CH_2)_{15}CH_3)$ in 95% ethanol and not stored for longer than two days before use. The surface molecular organization and chemistries have been described previously [47, 48]. Immediately prior to use, the gold surfaces were placed in 99.5% ethanol for 30 min, sonicated for 3 min and rinsed three times in 99.5% ethanol. The thiolated surfaces were sonicated for 3 min in ethanol and rinsed three times in ethanol. All surfaces were then placed in sterile polystyrene tissue culture dishes containing sterile Hank's balanced salt solution (HBSS: 0.44 mmol KH_2PO_4 and 0.33 mmol Na_2PO_4; 0.15 mol NaCl and 1 g l^{-1} glucose) supplemented with Ca^{++} (0.1 g l^{-1}) and used within 2 h. The implants were carefully handled in order to minimize surface damage.

2.2. Incubation, desorption and collection of proteins

In vitro plasma protein adsorption experiments were performed with thawed, heparinized and pooled whole rat plasma. The experiments were approved by the Local Ethical Committee for Laboratory Animals (143/96). Each disc ($n = 5$ per modification) was incubated for 1 min in pre-warmed rat plasma, 37 °C, rinsed three times in 10 mmol Tris, pH 7.4 (Merck, Germany) and placed against a filter paper to remove excess buffer. Bound proteins were dissolved and collected by rubbing the surface with four mini-sponge applicators (polyurethane sponge, 3M Dental Products, St Paul) each containing a 5 µl droplet of 2% SDS (ICN Biomedicals Inc.,) in 10 mmol Tris including enzyme inhibitors (0.5 mmol Pefabloc, Boehringer Mannheim, Switzerland; 10 µmol Leupeptin, Sigma; 0.15 µmol Aproteinin, Sigma-Aldrich, Sweden). Finally, a dry mini-sponge was drawn over the surface. All mini-sponges (five per surface) were placed in a mini Eppendorf centrifuge tube with a 0.6 mm hole (Microlane syringe 0.6 × 25) in the bottom and the hat, and the proteins were retrieved from the sponges by centrifugation (1500 g, 10 min) into a new, outer Eppendorf tube. Samples from the same surface modifications were pooled, and 10 µl was removed for the determination of total amount of proteins by spectrophotometric bicinchoninic acid assay at 562 nm (BCA micro assay, Pierce, Rockford) using rat albumin as a standard. The remaining sample was prepared for SDS–PAGE and immunoblotting analysis.

2.3. Gel electrophoresis, immunoblotting and densitometric analysis

Each sample was diluted with sample buffer pH 6.8 (0.50 mol Tris-HCl, Merck, Germany; 10% glycerol, Sigma; 7.5% β-mercaptoethanol, Sigma; 2% (w/v) SDS; 2% bromo-phenol blue (The British Drug Houses Ltd). The *in vitro* samples were diluted 3 : 1 with sample buffer (× 4). A plasma control, which was prepared from heparinized pooled whole blood, was diluted 1 : 99 in sample buffer and standard rat proteins were diluted in the sample buffer as follows: fibronectin, FN (0.2–0.01 g l^{-1}, Calbiochem), fibrinogen, Fib (0.6–0.025 g l^{-1}, Sigma), albumin, Alb (0.4–0.025 g l^{-1}; Nordic Immunological Laboratories, Netherlands) and immunoglobulin G, IgG (0.4–0.025 g l^{-1}; Serotec). All samples were heated to >97 °C for 7 min, cooled and kept frozen (−20 °C) until use. The prestained broad range molecular weight standard (SDS–PAGE standard) was obtained from Bio-Rad. Gel electrophoresis was performed on 4–15%, precasted acryl amide gradient gels at 150 V constant (Mini Protean II Page, 70–40 mA, Power200; Bio-Rad) for approximately 45 min in a

buffer (pH 8.3) with Tris (15 gl^{-1}), glycine 72 gl^{-1} (ICN Biomedicals Inc.) and 5 gl^{-1} SDS dissolved in deionized water. After immersion of the gels for 3 × 10 min in transfer buffer (25 mmol Tris, 192 mmol Glycine and 20% methanol in deionized water) proteins were electroblotted to nitro-cellulose membrane (0.45 μm, Bio-Rad) at 70 V constant (for 3 h), using a transfer buffer supplemented with 0.02% SDS. Prior to antibody incubation, the membrane was blocked for a minimum of 1 h, in 3% gelatine (Sigma) in Tris buffered saline (TBS), pH 7.5 (20 mmol Tris and 0.5 mol NaCl in deionized water). The primary antibodies were polyclonal IgG fractions of: sheep anti rat Immunoglobulin G, (IgG γ-chain, 1 : 15 000; The Binding Site Ltd), sheep anti rat albumin (Alb 1 : 15 000; The Binding Site Ltd), goat anti rat fibrinogen (Fib. 1 : 10 000, Nordic Immunological Laboratories, The Netherlands) and rabbit anti rat fibronectin (FN, 1 : 30 000; Calbiochem). The membranes were incubated for a minimum of 1 h in primary antibody diluted in 0.1% gelatine in TTBS (TBS with 0.05% Tween-20, polyoxyethylene sorbitan monolaurate; INC Biomedical). Thereafter, the membranes were incubated for 1 h in secondary antibody solution: biotinylated anti sheep (1 : 10 000; The Binding Site) or anti rabbit (H&L, 1 : 10 000; Vector Laboratories Inc.) in 1% gelatine in TTBS, and finally with the steptavidin conjugated alkaline phosphatase (1 : 30 000; Vector Laboratories Inc.) in 1% gelatine in TTBS. Between each incubation step the membrane was rinsed 2 × 5 min in TTBS. Color development of the proteins was performed by the combination of 5bromo-4-chloro-3-indyl-phosphate-p-toluidine salt and 4-nitro blue tetrazolium chloride (BCIP and NBT, Bio-Rad Laboratories AB, Sweden) as recommended by the manufacturer, after an extra rinsing of the membrane in TBS (5 min).

Color reaction on the membranes was analyzed using the ''Image'' public domain program (Macintosh; V. 1.57) and processed further by computer analysis. The amount of specific proteins was determined from the standard curves obtained from the purified proteins.

2.4. Control experiments

Control experiments were performed in order to determine how effectively the proteins were desorbed and collected. Samples were obtained from four extra rubbings and four extra washings of the sponges and analyzed using the BCA assay and immunoblot techniques. In addition, ellipsometry *in vitro* techniques were used to determine proteins remaining on the surface after the desorption and collection procedures (Rudolph Research III ellipsometer, laser wavelength 632.8 nm and 70° angle of incidence). Five surfaces of each material surface modification were used and delta and psi were determined at the same spot *in situ* in a cuvette. The measurements were done at 37 °C in the following order: calibration measurement after surface exposure to 0.05 mol Tris buffer for 5 min; plasma thickness measurement after rat plasma incubation for 1 min followed by three rinsings with Tris buffer; immediate measurement at 2% SDS (in Tris buffer) injection in the cuvette; continuous measurement during SDS incubation up to 5 min and final thickness measurement after one rinsing with Tris. The mean organic thickness value and the mean adsorbed mass value were calculated according to McCrackin [49] and de Feijter *et al*. [50], respectively. In order to validate the molecular weights of the components in the samples and to confirm proper transfer of the proteins during electroblotting, staining of the gels was done after separation and after electroblotting. The gels were stained with Coomassie brilliant blue R-250 stain (0.1%, Svenska ICI AB, Sweden) in 40% methanol and 10% acetic acid in deionized water for a minimum of 30 min or stained with silver stain according to the supplier of the kit (Bio-Rad). Possible cross-reactions of the primary and secondary antibodies in the immunoblot system were tested by probing all antibodies against all standard proteins.

3. Results

3.1. Protein collection and methodology

The rubbing technique applied for the desorption and collection of adsorbed proteins (repeated rubbing with sponges containing SDS) resulted in detectable amounts of proteins as determined by the BCA assay, Coomassie brilliant blue and silver stain of the gels. Thus, protein concentration steps or the use of particles/beads with an increased surface area were not necessary for the analysis of surface bound proteins with this technique. The BCA assay revealed small amounts of proteins from all surfaces, although the pure gold surface adsorbed most plasma proteins followed by methylated and hydroxylated surfaces, respectively. Similar adsorption patterns of plasma proteins were found in studies with *in vitro* ellipsometric measurements [51]. The results of the control experiment with SDS *in situ*, measured with ellipsometry at the same spot, showed that preadsorbed plasma proteins were immediately desorbed from functionalized surfaces (within 1 min) but not from gold surfaces (Table I). Gold surfaces have previously been reported to adsorb and retain plasma proteins very strongly, due to electric interactions [3, 52]. The additional control test, where surface adsorbed proteins were

TABLE I A summary of the results obtained from ellipsometric measurements of proteins in rat plasma (μg cm^{-2}), retained after SDS elution on Au, −OH and $-CH_3$ surfaces

Surface	Amount after plasma incubation	Amount after SDS elution 0 min	Amount after SDS elution 1 min	Amount after SDS elution 5 min + rinsing
Au	0.370 ± 0.05	0.336 ± 0.05	0.378 ± 0.02	0.336 ± 0.03
−OH	0.084 ± 0.04	0.005 ± 0.00	0.014 ± 0.01	0
$-CH_3$	0.175 ± 0.05	0.002 ± 0.00	0.004 ± 0.01	0

eluted and collected by rubbing with SDS-containing sponges four extra times, however, showed no remaining proteins. Therefore, described mechanical rubbing of the surface enhanced the total desorption of proteins from gold surfaces. Also, no further proteins were eluted from the sponges after the extra washing steps.

The proteins were well separated in the gradient gels and allowed a molecular weight approximation of the silver stained proteins in the *in vitro* samples and in the standards (Fig. 1). Analysis of the standard proteins revealed slightly heavier molecular weights for IgG, albumin and fibrinogen than expected. Fibronectin was detected above the 206 000 marker, which is in accordance with the reported 220 000 rat fibronectins (Fig. 1, lane 7). We have no explanation for the tendency of fibrinogen to be about 10 000 too heavy (61 000 and 56 000 for the $\alpha+\beta$ and γ bands, respectively, Fig. 1, lane 5). This could, however, be an effect of the gradient gel or due to improper reduction of the molecule. When silver stained, the test samples (Au, —OH and $—CH_3$) showed clear bands at the molecular weight for albumin and more diffuse bands at the IgG level. These proteins are the major components in plasma and probably in our sample as well (in Fig. 1 compare lanes 1 = Au, 2 = —OH and 3 = $—CH_3$ with lanes 4 = IgG and 6 = Alb). Coomassie staining of the gels after the electroblotting showed small amounts of proteins persisting from the highest standard concentrations of fibrinogen ($> 0.3\,gl^{-1}$), albumin ($> 0.2\,gl^{-1}$) and IgG ($> 0.1\,gl^{-1}$) but not for fibronectin. By addition of 0.02% SDS in the blotting transfer buffer we were able to reduce the amount of proteins remaining in the gels. Our test samples never showed proteins persisting in the gels after electroblotting, most probably because of a low total protein content.

The immuno visualization with antibodies directed towards the specific proteins of electroblotted *in vitro* samples showed colored proteins on the nitro-cellulose membranes, except for fibronectin. The binding of proteins to nitro-cellulose membranes is not known in detail but is predominantly hydrophobic [53] and very stable. The following antibody detection and visualization of membrane bound proteins is a very sensitive method and in many ways dependent on the specificity and affinity/avidity (amplification) of the antibodies used. Since our antibodies were all polyclonal, possible cross-reactivity was investigated. Evaluation was done with all antibodies against all protein standards. Colored bands of purified fibronectin were seen with its own antibody only (Fig. 2b, lanes 5–8). The IgG standard showed a weak cross-reactivity with all antibodies (Fig. 2a, lanes 1–4), at the levels of IgG heavy and light chains, respectively. The cross-reactivity could be due to unspecific binding (affinity) of the primary (sheep a-rat) or secondary (donkey a-sheep IgG) antibodies in the system. The albumin standard induced no cross-reaction with anti-fibrinogen or anti-fibronectin (Fig. 2a, lanes 6 and 7). Weak bands were seen at the level of whole albumin (top band) and smaller albumin related products (bottom band) when anti IgG was used (Fig. 2a, lanes 5–

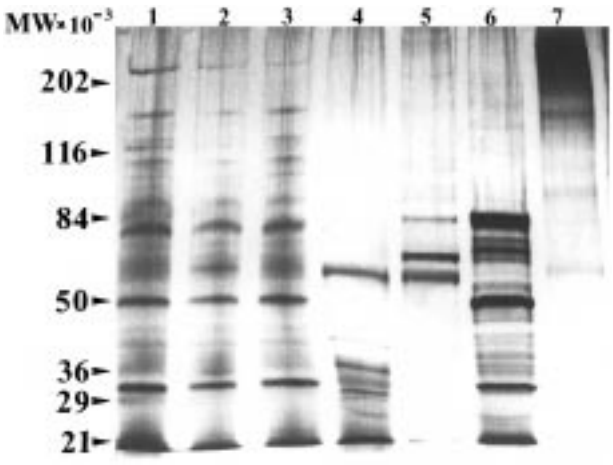

Figure 1 Silver stained gels after SDS–PAGE (4–15% gels) of proteins recovered from test surfaces, purified rat proteins and of a pooled plasma sample. Some 7 µl portions were loaded in each well according to: Au, lane 1; —OH, lane 2; $—CH_3$, lane 3; IgG ($0.1\,gl^{-1}$), lane 4; fibrinogen ($0.1\,gl^{-1}$), lane 5; albumin ($0.1\,gl^{-1}$), lane 6; fibronectin ($0.1\,gl^{-1}$), lane 7. Arrows indicate the standard molecular-weight markers: myosin (202 000), β-galactosidase (116 000), bovine serum albumin (84 000), ovalbumin (50 000), carbonic anhydrase (36 000), soybean trypsin inhibitor (29 000) and lysozyme (21 000).

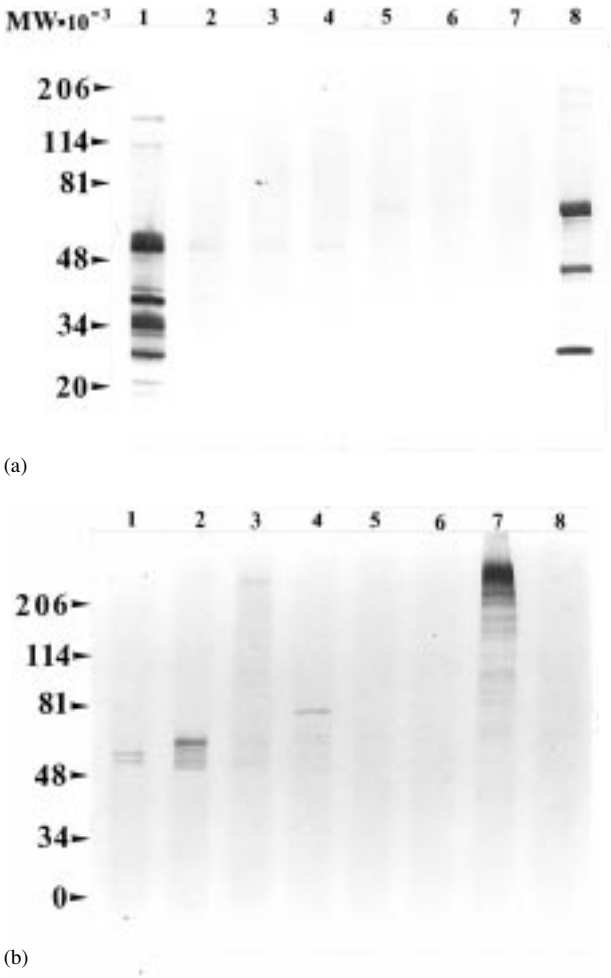

Figure 2 Western blots of standard rat proteins reacted with different antibodies. (a) Reaction of $0.4\,gl^{-1}$ of purified IgG (lanes 1–4) and albumin (lanes 5–8) reacted with antibodies against IgG, lanes 1 and 5; fibrinogen, lanes 2 and 6; fibronectin, lanes 3 and 7; and albumin, lanes 4 and 8. (b) Reaction of $0.6\,gl^{-1}$ of purified fibrinogen (lanes 1–4) and $0.3\,gl^{-1}$ of purified fibronectin (lanes 5–8) with antibodies against IgG, lanes 1 and 5; fibrinogen, lanes 2 and 6; fibronectin, lanes 3 and 7; and albumin, lanes 4 and 8. Arrows indicate the standard molecular-weight markers: myosin (206 000) β-galactosidase (114 000), bovine serum albumin (81 000), ovalbumin (48 000), carbonic anhydrase (34 000) and lysozyme (20 000).

8). The fibrinogen standard produced colored bands irrespective of antibody (Fig. 2b, lanes 1–4). The cross-reactions appeared at the molecular weight of the respective protein, most probably indicating improper purification of the fibrinogen standard. Furthermore, the fibrinogen γ-chain appeared at approximately the same molecular-weight level as the heavy chain of IgG. Therefore, cross-reactivity of anti IgG with fibrinogen cannot be excluded. In summary, all antibodies used reacted with the purified IgG standard, and the anti IgG antibody seemed to react with the rat fibrinogen γ-chain.

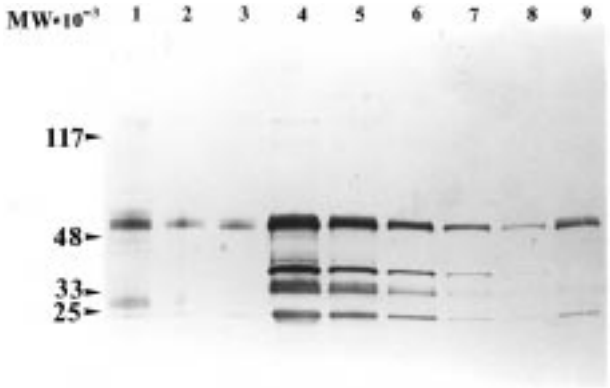

Figure 3 Staining of IgG in Western blots of samples from pure gold, —OH and $-CH_3$ functionalized surfaces, pooled plasma and purified rat IgG. Some 15 µl samples from the test surfaces were loaded in each well in the following order: Au, lane 1; —OH, lane 2; $-CH_3$, lane 3. The titrated rat IgG standard (10 µl in each well) was added according to: 0.2 gl^{-1}, lane 4; 0.1 gl^{-1}, lane 5; 0.05 gl^{-1}, lane 6; 0.025 gl^{-1}, lane 7; 0.012 gl^{-1}, lane 8. The pooled plasma reference (5 µl, 1 : 100) was added to lane 9. Arrows indicate the molecular weights estimated from the prestained marker: β-galactosidase (117 000), ovalbumin (48 000), carbonic anhydrase (33 000) and soybean trypsin inhibitor (25 000).

3.2. Densitometry

Western blotted proteins desorbed from the *in vitro* plasma incubated surfaces were image-processed by digitalized video imaging and analyzed using computer software. Similar amounts of the specific proteins and similar intermaterial relationships were obtained in repeated experiments. Except for albumin, which fitted to an exponential curve, the titrated rat standards fitted to a linear line ($R > 0.99$). The densitometric results are summarized in Table II.

3.2.1. Immunoglobulin G

An imaged nitro-cellulose membrane, analyzed with respect to IgG is shown in Fig. 3. Almost twice as much IgG was retrieved from the gold surface (about 0.03 gl^{-1}) than from the two functionalized surfaces (—OH showed 0.013 gl^{-1} and $-CH_3$ 0.014 gl^{-1} Table II). The amount of IgG in the plasma reference was approximately 7 gl^{-1}, which is in accordance with the literature [54]. The IgG was effectively reduced, since no native IgG molecules with molecular weights of approximately 150 000 were found on the membranes. The visualized top and bottom bands correspond to the heavy and light chains found at molecular weights of approximately 50 000 and 25 000, respectively. Only these bands were densitometrically analyzed. The small protein bands in conjunction with and in between the heavy and light chain of the IgG standard refer to IgG subclasses. According to the manufacturer, differently sized κ- and λ-chains may occur, as well as breakdown products. The band between molecular weights of 25 000 and 33 000 is, plausibly, albumin (see below).

3.2.2. Albumin

Three clear bands were observed on the anti albumin incubated membrane (Fig. 4). The largest structure was detected at a molecular weight slightly above 70 000 (representing the whole albumin molecule), followed by approximately 50 000 and 30 000 proteins. These may represent prealbumin, subunits or degradation products. A band of putative cross-reactions with IgG heavy chain at approximately 50 000 in the gold sample was excluded prior to the densitometric analysis (Fig. 4, lane 1). Similarly for the pattern seen for IgG, pure gold adsorbed twice the amount of albumin compared with the hydroxylated and methylated surfaces. Approximated numerical values were for Au 0.042, —OH 0.018 and $-CH_3$ 0.019 gl^{-1}, respectively (Table II). The albumin value for the plasma sample (about 19 gl^{-1}) is low compared with that given in the reference literature [54], giving normal values ranging between 27 and 35 gl^{-1}. One possible reason for the low value could be so-called side-effects during electrophoresis, which resulted in altered conditions for that particular sample.

TABLE II A summary of the results from densitometric analysis of the stained bands on immunoblots obtained with rat plasma proteins desorbed (using a combination of SDS elution and mechanical rubbing) from pure gold, —OH and $-CH_3$ functionalized surfaces. Densitometric results applied to a pooled rat plasma sample are given. Mean values ± standard deviation (gl^{-1}) were calculated from the staining obtained with purified protein standards (in the same experiment). The compiled literature reference values of normal rat plasma proteins were obtained from [54]

Surface	IgG	Albumin	Fibrinogen	Fibronectin
Au	0.030 ± 0.006	0.042 ± 0.02	0.053 ± 0.015	ND[a]
—OH	0.013 ± 0.002	0.018 ± 0.02	0.023 ± 0.003	ND
$-CH_3$	0.014 ± 0.001	0.019 ± 0.02	0.026 ± 0.003	ND
Pooled plasma	6.6 ± 0.85	19.3 ± 4.3	5.1 ± 0.14	ND
Normal plasma	4–9	27–35	1.8–2.3	NA[b]

[a]ND, not detected.
[b]NA, not available.

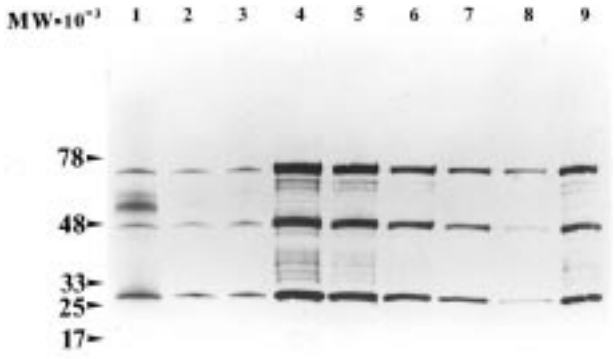

Figure 4 Staining of albumin in Western blots of samples from pure gold, —OH and $-CH_3$ functionalized surfaces, pooled plasma and purified rat albumin. Some 15 µl samples from the test surfaces were loaded in each well in the following order: Au, lane 1; —OH, lane 2; $-CH_3$, lane 3. The titrated rat albumin standard (10 µl in each well) was added according to: 0.4 gl^{-1}, lane 4; 0.2 gl^{-1}, lane 5; 0.1 gl^{-1}, lane 6; 0.05 gl^{-1}, lane 7; 0.025 gl^{-1}, lane 8. The pooled plasma reference (5 µl, 1 : 100) was added to lane 9. Arrows indicate the molecular weights estimated from the prestained marker: bovine serum albumin (78 000), ovalbumin (48 000), carbonic anhydrase (33 000), soybean trypsin inhibitor (25 000) and lysozyme (17 000).

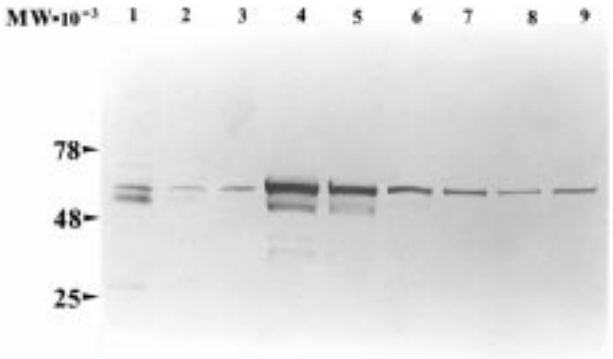

Figure 5 Staining of fibrinogen in Western blots of samples from pure gold, —OH, and $-CH_3$ functionalized surfaces, pooled plasma and purified rat fibrinogen. Some 10 µl samples from the test surfaces were loaded in each well in the following order: Au, lane 1; —OH, lane 2; $-CH_3$ lane 3. The titrated rat fibrinogen standard (10 µl in each well) was added according to: 0.6 gl^{-1}, lane 4; 0.3 gl^{-1}, lane 5; 0.1 gl^{-1}, lane 6; 0.05 gl^{-1}, lane 7; 0.025 gl^{-1}, lane 8. The pooled plasma reference (10 µl, 1 : 100) was added to lane 9. Arrows indicate the molecular weights estimated from the prestained marker: bovine serum albumin (78 000), ovalbumin (48 000) and soybean trypsin inhibitor (25 000).

3.2.3. Fibrinogen

The estimated concentrations of fibrinogen for Au, —OH and $-CH_3$ were 0.053, 0.023 and 0.026 gl^{-1}, respectively (Table II). The amount of fibrinogen in plasma was 5.1 gl^{-1}, which is higher than found in the reference literature (approximately 2 gl^{-1}) [54]. The molecular weight analysis revealed that fibrinogen γ-chains and IgG heavy chains migrated to similar levels in the gel used, and the cross-reactivity experiments indicated reactions of anti-fibrinogen antibody with the IgG heavy chain. Therefore, the band at the level of γ-chain fibrinogen in the gold sample was excluded before densitometric analysis. The two functionalized surfaces showed no γ-chain after plasma incubation for 1 min, and the amount of α + β-chains together was lower than for the pure gold surface (Fig. 5, lanes 1–3. Table II).

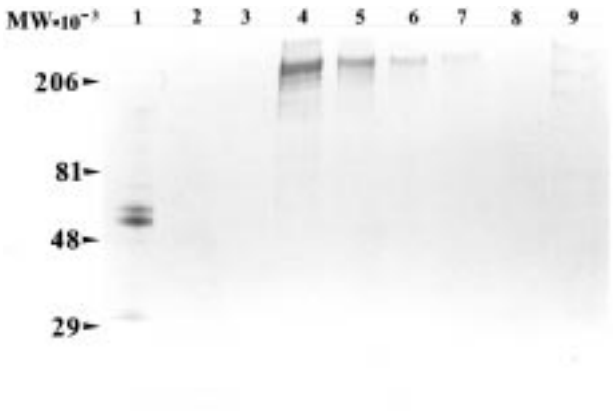

Figure 6 Staining of fibronectin in Western blots of samples from pure gold, —OH and $-CH_3$ functionalized surfaces, pooled plasma and purified rat fibronectin. Some 10 µl samples from the test surfaces were loaded in each well in the following order: Au, lane 1; —OH, lane 2; $-CH_3$, lane 3. The titrated rat fibronectin standard (10 µl in each well) was added according to: 0.2 gl^{-1}, lane 4; 0.1 gl^{-1}, lane 5; 0.05 gl^{-1}, lane 6; 0.025 gl^{-1}, lane 7; 0.012 gl^{-1}, lane 8. The pooled plasma reference (10 µl, 1 : 100) was added to lane 9. Arrows indicate the molecular weights estimated from the prestained marker: myosin (206 000), bovine serum albumin (81 000), ovalbumin (48 000) and soybean trypsin inhibitor (29 000).

3.2.4. Fibronectin

The purified fibronectin standard solution showed diffuse bands on the membrane, probably due to a high salt level in the standard solution (Fig. 6). Although three high molecular bands were stained in the plasma sample, no band typical of fibronectin at 220 000 was seen in the plasma or *in vitro* samples (Fig. 6, lanes 1–3 and 9). This is probably due to the low amount of fibronectin in the plasma. An estimated normal amount of fibronectin in rat plasma could not be obtained from the literature, however, the physiological concentration in human plasma is 0.17 gl^{-1} [26, 55]. Providing that the level of fibronectin in rat and human plasma is similar, plasma diluted 1 : 100 (used in our test) would correspond to a concentration of approximately 0.0017 gl^{-1}. This level is below the detection limit of the assay, seen from the staining of the fibronectin standards.

4. Discussion

Our results showed that pure gold had a two-fold adsorption of IgG compared to the functionalized methylated hydrophobic and hydroxylated hydrophilic surfaces. The result could be explained by the state of the chemical interactions between gold and proteins i.e. electric interactions, and the weaker binding of hydrophobic $(—CH_3)$ or electrostatic interaction $(—OH)$ on the surfaces. Comparison of our results of IgG adsorbed to surfaces and other reported experiments using whole plasma stresses the importance of the surface end group functionality in the adsorption of IgG. Studies of the adsorption kinetics of radioactively labeled IgG (in

whole human plasma) on polymeric beads with different surface functionalities, show that sulfonated polystyrene ($PSSO_3$) adsorbed more IgG than controls of glass and polystyrene, and thyrosyl and methyl sulfamide polystyrene beads [29]. In contradiction to the present results, experiments with rat plasma *in vitro* using ellipsometry/antibody techniques revealed no IgG on the gold or methylated surface but small amounts on the hydroxylated surface [51]. Using ellipsometry/antibody techniques when analyzing human plasma, IgG was detected on the gold surface as previously stated [7, 17]. Earlier studies show that human plasma proteins, which are expected to adsorb to pure gold [33], are not detected by ellipsometry/antibody techniques but by radioactive labeling techniques [32]. Taken together, IgG may be present on rat plasma-incubated gold and functionalized surfaces, although, only biologically accessible *per se* on the hydroxylated surface.

Surfaces preincubated with albumin have been reported to passivate platelet activation [56], the inflammatory reaction measured as phagocyte accumulation on surfaces [9, 36] and reduce cellular activity *in vitro* [24]. However, contradictory results have also been reported. More erythrocytes adhered and spread on albumin pre-coated surfaces than on fibrinogen and fibronectin pre-coated polymeric surfaces [26]. In our study semiquantitative analysis of the amount of albumin incorporated in the adsorbed plasma layer on the differently treated surfaces showed that more albumin adsorbed to the pure gold than to the functionalized surfaces. It is well known that albumin adsorbs to metal surfaces *in vitro* present alone in solution, or together with other proteins [18, 21, 25]. Studies on adsorption from solutions containing albumin and other proteins, have shown that the proteins compete for binding, particularly on a hydrophobic surface. Also, the conformational changes of albumin were more pronounced on the hydrophobic surface compared with the hydrophilic one [25]. It has further been suggested that the positive charge of gold binds negatively charged albumin by electric interaction [18]. Studies on albumin adsorption have been done by ellipsometry/antibody methods showing no or low adsorption of albumin to gold, hydroxylated and methylated surfaces from human [7] or rat plasma [51]. Differences in, for example, conformation and steric arrangement may explain why albumin was not detected using the ellipsometry/antibody technique, but was detected using the reported SDS method. Such factors may also be responsible for the different cellular responses observed *in vivo* on these surfaces [17].

Fibrinogen is considered to be the prime protein responsible for recruitment and accumulation of inflammatory cells to implants [57]. It may also be involved in delayed-type hypersensitivity reactions as well as in general inflammatory responses via the Integrin Mac-1 receptor, CD11b/CD18 [58]. In the present study pure gold surfaces collected twice the amount of fibrinogen compared with the functionalized surfaces. Conformation and access to particular epitopes on the surface-bound fibrinogen may, however, be more important for cellular response than the total amount [26, 59]. Our finding of fibrinogen on the different surfaces is in accordance with results obtained with targeted fibrinogen in whole rat plasma measured with ellipsometry/antibody techniques *in situ* [51]. In the same ellipsometry/antibody system no fibrinogen was, however, detected from human plasma on the hydrophilic —OH surface [51]. Other studies [3] show that gold surfaces adsorb significantly more fibrinogen than other metal surfaces from single protein solutions possibly due to the lack of a protecting oxide layer.

The high molecular weight fibronectins are a family of glycoproteins found on cell surfaces, in plasma and in connective tissue. Their function is mainly related to the adhesive ligand-like structure. Preadsorption of fibronectin may therefore alter the cellular responsiveness at implant surfaces [10, 22, 25] and may play a pivotal role for cell attachment and tissue organization at biomaterial surfaces. The results showing that no fibronectin adsorbed to the functionalized surfaces are in agreement with previous ellipsometry *in situ* measurements using rat and human plasma [17, 51]. Small amounts (using the same technique) were, however, found on the gold surface from both systems. We found colored bands at approximately 50 000 and 30 000, respectively, in the sample from pure gold. These bands probably represent cross-reactions with IgG. The surface chemistries may influence the adsorption of fibronectin, and different antigenic sites were exposed on the fibronectin bound to hydrophilic and hydrophobic surfaces [10] and on heparinized versus non-heparinized catheters [60].

A study of the fibronectin immunoreactivity of selected macromolecules surrounding titanium and polytetrafluoroethylene (PTFE) implants in the rat soft tissue after one week of implantation [61] are in accordance with our results on the functionalized surfaces, i.e. no fibronectin was found on the surface. In our *in vitro* study, IgG was detected in small amounts on the functionalized surfaces, which might corroborate with weak immunoreactivity of IgG close to the implants in the rat. The absence of albumin and fibrinogen immunoreactivity at the titanium and PTFE surfaces *in vivo*, does not correspond with our results indicating both albumin and fibrinogen on the gold and functionalized surfaces.

We have discussed a number of events involved in the adsorption of proteins to a surface. Surface characteristics, such as charge and pH, influence the binding [62], and upon adsorption, proteins may undergo conformational changes [25, 60, 62] and displace each other [29, 63, 64]. All these factors contribute to the complexity and difficulties in the evaluation of proteins binding to surfaces *in vivo*. Furthermore, our findings did not always corroborate with results obtained by ellipsometry/antibody techniques, and it is therefore important to use different methods for the analysis of protein–surface interactions. The ellipsometry/antibody techniques are, however, not suitable for evaluation of proteins from samples *in vivo*, wherefore the present SDS method was applied to an *in vivo* rat model, for examination of the composition of biofilms on implants. The analysis of proteins adsorbed to Au, —OH and $—CH_3$ after one day of implantation are under evaluation *ex vivo*. Preliminary results indicate high amounts of proteins eluted from the surfaces.

5. Conclusions

he aim of the present study was to apply sensitive techniques for the analysis of surface-adsorbed proteins. Rat plasma proteins were adsorbed to functionalized metallic substrata, desorbed by SDS combined with mechanical rubbing and analyzed with SDS–PAGE and immunoblotting techniques. By rubbing the surface with SDS, adsorbed proteins could be properly collected from all surfaces tested. The desorbed proteins were quantified by image analysis of the immunoblots. IgG, albumin and fibrinogen were found on all tested surfaces. Fibronectin was not detected on any of the surfaces. Gold surfaces adsorbed more proteins than the —OH and $—CH_3$ functionalized surfaces. The methods may be applied to situations involving comparisons between protein adsorption/desorption *in vitro* or *ex vivo*. The image analysis and semiquantification of proteins by densitometry can be used, together with other techniques, to determine the relative amount of proteins on biomaterial surfaces.

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