**Adsorption of HSA, IgG and laminin-1 on model hydroxyapatite surfaces –
effects of surface characteristics**

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Short title: Protein adsorption on HA surfaces

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Abstract

Ellipsometry and mechanically assisted sodium dodecyl sulphate elution was utilized to study the adsorption of human serum albumin (HSA), human immunoglobulin G (IgG), laminin-1, as well as competitive adsorption from a mixture of these proteins on spin-coated and sintered hydroxyapatite (HA) surfaces, respectively. The HA surfaces were characterized concerning wettability and roughness by means of water contact angles and atomic force microscopy, respectively. Both surface types were hydrophilic, and the average roughness (Sa) and surface enlargement (Sdr) were lower for the sintered compared to the spin-coated HA surfaces. The adsorbed amounts on the sintered HA increased as follows: HSA < laminin-1 < IgG < protein mixture. For the competitive adsorption experiments, the adsorbed fractions increased accordingly: HSA < laminin-1 < IgG on both types of HA substrata. However, a higher relative amount of HSA and laminin-1 and a lower relative amount of IgG was found on the spin-coated surfaces compared to on the sintered ones. The effects observed could be ascribed to differences in surface roughness and chemical composition between the two types of HA substrata, and could have an influence on future implant surface coatings.

Keywords:

dental implants, protein mixture, calcium phosphate, nanotopography, elutability, ellipsometry, gel electrophoresis

Introduction

When implants are introduced into the human body they will immediately interact with plasma proteins (Brunette et al. 2001) which will adsorb onto the surface of the implant. In nature, this event precedes but is otherwise concurrent with the presence of potentially adhesive cells (Mendonca et al. 2008). The arrangement and composition of the initially adsorbed protein layer is of major importance for the following cellular reactions which in turn is decisive for the host response to the biomaterial (Mendonca et al. 2008, Flemming et al. 1999, Tagaya et al. 2011). This study aimed at gaining a deeper understanding of the influence of the characteristics of the biomaterial on plasma protein adsorption. Such knowledge would be beneficial for the design of successful biomaterials.

Implant surface characteristics, such as roughness (Flemming et al. 1999, Wennerberg & Albrektsson 2009) and surface free energy (Mendonca et al. 2008, Zhao et al. 2005) are known to influence protein adsorption, as well as subsequent events leading to osseointegration, i.e. the connection between living bone and an implant surface. Influence of roughness at the micrometer level has been well documented and shown favorable osseointegration with an optimal average height deviation parameter (Sa) of 1-1.5 μm (Wennerberg & Albrektsson 2000). Recent reports further indicate that also nanometer roughness may be of importance for this process (Mendonca et al. 2008, Meirelles et al. 2008, Elias & Meirelles 2010). Wettability of the implant has also been shown to influence cell response and attachment to the surface. Hydrophilic surfaces have shown a higher degree of bone-to-implant contact and also a higher degree of adhered cells compared to hydrophobic surfaces (Zhao et al. 2005, Rupp et al. 2006, Zollner et al. 2008). Surface modifications such as coating of the implant surface with bioactive

molecules, e.g. hydroxyapatite (HA), have further indicated an effect on bone formation and soft tissue healing (Meirelles et al. 2008, Jimbo et al. 2011). HA exhibits good biocompatibility and as it is the mineral component of biological hard tissues like bone and teeth, there is a vast interest in using it as a coating on implants. In particular, such enhancement of osseointegration would be beneficial for challenging cases as e.g. patients in need of bone augmentation or with non-optimal bone quality, as well as vulnerably patients who have diminished wound healing ability due to e.g. multiple diseases or irradiation treatment in the head and neck region. By investigating the influence of surface characteristics on plasma protein adsorption, it would be possible to strive at designing an implant surface that will promote the adsorption of specific plasma proteins which in turn would be beneficial for enhanced cell attachment and growth during osseointegration and mucosal healing. In the same context, it would also be favorable to suppress the adsorption of other types of proteins that may interrupt such processes. Thus, the present study aimed at testing the hypothesis that the adsorption of selected plasma proteins, as single proteins as well as in a mixture, is affected by the characteristics of hydroxyapatite substrata, as e.g. surface roughness, and that the adsorption pattern of the investigated proteins is different between hydroxyapatite and pure titania substrata. In the current investigation three plasma proteins were studied; human serum albumin (HSA; 67 kDa, pI ~4.7), human immunoglobulin G (IgG; 150 kDa, pI's ranging between 5 and 8) and laminin-1 (from Engelbreth-Holm-Swarm murine sarcoma basement membrane; approx. 900 kDa, pI ~6.8). HSA and IgG have been shown to reduce cell attachment (McFarland et al. 1998, Sousa et al. 2008), whereas studies on laminin have indicated improved soft tissue response and implant integration when coated on a titanium implant (Dean et al. 1995, El-Ghannam et al.

1998, Werner et al. 2009). The proteins were thus chosen for this study based on their documented (positive or negative) effect on early stages of osseointegration and soft tissue regeneration.

In situ ellipsometry and mechanically assisted sodium dodecyl sulphate (SDS) elution were used to study adsorption of the proteins to two types of HA surfaces; one model HA surface (sintered) and one with HA spin-coated on a titanium substratum. Single protein systems, consisting of HSA, IgG and laminin-1, respectively, were studied. Also the competitive adsorption from a mixture of these three proteins was studied. Adsorption from a defined mixture of proteins represents a complex model that is closer to the *in vivo* situation of plasma protein adsorption, while the model is still simple enough to interpret competitive adsorption behavior. As surface roughness and wettability are key factors that influence the integration of an implant, the studied HA surfaces were characterised with respect to surface topography using atomic force microscopy (AFM) as well as by water contact angle measurements.

Materials and methods

General

A 10 mM phosphate buffer supplemented with 50 mM NaCl (pH 7.0), denoted PBS, was used for all adsorption experiments. HA is a complex surface with both Ca^{2+} and PO_4^{2-} at the interface which could result in some degree of dissolution of these ions into the solution leading to an effect on the surface charge as well as on the adsorption behaviour. In a previous study utilising the same type of sintered HA substrata and with the same buffer composition but also containing 1 mM CaCl_2 (Hahn Berg et al. 2001), it was shown that calcium phosphate nucleation occurred on the substrata resulting in surface instabilities and thus failure to record protein adsorption. Therefore no calcium was included in the buffer in the present study. Any significant changes in the calcium phosphate composition of the surface would have been evident from the ellipsometric studies and resulted in unstable values for surface characterization. As this was not observed, such changes were considered to be negligible.

The anionic surfactant sodium dodecyl sulphate, SDS, (L6026) was purchased from Sigma-Aldrich Sweden AB (Stockholm, Sweden), and used without any further purification. It was diluted in PBS to a final concentration of 17 mM (i.e. 0.5 % (w/v)) for the elution trials. All water used was of ultra high quality (UHQ), processed in an Elgastat UHQ II Model UHQ-PS-MK3 unit (Elga Ltd, High Wycombe, Bucks, England). All other chemicals used were of at least analytical grade (VWR International, Stockholm, Sweden).

Proteins

HSA, (A3782), and IgG, (I4506), and laminin-1 (L2020), were all obtained from Sigma-Aldrich Sweden AB (Stockholm, Sweden), and used as received. All proteins were diluted in PBS. The experiments were performed at concentrations of $25 \mu\text{g ml}^{-1}$ of HSA, $50 \mu\text{g ml}^{-1}$ of IgG, and $2.5 \mu\text{g ml}^{-1}$ of laminin-1, respectively, as well as from a mixture of the three proteins at the above mentioned concentrations. The protein concentrations used in the present study were based on a previous study on model titania substrata, using the same experimental outline (Santos et al. 2011). The concentrations were chosen so that approximately equal molar concentrations of HSA and IgG were used ($25 \mu\text{g ml}^{-1}$ of HSA = 373 nM, $50 \mu\text{g ml}^{-1}$ of IgG = 313 nM). As laminins are known to be present in plasma at only trace amounts, the concentration of laminin-1 was chosen to be 100 times more dilute ($2.5 \mu\text{g ml}^{-1}$ of laminin-1 = 3 nM) compared to HSA and IgG. The chosen concentrations does not resemble the physiological relationship or concentrations in human plasma, but are still considered relevant when characterizing the physicochemical adsorption behaviour from a mixture (Santos et al. 2011).

Surfaces

Two types of hydroxyapatite (HA) surfaces were used; I) polished titanium surfaces with spin-coated hydroxyapatite (Promimic, Göteborg, Sweden) and II) sintered hydroxyapatite discs (HA, Ø 16 mm, Calcitek, Carlsbad, CA, USA).

For the spin-coated HA surfaces, the calcium/phosphorous ratio (Ca/P) was confirmed by XPS to be 1.6 (data not shown), which is close to the theoretical value of hydroxyapatite (1.67). The thickness of the HA layer was between 200-300 Å. In the case of HA coated implants, the integrity of the coating is of highest relevance, as this will directly influence the protein adsorption pattern and cell adhesion (see

comparison below between HA and titania in the results and discussion section). These newly developed spin-coated surfaces used in the present study have been shown to retain its characteristics, compared to other earlier types of implants with a thicker HA layer, resulting in a higher success rate of the implants (Lee et al. 2000). Prior to use the surfaces were soaked in water for 5 minutes followed by rinsing in water, ethanol and water. The surfaces were then dried under nitrogen, and stored individually in sealed Petri dishes until use.

The sintered HA surfaces had a Ca/P ratio of 1.2 as confirmed by XPS (data not shown). The substrata were polished according to the procedure in Hahn Berg *et al* (Hahn Berg et al. 2001). The surfaces were cleaned by rinsing in ethanol and then sonication in ethanol for 30 minutes. Thereafter they were rinsed extensively in water, treated in dilute household detergent solution (YES Original, Procter and Gamble, Stockholm, Sweden: 15-30 % anionic surfactants, 5-15 % non-ionic surfactants, methylisothiazolinone, phenoxyethanol) and finally rinsed extensively in water, ethanol and water. The cleaned surfaces were dried under nitrogen and kept individually in a sealed Petri dish.

Prior to the adsorption measurements both surface types were cleaned for 5 minutes in radio frequency plasma (model PDC-32G, Harrick Scientific Corp., USA) in residual air at 0.03 mbar at a power of 30 W. Glow discharge treatment would also remove non-crystalline debris from the surfaces. The surfaces were used immediately after glow discharge treatment.

Contact angle measurements

A Kruss Goniometer (model DSA 100) (Kruss GmbH, Hamburg, Germany) was used to measure the water contact angles on the surfaces by means of the sessile drop

method. The measurements were carried out in air at room temperature, using UHQ as a probe liquid. The application of the droplet from the syringe onto the surface was done manually and the contact angle measured immediately. The spreading process was recorded using a CCD camera connected to an image analyser. The images were analysed using the software DSA1. A mean of 5 measurements on two separate discs of each surface type (n=10) was used for the determination of the contact angle. The measurements were done after surface cleaning and glow discharge treatment.

Atomic force microscopy (AFM)

AFM imaging was performed using a commercial set-up (Multimode SPM with a Nanoscope IV control unit, Veeco Instruments Inc., Santa Barbara CA). Silicon rectangular cantilevers with integrated pyramidal tips, and with a nominal resonance frequency between 320 kHz and 364 kHz, were used (model RTEP7, Veeco Instruments Inc., Santa Barbara CA). Imaging was performed by operating in the tapping mode, in air and at room temperature. Two different parameters were selected to characterize the surface topography, one height descriptive (S_a) and one hybrid parameter; surface developed area ratio or surface enlargement (S_{dr}), i.e. the ratio between the 3D measurement and the 2D area. These parameters are presented as a mean and a standard deviation values calculated from at least three measurements over an area of $5 \times 5 \mu\text{m}^2$ area. The different roughness parameters were calculated with the NanoScope v6.13 software (Veeco Instruments Inc., Santa Barbara CA). Three-dimensional representations of the surfaces (Figure 1) were generated with the WSXM software (Horcas et al. 2007).

Ellipsometry

In situ null ellipsometry was used to study the adsorption of the individual proteins HSA, IgG, laminin-1, as well as competitive adsorption from the mixture of the three proteins. An automated Rudolph thin film ellipsometer type 43 603-200E (Rudolph Research, Fairfield, N.J., USA) was used, with a xenon light filtered to a wavelength of 4429 Å, incident at an angle of 67.83° to a plane normal to the surface. Ellipsometry is an optical technique based on the fact that polarized light changes its state of polarization when reflected at a surface. In null ellipsometry the polarizer and analyzer positions corresponding to the minimum transmission of light to the detector are measured. Since the measurements are made every ~6 sec in the present null ellipsometer, the method allows dynamic studies of film growth/removal with a time resolution relevant for many biological processes. A detailed description of the theory, instrumentation and applications of ellipsometry is given by Azzam and Bashara (Azzam & Bashara 1989). To reduce the effect of imperfections in the optical components, a four-zone surface calibration was performed on the clean surface in both air and PBS, which consists in averaging the azimuths obtained in the four settings (zones) of polarizer, analyser and compensator that give light minima. According to a three-layer optical model, where the three layers are the ambient solution, the protein film, and the bulk surface, the ellipsometric angles Δ (relative phase shift given by the polarizer position) and Ψ (relative amplitude change given by the analyzer position), were measured in PBS. Assuming isotropic media and planar interfaces the complex refractive index of the HA ($N_2 = n_2 - ik_2$) can be determined. Typical values of the optical parameters of the HA surfaces were: $n_2 = 1.653 \pm 0.002$ and $k_2 = -0.02 \pm 0.005$. After adding the protein solution, the ellipsometric angles are used to calculate the mean refractive index and mean

thickness of the film. When the difference between the refractive index of the surface and adsorbed film is low the resolution in the Ψ and the Δ angles does not allow an accurate determination of the refractive index and thickness of the film. However, the adsorbed amount can be accurately determined by imposing a constant refractive index (of 1.5 in the present study) for the protein film (Arwin et al. 1993). This is a procedure that has been used in previous studies (Santos et al. 2008, Santos et al. 2010) and it has been shown that applying a constant refractive index of 1.5 is close to the real situation (Tengvall et al. 1998). The refractive index of the ambient medium used in the calculations was 1.339. From the film thickness and the refractive index, the adsorbed amount (Γ ; mg m^{-2}) can be calculated according to de Feijters formula (de Feijter et al. 1978), which assumes a linear increase of the refractive index with the concentration. The refractive index increments used were 0.187 for HSA, laminin-1 and the protein mixture and 0.188 for IgG (Santos et al. 2011). Only the sintered HA substrata were reflective enough to be used in these measurements.

The surface was mounted vertically in a 5 ml quartz cuvette, thermostated to 37° C and with a magnetic stirrer at constant stirring rate (325 rpm). After surface characterization, solutions of the individual proteins were added to the cuvette at the final concentrations of 25 $\mu\text{g ml}^{-1}$ of HSA, 50 $\mu\text{g ml}^{-1}$ of IgG, and 2.5 $\mu\text{g ml}^{-1}$ of laminin-1, respectively, as well as from a mixture of the three proteins at the above mentioned concentrations. This mixture of the proteins was prepared one hour prior to addition to the cuvette in order to stabilize. The adsorption was monitored for 60 minutes after which a 5 minutes rinse with PBS was performed using a continuous flow of 18 ml min^{-1} . After rinsing, the film was allowed to stabilize for 30 minutes followed by addition of SDS solution. After either 5 or 30 minutes, a second rinse

with PBS was performed using the same settings as above. The film stability was then monitored for a final 30 minutes. All experiments were performed at least twice with a deviation less than 0.1 mg m^{-2} from the mean.

Collection of adsorbed proteins from HA surfaces

The compositions of the adsorbed films from the competitive adsorption experiments were obtained for both the sintered HA and the HA spin-coated titanium surfaces by means of mechanically assisted SDS elution. This technique has been described in a previous publication (Santos et al. 2011). Briefly, each type of surface was submerged in a tube containing 5 ml of PBS and the protein mixture was added to the final concentrations given in the ellipsometry section. After 60 minutes of adsorption on a shaking table at room temperature, the surface was removed from the solution and rinsed with 5 ml of PBS and then dried with nitrogen gas. The experimental outline used for adsorption studies in this section was carried out to resemble the experimental outline used in the ellipsometer as closely as possible. Subsequently, the surface was rubbed with polyamide fibre pellets (Quick-sticks™, Dentonova AB, Huddinge, Sweden) soaked in 17 mM SDS solution. Five consecutive rubbings were performed followed by rubbing with two dry fibre pellets. The fibre pellets were then collected in a test tube and 10 µl of the SDS solution was added. The tubes were then heated to 80 °C for 7 minutes (Carlen et al. 1998) followed by perforation of tubes, placement in an outer test tube and centrifuged for 2 minutes at 1000 rpm in a table-top centrifuge (Mini centrifuge C-1200, Labnet International Inc, Woodbridge, NJ, USA). The eluted SDS solutions containing the adsorbed proteins were stored at -20 °C until subjected to gel electrophoresis.

Gel electrophoresis, staining and image analysis

7.5 % Tris-HCl Ready Gel[®] Precast SDS-PAGE gels (Bio-Rad Laboratories, Sundbyberg, Sweden) were used to separate and visualize the adsorbed proteins. A volume of 20 µl (corresponding to the maximum sample volume per well) was loaded on the SDS-PAGE. Laemmli sample buffer (161-737), and Tris/Glycine/SDS buffer (161-732) were used for the gel electrophoresis and were obtained from Bio-Rad (Bio-Rad Laboratories, Sundbyberg, Sweden). Gels were run at a constant voltage (200 V) for approximately 40 minutes. Subsequently, the gels were stained according to “Sypro Ruby protein gel stain” staining protocol (Molecular Probes, Leiden, the Netherlands, no. S-12000).

The elution from the quicksticks was tested as to see if all proteins had been released from the fibre material, and this was done by placing a fibre pellet directly on the gel. The data showed no trace of any of the investigated proteins (data not shown), thus indicating that all proteins had been eluted from the fibre pellets. A previous publication (Siqueira et al. 2007) showed a 97 % recovery of salivary proteins loaded on electrode wick filter paper strips using a similar technique, thus further indicating the complete elution of proteins from the collection pellets.

A Chemi Doc[™] XRS unit (Bio-Rad Laboratories, Sundbyberg, Sweden) was used to scan the stained gels and the visualized protein bands were then quantified by means of the software Quantity One[®], also supplied by Bio-Rad. The relative amounts presented were calculated based on the ratio of the density of the individual proteins to the total density of all proteins in the mixture.

Result and discussion

Surface characteristics

Two different types of HA surfaces were chosen for comparison in the present study. One was a titania substratum with a thin layer of spin-coated HA. The reason for using the spin-coated HA surface was that this surface coating has shown promising results in enhanced osseointegration in animal studies *in vivo* (Meirelles et al. 2008) and might consequently be a candidate for the market. Additionally, this surface was interesting to incorporate in this study due to its surface roughness, as it has been shown that surface roughness itself in the nanometer scale also enhances osseointegration (Mendonca et al. 2008). As this surface has great potential as implant substrata, no modifications (polishing or heavy cleaning) were performed, to keep the surface characteristics as close to the state it would be in if implanted into the body. The other HA surface was a sintered HA disc, which served more as a model substrata than as a possible implantable surface. This model HA surface has been used in several studies (Hahn Berg et al. 2001, Santos et al. 2011, Santos et al. 2008, Santos et al. 2010), and has recognized cleaning and polishing procedures to obtain stable and reproducible results with ellipsometry. The reason for choosing these two substrata was merely to obtain fundamental data on how different characteristics of the surfaces would influence the protein adsorption. The comparison with titania ((Santos et al. 2011)) was done as HA-covered implants have the potential to be as successful as or even replace the well-known and studied titania.

The water contact angles were determined to be $15^{\circ} \pm 2$ (sd) for the HA spin-coated titanium (n=10) and $8^{\circ} \pm 1$ (sd) for the sintered HA surfaces (n=10). The contact angle values showed that both surface types have a hydrophilic character, in line with

previous reports (Kawasaki et al. 2003). As the surface roughness may induce hysteresis even on rough hydrophilic substrata, the advancing and receding contact angles were also measured. They were shown to exactly resemble each other (data not shown), verifying that no such hysteresis was present on the studied substrata.

Two different roughness parameters obtained from AFM measurements are shown in Table 1 for the two studied HA surfaces as well as for the HA spin-coated underlying polished titanium surface. The average height deviation parameter S_a , and the developed surface area ratio (S_{dr}), were lower for the sintered HA than for the spin-coated HA substrata, revealing that the latter surface is clearly rougher. This could also be seen in the 3D representation of both surface types in Figure 1. The difference in roughness may be explained by the pre-treatment of the sintered surface by polishing before the cleaning process. This polishing procedure of the surface made from sintered HA was needed for the ellipsometry technique, which requires a very smooth and reflective surface.

As the surface roughness was found to differ between the two HA surfaces this may influence the protein adsorption pattern and subsequently the biological response since more adhesion sites will be available on the spin coated surface (Mendonca et al. 2008, Aryal et al. 2009, Blind et al. 2005, Scopelliti et al. 2010).

Adsorption & desorption determined by ellipsometry

One of the main aims of the paper was to investigate differences in adsorbed amounts between different plasma proteins as well as how the adsorption differed on different HA surfaces. In general, the amount of adsorbed proteins will influence the subsequent particle/cell adhesion. If the surfaces are covered by a monolayer or more, the approaching cells will interact mainly with the protein film. A thicker

adsorbed film (i.e. higher number of adsorbed protein layers) will more sufficiently screen the surface characteristics. On the contrary, cells approaching surfaces covered with less than a monolayer will naturally be more affected by the implants characteristics.

Total adsorbed amounts

Adsorption of HSA, IgG and laminin-1 on HA

The plateau values of adsorbed amounts of HSA, IgG, laminin-1 and protein mixture after 60 minutes of adsorption measured by ellipsometry, are illustrated in Figure 2. The low adsorbed amount of HSA on sintered HA (Figure 2) could be due to repulsive electrostatic interactions between the slightly negatively charged HSA and the negatively charged HA surface (Kawasaki et al. 2003). The Debye screening length was approximately 15 Å in the buffer used (ionic strength 0.08 M (Hahn Berg et al. 2001)), and thus lower than the protein size (dimensions of HSA: heart shaped: 80*80*80 Å with a thickness of 30 Å (Ferrer et al. 2001)) resulting in an efficient screening of most of the long-ranged electrostatic interactions between proteins and substrata. The low adsorption of HSA was in agreement with earlier reports (Iafisco et al. 2010). For laminin-1, the adsorbed amounts approached a side-on monolayer coverage (corresponding to 4.1 mg m⁻²; (Santos et al. 2011)), and for IgG, a mixed monolayer of side-on and end-on (corresponding to 2.5 and 13.1 mg m⁻² respectively; (Santos et al. 2011)) adsorbed molecules seemed to exist on the surface. To the authors knowledge, this is the first *in situ* ellipsometric study of laminin-1 adsorption to HA substrata. Laminin-1 resembles a crucifix type of structure (Beck et al. 1990) with one long flexible arm of 1100 Å in length and 30 Å in width. This length and width was used for the monolayer coverage calculations, and thus it

seems likely that laminin-1 was lying flat on the surface, similar to what was found on titania substrata (Santos et al. 2011). The results for IgG differed from previous reports, where the adsorption was only 0.5 mg m^{-2} (Kandori et al. 2004). However comparisons are difficult due to different experimental procedures, as well as different buffers and adsorption times.

One should keep in mind that although the adsorbed amounts can closely be related to the estimated monolayer coverage, there are no evidence that the adsorbed amounts are monolayers, but could also resemble that of incomplete bilayers/multilayers with naked patches of the surface in between since the thickness obtained with ellipsometry is an average thickness, based on a model that assumes a homogenous layer. Thus, the proteins may interact with already adsorbed components and not only with the HA substrata.

Comparing the adsorbed amounts in molar amounts to the molar concentrations of each protein gives a rough approximation of the affinity of the proteins at the investigated concentrations, and also disregards the molecular mass of the proteins. For HSA this resulted in an affinity factor of $0.008 (0.2 \text{ mg m}^{-2}/67000 \text{ g mole}^{-1})/(25 \text{ mg l}^{-1}/67000 \text{ g mole}^{-1})$, IgG: 0.080 and laminin-1: 1.44, thus indicating that laminin-1 had the highest affinity of the investigated proteins for the sintered HA surface.

Adsorption from the mixture

The total adsorbed amount from the protein mixture was 4.6 mg m^{-2} (Table 2), which is much lower than the total of the individual proteins added together (HSA: 0.2 mg m^{-2} , IgG: 4 mg m^{-2} , laminin-1: $3.2 \text{ mg m}^{-2} = 7.4 \text{ mg m}^{-2}$, Table 2) which suggests that the proteins in the mixture interacted and competed for the adsorption sites. It should

be pointed out that the proteins may adsorb both to sites on the surfaces as well as to other proteins which are present both on the surface as well as in solution.

Comparison of total adsorbed amounts between HA and titania

HSA and laminin-1 adsorbed less on HA compared to titania, whereas the adsorbed amount of IgG as well as adsorption from the mixture were higher on HA substrata (Table 2). HA plates have a reported zeta potential of -25 mV in 10 mM phosphate buffer pH 7.0 (Kawasaki et al. 2003) compared to titania which have been reported to have a zeta potential of -32.6 mV in 20 mM $\text{NaC}_2\text{H}_3\text{O}_2$ buffer, pH 7.15 (Wassell & Embery 1996). Although the buffers used for the zeta potential determinations differed for the two types of substrata, the pH was similar, and it would be expected that the surfaces would differ in the magnitude of the charge at the ionic strength used in this investigation. The differences in charge, as well as the differences in nanometer roughness (Table 1) and chemical composition between the surface types are likely reasons for this difference in adsorption behaviour. Additional reasons concerning the IgG adsorption is the fact that the protein solution contains multiple fractions of molecules with varying pI's. A shift in the composition of the film might thus also be likely. The same could be argued for the mixture, which also consists of different fractions.

Effect of PBS Rinsing and SDS elution on protein adsorption

Rinsing with PBS did not induce any visible change in adsorbed amounts for HSA or laminin-1 (Figure 2). For IgG a decrease of approximately 12.5 % could be observed and for the protein mixture a 11 % decrease of the adsorbed amount was seen (which could be due to the high content of IgG in the mixed protein layer, see below and

Table 2). Treatment with SDS reduced the adsorbed amounts considerably for all studied proteins as well as for the mixture. The reduction in adsorbed amount after 5 minutes exposure to SDS followed by buffer rinse was for HSA: 50 %, IgG: 90 %, laminin-1: 80 % and for the protein mixture: 90 % (Figure 2) (again the high content of IgG in the mixed film may be the cause of this behavior, see below and Table 2). As the SDS concentration (17mM) was 8.7 times the critical micelle concentration (cmc) when dissolved in the buffer used (Hahn Berg et al. 2001), this was expected. Additionally, we tested a prolonged exposure time with SDS of 30 minutes, but only the amount of laminin-1 was affected and decreased further to 97 % (Figure 2). This could be due to the large size of laminin, where more adsorptions sites needed to detach simultaneously for desorption of the whole protein, and a prolonged SDS elution might have facilitated such desorption. The results showed that IgG was easier to remove from the HA surface, both with PBS rinsing and with SDS elution compared to HSA and laminin-1. IgG is a rather large protein which would anticipate that it would adsorb with many anchoring points. However, these attachment sites seem to be rather easily disconnected upon dilution/elution. The reason may be that different fractions of IgG with different pI's may adsorb with different strength to HA (see above). Further, the different fractions may interact, forming different types of complexes on the surface that are more or less easily desorbed. A contributing factor could also be the stable structure of IgG. Previous studies have shown that IgG experiences less unfolding upon adsorption to hydrophilic surfaces compared to HSA (Vieira et al. 2009). As minor unfolding of IgG may occur, it would be easier to desorb from the surfaces (as unfolding generally results in increased anchorage to the surface).

Adsorption kinetics

In Figure 3, a representative example of the kinetics of adsorption from the competitive adsorption experiments of HSA, IgG and laminin-1 is shown. It is clearly seen that the initial adsorption is fast and levels out to a plateau within the monitored 60 minutes of adsorption. Also the elution kinetics are illustrated.

Comparison of adsorption kinetics between HA and titania

Compared to previous studies of the same system on titania model substrata (Santos et al. 2011), the initial adsorption kinetics were similar for the two types of surfaces, as well as the fact that a plateau was reached within 60 minutes. There was also a good agreement between the total adsorbed amounts at that time. A previous investigation showed that there was no statistical difference in total amount of plasma protein adsorbed to HA-coated titanium surfaces compared to pure titanium (Lee et al. 2011). However, lower amounts remained adsorbed after SDS treatment on HA (approximately 0.3 mg m^{-2} ; Figure 3) compared to titania (approximately 1.8 mg m^{-2} (Santos et al. 2011), which indicated that the film formed on HA was not as strongly adsorbed as on titania. This could, apart from weaker surface-protein interactions, also be due to different fractions of the proteins adsorbed on the two types of substrata (see below and Table 2) and protein-protein interactions at the surfaces. HSA and some fractions of IgG are negatively charged at neutral pH, and laminin-1 is also slightly negatively charged, and thus interactions/competition between the adsorbed proteins depending on substratum characteristics is expected. In previous studies on competitive adsorption from HSA, IgG and fibrinogen mixtures (Lassen & Malmsten 1997, Lassen & Malmsten 1996) it was shown that on methylated substrata HSA and IgG were the dominating components in the adsorbed

film, while on hydrophilic substrata fibrinogen was dominating the adsorbed layer with only traces of HSA and minor amounts of IgG.

Adsorbed proteins from protein mixture determined by gel electrophoresis

The technique based on mechanically assisted SDS elution of adsorbed proteins has been used in several previous publications and shown to be efficient in removal of different types of protein films (Santos et al. 2011, Carlen et al. 1998, Kalltorp et al. 2000, Milleding et al. 2001, Svendsen et al. 2008). Quantification using gel electrophoresis in combination with Sypro Ruby staining is also a technique used in the literature (Berggren et al. 2000, Chevalier et al. 2004, Wheelock et al. 2006) and Sypro staining is known to be very sensitive with linearity over 3 orders of magnitude, making it one of the most trustworthy stains for quantifying proteins in gel electrophoresis. The above mentioned characteristics justify the use of this technique for the protein quantification performed in the present study.

Adsorbed fractions of the proteins from the mixture; comparison between spin-coated HA and sintered HA.

Table 2 illustrates the eluted amounts from the competitive adsorption experiments performed with mechanically assisted SDS elution, after rinsing with PBS to remove the loosely bound layer, on sintered HA as well as HA spin-coated titanium surfaces. As the relative amounts were compared, the factor of actual surface area of the different substrata is eliminated. When comparing the relative adsorbed amounts obtained by gel electrophoresis of the respective proteins from the mixture, there is an obvious difference in the composition of the films between the two types of HA surfaces. HSA showed twice the relative amount on the rougher spin-coated surface

compared to on the sintered one. Differences in pure HSA adsorption between different HA substrata (plate-shaped and needle-shaped HA nanocrystals) have been shown previously, corroborating differences in adsorption behavior depending on HA surface (Iafisco et al. 2010). Further, Iafisco et al. also showed differences in HSA protein conformation when adsorbed to the two HA substrata. An increase in the relative fraction of laminin-1 was also observed on the spin-coated HA substratum. However, for IgG the trend was the opposite with approximately half the relative amount on the HA spin-coated titanium surface compared to on the sintered HA. However, for both types of substrata, a trend could be observed where IgG played a significant role in the adsorbed films followed by laminin-1, while HSA was present at lower fractions (Table 2). However, the high affinity of laminin-1 is reflected in its high content in the film while it constitutes less than 1 % of the protein molecules in the solution.

Relation between HA surface characteristics and competitive protein adsorption

Both HA surface types are considered to be of hydrophilic character as shown by the determined water contact angles, indicating that other factors are of importance for the observed difference in protein adsorption behaviour (Table 1). Previous studies on nanorough titanium substrata showed a large increase in bovine serum albumin (BSA) and fibrinogen adsorption when increasing the roughness from 150 to 300 Å (Scopelliti et al. 2010). Another factor contributing to the observed differences could e.g. be differences in chemical composition of the two surfaces. The Ca/P ratio differ between the two surfaces (1.6 for spin-coated and 1.2 for sintered HA), but previous studies have shown that adsorption of IgG and BSA are only weakly dependent on the Ca/P ratio (Kandori et al. 2004). However, from the XPS data (not shown) it was

revealed that the spin-coated surface contains titanium and aluminium, which is absent for the sintered one. Further, the sintered surface contains sodium which is absent in the spin-coated HA substrata. The presence of trace elements on the surfaces could influence the adsorption behaviour as they may impose new charges and thus possibilities for interactions.

Adsorbed fractions of the proteins from the mixture; comparison between HA and titania

The model (sintered) HA substrata differed the most from the other types of substrata. For these surfaces, the protein layers consisted of only 24 % of laminin-1 and as much as 65 % IgG. Spin-coated HA substrata also has a lower laminin-1 fraction (35 %) compared to the titania substrata (67 %) but not as low as that on sintered HA, possibly because it is sprayed onto a titania substratum, and as described above the XPS data revealed titanium on the surface of this substratum.

Differences in adsorption behavior between a mixture of proteins and the proteins individually have been observed in previous studies (Santos et al. 2011, Horbett 1993, Klein et al. 1980, Brash 2000, Arvidsson et al. 2007) and could be influenced by both the underlying substratum as well as the combination of proteins.

The differences in adsorption behavior observed on the two types of surfaces in the present study may indicate differences in e.g. osseointegration depending on HA surface type. Thus it is of great importance to use well-characterized HA substrata for optimal protein adsorption and finally successful implant retention.

Conclusions

The present study showed different adsorption as well as buffer and surfactant elution behavior of HSA, laminin-1 and IgG upon adsorption to sintered HA surfaces. The studies on stability of the films demonstrated that the HSA and laminin-1 films were unchanged when exposed to PBS rinsing mimicking physiological conditions. However, the laminin-1 film was the least stable to surfactant rinsing. The present study further demonstrated differences in the competitive adsorption from mixtures of these proteins on hydroxyapatite surfaces with different characteristics (sintered and spin coated). These variances were manifested as differences in the relative fractions of the proteins adsorbed on the different surfaces. The effects observed could be ascribed to differences in surface roughness and chemical composition. The hypothesis stated in the introduction thus showed to be true. The surface roughness and the chemical composition of the substrata will influence the initial protein film composition. These differences in protein film quality may further affect subsequent events of cell adhesion. This study contributes to a deeper knowledge in understanding protein-material interactions which is of fundamental importance for successful implant surface coating designs.

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Figure captions

Figure 1. Representative three-dimensional AFM topography images of **a)** spin-coated and **b)** sintered hydroxyapatite surfaces. Both images have the same height and color scales. Color ranges from 0 Å (black) to 340 Å (white). For more details on the pure titania substratum, the reader is referred to Santos et al 2011 ((Santos et al. 2011)).

Figure 2.

Adsorbed amount (mg m^{-2}) of HSA, IgG, laminin-1 and from the protein mixture before (adsorption 60 min) and after 5 minutes of buffer rinse (R), as well as after SDS addition, of either 5 minutes or 30 minutes, followed by PBS rinsing. Variations in adsorbed amounts between duplicates were below 0.1 mg m^{-2} . Data obtained with *in situ* ellipsometry. Experiments were performed at least twice with a deviation less than 0.1 mg m^{-2} from the mean.

Figure 3.

Representative graph of adsorbed amounts (Γ ; mg m^{-2}) vs. time (minutes) during build-up from the protein mixture on sintered hydroxyapatite surfaces measured by *in situ* ellipsometry. The protein solutions are added at time 0 minutes. R indicates buffer rinsing, D indicates desorption (stabilization) and SDS indicates addition of 0.5 % SDS to the cuvette for 5 minutes (a) and for 30 minutes (b). Experiments were performed at least twice.

Figure 1

Figure 2

Figure 3a

Figure 3b

Table 1.

Average height deviation, (Sa) and developed surface area ratio, (Sdr), of spin-coated HA and sintered HA surfaces as measured by AFM. The parameters are presented as a mean value and a standard deviation value calculated by averaging at least three $5 \times 5 \mu\text{m}^2$ images at different positions on the samples.

Surface type	Sa (Å)	Sdr
Sintered HA	14±4	0.15±0.05
Spin coated HA	63±9	1.5±1.1
Titania	56±5	1.1±0.3

Table 2.

Comparison between adsorbed amounts (Γ ; mg m^{-2}) and relative adsorbed amounts (Γ ; weight fraction) obtained for titania, sintered HA and spray coated HA surfaces. Experiments were repeated at least twice, with a variation below 10 % for the relative adsorbed amounts in the mixture.

Surface type	Γ (mg m^{-2}) after 60 minutes obtained with ellipsometry				Relative Γ (weight fraction) in the mixture obtained with gel electrophoresis		
	HSA	IgG	laminin-1	mixture	HSA	IgG	laminin-1
Sintered HA	0.2	4	3.2	4.6	0.11	0.65	0.24
Spin coated HA	-	-	-	-	0.27	0.38	0.35
Titania	1.4 ^a	3.4 ^a	3.8 ^a	4.0 ^a	0.05 ^a	0.28 ^a	0.67 ^a

^a(Santos et al. 2011).

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