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Protein adsorption on thin films of carbon and carbon nitride monitored with *in situ* ellipsometry

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Thin films of amorphous carbon and amorphous, graphitic and fullerene-like carbon nitride were deposited by reactive magnetron sputtering and optically characterized with spectroscopic ellipsometry. Complementary studies using scanning electron microscopy and atomic force microscopy were performed. The films were exposed to human serum albumin (HSA) and the adsorption was monitored *in situ* using dynamic ellipsometry. From the ellipsometric data the adsorbed amount of proteins was quantified in terms of surface mass density using de Feijter's model. The results indicate larger adsorption of proteins onto the amorphous films compared to the films with a more textured structure. Complementary studies with ¹²⁵I-labeled HSA showed an apparent protein adsorption up to 6 times larger compared to the ellipsometry measurement. In addition, the four types of films were incubated in blood plasma followed by exposure to anti-fibrinogen, anti-HMWK or anti-C3c revealing the materials response to complement and contact activation. The amorphous and graphitic carbon nitride exhibit rather high immune activity compared to a titanium reference, whereas the amorphous carbon and the fullerene-like CN_x show less immune complement deposition. Compared to the reference, all films exhibit indications of a stronger ability to initiate the intrinsic pathway of coagulation. Finally, the surfaces bone bonding ability was investigated by examination of their ability to form calcium phosphate crystals in a simulated body fluid, with a-CN_x depositing most calcium phosphate after 21 days of incubation.

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1. INTRODUCTION

Carbon-based materials receive a growing interest due to their mechanical, tribological, electronic, and optical properties [1-3]. However, not that much effort has been put into their use in biotechnology. A majority of the investigations regarding biointeractions on carbon films have been carried out on diamond-like hydrogenated carbon (DLHC) and pyrolytic carbon, which already are in use in artificial heart valves [4]. Some bioadsorption studies on amorphous carbon (a-C) and diamond like carbon (DLC) films have been reported [5,6], but only a few adsorption tests of biomolecules on carbon nitrides (CN_x) have so far been presented [7-9]. A few studies on haemocompatibility of CN_x -surfaces have also been published [10-12]. As an integrated part of our search for improved materials for life science applications like biomaterials and biosensors, the objective of the present study is to investigate the interaction of carbon and carbon nitride surfaces with blood proteins. Four types of carbon and carbon nitride films were grown by physical vapor deposition in a magnetron sputtering system and optically characterized with spectroscopic ellipsometry. The films are representatives of amorphous carbon (a-C), amorphous carbon nitride (a- CN_x), graphitic (g) CN_x , and fullerene-like (FL) CN_x . The two amorphous films exhibit a microstructure without order, whereas the graphitic structure is described with large graphitic domains with less curvature as compared to the FL structure, which consists of smaller domains and a more pronounced curvature. These materials exhibit high hardness and in addition the g- CN_x and FL- CN_x have shown an extreme elasticity [13, 14]. Depending on how the materials act *in vivo* they might be good candidates for bioactive coatings as inter-layers between inorganic materials and tissues, perhaps as a thin film on bone replacement materials to reduce wear particle formation.

Adsorption of proteins onto implant surfaces is a rapid process that strongly influences subsequent cellular response. The cellular response determines if a material is accepted by the body or not. In this work, protein adsorption on carbon and CN_x surfaces has been investigated using spectroscopic ellipsometry (SE), utilizing the *in situ* capability and sub-nanometer thickness resolution of this surface sensitive technique. Compared to single-wavelength ellipsometry, spectroscopic ellipsometry provides enhanced accuracy when measuring adsorption of biomolecules, e.g., native proteins. Prior to protein adsorption, the structures of the three CN_x films and the a-C film were optically characterized with

spectroscopic ellipsometry in the wavelength range 350-1200 nm to determine their complex-valued refractive index $N=n+ik$.

Static contact angles were measured with water and the morphology was examined using Atomic Force Microscopy (AFM) and Scanning Electron Microscopy (SEM). The films were exposed to human serum albumin (HSA) and the dynamics of adsorption was monitored *in situ* using ellipsometry. From the ellipsometric data the adsorbed amount of protein was quantified in terms of surface mass density using de Feijter's model [15]. Complementary studies with HSA labeled with iodine, ^{125}I -HSA (HSA*), a technique often used for quantification of adsorbed proteins from single protein solutions, has also been done.

The activation of immune complement and/or coagulation may be initiated by an artificial material implanted in the body. An uncontrolled regulation of the haemostatic and inflammatory processes upon contact with blood may lead to thrombus formation, and altered healing processes in tissues. Inflammation triggered by a foreign material is not necessarily negative, but is most undesirable in blood contacting applications. However, the fibrous capsule formed around a tissue-located implant, due to a prolonged inflammation process, reduces the ability for the implant material to adhere to the surrounding tissue and may increase the risk of impaired functionality. In order to get information about the response to contact activation and complement, the carbon and carbon nitride films were incubated in blood plasma, followed by incubations in anti-high molecular weight kininogen (a-HMWK), anti-fibrinogen (a-fib) and anti-C3c (a-C3c) solutions while monitored *in situ* using null ellipsometry (NE).

Bone-like bioactivity of the carbon and CN_x films were tested using simulated body fluid (SBF) with ion concentration and pH equal to that of human blood plasma.

2. MATERIALS AND METHODS

2.1 Surfaces and characterization

Thin films of a-C and a- CN_x , g- CN_x , and FL- CN_x , were grown to a nominal thickness of 200 nm on Si(001) wafers in a d.c. magnetron sputter deposition system and used as substrates for protein adsorption studies. Further details about the deposition technique, the microstructure and optical properties of the films are provided elsewhere [1,16,17].

Surface roughness of the films was determined with AFM using a Nanoscope IIIa from Digital Instruments operating in tapping mode and equipped with a Si tip having a cone angle

of 22°. Root mean square (RMS) roughness and average roughness (Ra) were evaluated from 1x1 μm^2 scans. The morphology was also studied with SEM using a Leo 1550 FEG SEM Gemini. Before protein adsorption, the contact angles of the films were measured with water droplets in static mode using an optical contact angle meter (CAM 200) from KSV Instruments Ltd.

Hydrophilic Si wafers (with native oxide) cut in pieces were used as reference surfaces in the ellipsometric protein adsorption study as well as in the experiments with labeled HSA, whereas Ti films (200 nm) evaporated on Si wafers were used as reference in the plasma study. For examination of the surfaces in SBF both Si and Ti samples were used as references. All Si samples were cleaned according to the RCA clean method with the SC-1 and the SC-2 steps [18]. After each cleaning step the samples were rinsed in Milli-Q water and dried in dry nitrogen gas. Due to this treatment the thin silicon dioxide layer on the samples became hydrated, giving the surfaces a hydrophilic character. The Ti samples were cleaned in ozone and UV light during 4 min.

2.2 Methods

Adsorption experiments with non-labeled HSA were performed *in situ* with a Variable Angle Spectroscopic Ellipsometer (VASE) from J. A. Woollam Co., Inc. in the spectral range 350 to 1200 nm at an incident angle of 68° at room temperature. The ellipsometer measures the spectral variation of the ellipsometric parameters Ψ and Δ defined by

$$\tan \Psi e^{i\Delta} = R_p / R_s$$

where R_p and R_s are the complex-valued reflection coefficients for light polarized parallel (p) and perpendicular (s), respectively, to the plane of incidence [19]. In addition to the ellipsometric measurement of protein adsorption, the surface mass density, Γ , was estimated by labeling of the protein in solution and measuring the radioactivity of the adsorbed protein in a gamma counter (Packard Cobra II, Canberra, USA). The plasma studies were performed and measured *in situ* at a wavelength of 633 nm and an angle of incidence of 70°, using a null ellipsometer of type Auto-El III from Rudolph Research, USA.

2.3 HSA, blood plasma, and antibodies

For *in situ* spectroscopic ellipsometry measurements, a stock solution (10 mg/ml) of HSA (Sigma) in phosphated buffered saline (PBS) at pH 7.4 was prepared and used at a final

concentration of 0.91 mg/ml. HSA was also labeled with ^{125}I using the Iodo-bead iodination method (Pierce Biotechnology, USA) [20] for measurements in a gamma counter. Adsorption experiments with labeled HSA were performed at a protein concentration of 0.2 mg/ml. Normal human blood plasma was prepared from blood of two healthy donors, mixed and frozen within 3 h to below -70°C until use. Sodium dodecyl sulphate (SDS) solutions were prepared by mixing SDS (Sigma) with PBS to a concentration of 0.3 %. Antibodies were obtained from DAKO Immunoglobulins A/S (rabbit anti-human C3c and rabbit anti-human fibrinogen) and Nordic AB (goat anti-human HMWK).

2.4 Ellipsometric procedure for HSA incubation

All HSA adsorption measurements on the a-C and CN_x films were performed at room temperature two months after film growth. A glass cell with a magnetic stirrer was used for the experiments as presented in Fig. 1. Before adsorption studies the a-C and CN_x thin film substrates were rinsed in alcohol and dried in flowing nitrogen whereas the cell was cleaned in three steps starting with SC-1 [18], followed by rinsing in alcohol and water and drying in nitrogen. The first two SE spectra in each experiment were recorded in air, without and with cell, respectively, to observe the effect of the cell windows. After addition of 3 ml PBS at pH 7.4, a dynamic scan at 500 nm was immediately recorded to verify a constant baseline. A spectral measurement in PBS was then performed. Another dynamic scan was started and after ~ 10 min, 300 μl HSA was added to give a final concentration of 0.91 mg/ml and data were recorded during 2 hours. A final spectral measurement of the sample, with the adsorbed HSA film, was performed.

2.5 Modeling of ellipsometric data

Acquisition and modeling of SE data were performed using the WVASE software from J.A. Woollam Co., Inc. [22]. The optical model consisted of a non-transparent carbon or CN_x film on top of a supporting substrate (Si), with the protein molecules forming a layer and PBS as ambient as schematically shown in Fig. 2. The a-C and CN_x films were sufficiently thick to be considered optically semi-infinite, and thus constitute the substrate in the optical model. Optical properties in terms of the complex valued refractive index $n_s + ik_s$ for the different carbon and CN_x films were obtained by wavelength-by-wavelength fits of experimental SE data for each film. The values of $n_s + ik_s$ for each individual film were then fixed and used in the subsequent fittings of Ψ and Δ recorded in presence of a protein layer.

Fig. 1. Schematic of the glass cell with magnetic stirrer used in ellipsometric *in situ* experiments with HSA.

The optical model for the protein layer includes the thickness d and a Cauchy model for the real-valued refractive index n_p according to

$$n_p = A + \frac{B}{\lambda^2}$$

where A and B are fitting parameters and λ is the wavelength in μm and B is in μm^2 . For the ambient PBS, earlier determined refractive index (n_a) data were used [23]. Experimental data were fitted to model-generated data using the Levenberg-Marquardt algorithm with d , A and B as fitting parameters. A multi-sample fitting procedure [24] was used to decrease the correlation between n_p and d in each protein film. In this procedure SE data from all four experiments are modeled simultaneously assuming the same (fitted) values of n_p , but with different thicknesses, for each protein film. A total number of six fitting parameter are thus used, the Cauchy parameters A and B and four thickness values. From the measurements in air with and without cell, the effects of the cell on the data were found to be small and less than 0.3° in Δ and negligible in Ψ . The recorded spectra can in principle be corrected for these window effects. However, we have chosen to include them in the analysis by solving for the substrate index $n_s + ik_s$ by using a two-phase model and SE-data recorded *in situ* prior to the start of each protein adsorption experiment. The advantage with this approach is that all systematic errors, like surface roughness, cell window effects, ambient index errors, beam

collimation errors, etc., will be included to first order. As quantification of the difference between experimental and model generated data, the standard-deviation biased mean squared error (MSE) as defined in [22] was used as a figure of merit of the quality of the fit. A value of 0.4 was found with the multi-sample procedure, which is a considerably low value.

Fig. 2. Schematic model (left) and optical model (right) used in the ellipsometric analyses. The blue ovals represent protein molecules.

The surface concentration Γ , i.e. the adsorbed surface mass density (ng/cm^2), was calculated using de Feijter's formula [15],

$$\Gamma = 100 \frac{d(n_p - n_a)}{dn/dc} \quad [\text{ng}/\text{cm}^2]$$

where d is the thickness (in nm) of the protein layer, n_p and n_a the refractive indices of the protein layer and PBS, respectively, and dn/dc the refractive index-increment (in cm^3/g) of HSA. A dn/dc value of $0.18 \text{ cm}^3/\text{g}$ was used, a value commonly used for protein adsorption [25].

Protein adsorption on Si was measured with NE in air and thickness values were calculated with the McCrackin algorithm [26]. Here $n_p=1.457$, which equals the refractive index of SiO_2 at 633 nm, was used to facilitate determination of Γ using a method developed by Stenberg et al. [27]. With this method, the apparent protein layer thickness d' in nm evaluated using the SiO_2 index, can be used to determine Γ according to the formula

$$\Gamma = 120 d' [\text{ng}/\text{cm}^2].$$

2.6 Radio labeling and experiment procedures

Labeling of HSA with sodium ^{125}I was performed using the Iodo-bead iodination method [20]. Sodium ^{125}I beads were dissolved in PBS at pH 6.5 and the solution was filtered and dialyzed. Finally, the activity of the solution was measured in a gamma counter. The HSA

concentration of the stock solution was determined by spectrophotometry measurements at 280 nm.

Before incubation in HSA* the a-C and CN_x films were rinsed in alcohol and dried in flowing nitrogen. The oxide thickness on each Si sample was measured with NE before incubation. Samples of CN_x, a-C and Si were incubated in a 0.2 mg/ml HSA* solution at room temperature during 60 min. Adsorbed protein layer thicknesses on Si samples were determined with NE before all samples were transferred to the gamma counter for radioactivity measurements. The activity of HSA* was measured during 10 min. Measured values of radioactivity were correlated with the amount of adsorbed protein by measuring known volumes with known HSA* concentration.

In addition, the elutability of HSA* on the samples was tested by incubation in unlabeled HSA during 24 h after which the remaining HSA* was measured. The samples were also incubated in SDS during 60 min and again the radioactivity of remaining HSA* was measured.

2.7 Procedure for blood plasma incubation using NE

All adsorption experiments with blood plasma and antibodies were performed with NE using a cell made of glass and steel. The cell was placed on an Al slab that could be resistively heated and maintained at a constant temperature of 37°C. The measurements were performed with NE *in situ* in veronal buffered saline supplemented with 0.15 mM CaCl₂ and 0.5 mM MgCl₂ (VB⁺⁺). The adsorbed protein layer thicknesses were calculated with the McCrackin algorithm [26] using $n_a = 1.335$ and $n_p = 1.465$ [28]. The surface mass density, Γ , was calculated using de Feijter's formula with $n_p = 1.465$ and $dn/dc = 0.18 \text{ cm}^3/\text{g}$.

Prior to adsorption in plasma, the substrate ellipsometric angles, Ψ and Δ , were determined for the Ti, a-C and CN_x samples immersed in VB⁺⁺ to define the substrate index. The cell was then emptied without letting the surfaces dry out and 100 % heparinized plasma was added. The samples were incubated during 20 min and thereafter the cell was rinsed with VB⁺⁺ (at 37°C) four times without flushing directly onto the samples, and again Ψ and Δ were measured after 5 min of equilibration in buffer. This was followed by incubation in 0.05% solutions of antibodies (a-HMWK, a-Fib or a-C3c) during 30 min. After rinsing and equilibration a last measurement was done for the determination of the presence of adsorbed antibodies on the samples.

2.8 Incubation in SBF

SBF was prepared by adding ions, to concentrations equal to those of human blood plasma, to deionized water and the pH of the solution was adjusted to 7.4. The solution was prepared according to a recipe by Kokubo [21]. The so called corrected SBF-solution was used.

Films of a-C, CN_x, and reference samples of Si and Ti were incubated during 3, 14, and 21 days in SBF. Three samples of each material were incubated in different tubes and stored in a water-bath at 37 °C until examination in SEM. Before examination in SEM, the surfaces were rinsed in distilled water and dried in flowing nitrogen.

3. RESULTS AND DISCUSSION

3.1 Characterization of a-C and CN_x films

Before exposure to proteins the a-C and CN_x films were examined with SEM and different surface morphologies were observed as shown in Fig. 3. The two amorphous films (a-C and a-CN_x) show very little contrast and are very smooth with small features. The g-CN_x and FL-CN_x films show a microstructure with spherical shaped nodules in the size of ~100 nm and ~40 nm, respectively. In addition, the SEM cross-section in Fig. 3 shows a columnar growth of the g-CN_x film and the nodular appearance of the surface is obvious.

Observations from plan-view and cross-sectional SEM images were confirmed by AFM measurements. The AFM surface plots in Fig. 4 indicate a low surface roughness for all films except the g-CN_x film showing a slightly rougher surface (RMS=13 nm). The amorphous films show a very smooth surface of 0.3 to 1 nm (RMS). The RMS and Ra values are listed in Table 1 together with angles from contact angle measurements. The a-C and FL-CN_x films could be described as slightly hydrophobic with contact angles up to 79°, whereas the two other films showed an intermediate behavior with contact angles around 65°.

Fig. 3. Plan-view SEM-images (a-d) of a-C and CN_x films before protein adsorption. The images were scanned at 2kV in secondary electron mode. In (e) a cross section of the $g-CN_x$ film is shown. The length scale in a-c is the same but is only shown in c.

Fig. 4. AFM surface plots from (a) an a-C film, (b) an a-CN_x film, (c) a g-CN_x film and (d) a FL-CN_x film, all with a scan size of 1x1 μm². The z-scale is 50 nm per division for all images except for (c) where the z-scale is 100 nm per division.

Table 1: Surface roughness as measured by AFM and contact angle measurements of the a-C and CN_x films.

Film	Ra [nm]	RMS [nm]	Contact angle [°]
a-C	1.0	1.5	79±1.6
a-CN _x	0.2	0.3	65±0.7
g-CN _x	11	13	67±2.9
FL-CN _x	2.0	2.6	76±2.0

3.2 Biointeractions – HSA incubation

Examples of primary data from HSA incubation experiments are shown in Fig. 5. Spectral SE data from the a-C film in terms of Ψ and Δ recorded before and after two hours of incubation in a 0.91 mg/ml HSA-solution is shown in Fig. 5 (a). Fig. 5 (b) shows a dynamic scan of the HSA incubation of the a-CN_x film. Results from modeling and analyses of the *in situ* HSA adsorption experiments are presented in Table 2 and in Fig. 6. The multi-sample procedure [24] used in modeling of the data is based on that the same protein index is fitted for all four films and it was found to be $n_p = 1.43 + 0.003/\lambda^2$. From de Feijter's formula, Γ was determined. Ideally, Γ should not depend on wavelength, but due to limitations in the de Feijter model, Γ shows a small variation with wavelength. To show the variation, Γ is presented at two wavelengths in Table 2. The thicknesses of adsorbed HSA differed among the samples in the range 1.3 to 3.8 nm and the corresponding differences in Γ was found to be in the range 75 to 220 ng/cm² at 633 nm and in the range 85 to 230 ng/cm² at 1200 nm. Γ at 633 nm is plotted in Fig. 6 together with results from the adsorption series performed with HSA*. The bars represent measurements with labeled HSA and the symbols are Γ -values obtained from ellipsometry measurements.

Fig. 5. (a) Ψ and Δ -spectra measured on the a-C film before and after HSA incubation. (b) Dynamics at 500 nm recorded on the a-CN_x film during 2 hours of incubation of HSA.

The results from SE and radioactivity measurements show a clear difference. In the experiment with labeled HSA, Γ is similar for all films whereas the ellipsometry measurements reveal a lower adsorption on the nano-structured surfaces, i.e. on the g-CN_x and FL-CN_x films. In addition, the radioactivity measurements show values in the range two to six times larger compared to Γ measured with SE. These differences might partly be explained by the surface

Table 2: Thickness and surface mass density at $\lambda=633$ nm and $\lambda=1200$ nm for the adsorbed protein films as evaluated by *in situ* ellipsometry.

Film	d [nm]	$\Gamma_{\lambda=633}$ [ng/cm ²]	$\Gamma_{\lambda=1200}$ [ng/cm ²]
a-C	3.5	200	210
a-CN _x	3.8	220	230
g-CN _x	1.3	75	85
f-CN _x	2.7	160	170

roughness of the films. In the SE experiment the surface mass density is normalized to the real microscopic surface area as seen by the protein molecules, whereas radioactivity measurements provide the surface mass density normalized to the macroscopic surface area as measured by a ruler and normalized with the surface area from AFM measurements. This difference was found in spite of that the adsorption measured in the gamma counter was performed from a 0.2 mg/ml solution (except for Si references) whereas the adsorption measured by *in situ* SE was from a 0.91 mg/ml solution. In addition, the incubation time for the labeled proteins was 60 min and for the ellipsometry measurements 120 min. The reason for using different parameters in the two experiments was not intentional. The concentration of the labeled protein solution was the highest possible. However, the different parameters are not likely to disturb the results. The HSA* and HSA adsorption on hydrophilic Si was comparable with result from other authors ranging from 50-200 ng/cm² depending on experimental conditions [29-31].

Another possible explanation of the differences of the results between the two measurement techniques seen in Fig. 6 is that the high ¹²⁵I signal for the a-C and CN_x samples originate from free ¹²⁵I absorbed from the HSA*-solution. This hypothesis is supported by the observation by Steinberg et al. that graphite, fullerene, and amorphous structures in carbon powder possess a high capacity for iodine sorption (higher than 450 mg iodine per gram) [32].

After the adsorption of HSA*, the samples were exposed to unlabeled HSA and SDS. After each step the radioactivity was measured to study the elutability (results in Fig. 7). In the case of HSA* molecules weakly adsorbed to the surface, these would be replaced by unlabeled HSA. Surfaces incubated in SDS would similarly loose most of the adhered HSA* and HSA molecules if they were not covalently bonded or firmly bound. Fig. 7 shows that the elutability of all films is very low with 60 to 80% of the original amount of HSA* still adsorbed to the

Fig. 6. Surface mass density of HSA and HSA* on C and CN_x films as well as on silicon references measured by in situ SE and in a gamma counter. Measurements on silicon references were performed with NE. Different a-C and CN_x samples were used for the radioactivity measurements and the in situ SE experiments, whereas the same silicon samples were used for NE and radioactivity measurements.

surface. This indicates very little reversible adsorption and could be a sign of firmly attached proteins. If this is true, the films showed very low elutability compared to the hydrophilic silicon reference which showed an elutability of more than 90%. Welin Klintström et al. [33] showed that SDS is more effective on hydrophilic surfaces compared to hydrophobic surfaces. However, a more probable explanation to the large signals from the carbon-based materials in Fig. 7 is that the signal to a large portion originate from high affinity absorbed ¹²⁵I on a-C and CN_x samples as suggested above. Buffer rinsing and SDS would simply not be able to desorb iodide from inside the materials [32,33]. This is a proof of non-specific uptake of free iodide by carbon materials and shows that the radioactivity measurement technique is not suitable on this type of materials.

The results from SE measurements display a decreased surface mass density on samples with increased surface roughness. The surface mass density does not follow the wetting

properties of the films, since the a-CN_x and g-CN_x films having the smallest contact angles (65-67) display the largest and smallest surface mass density as measured by SE.

However, the adsorption results can not only be correlated to one specific microstructural property. A complex situation with the interference of surface chemistry as, e.g., different surface groups, the isoelectric point, hydrophobic effects and surface charge as well as structural properties like bonding types, is more probable. Further investigation of those properties would be needed to be able to make any deeper analyses.

Fig. 7. Percentage of HSA* still adsorbed to the incubated surfaces after incubation in HSA and SDS.

3.3 Biointeractions - human blood plasma and antibody incubation

Native Ti, having a hydrophilic and negatively charged surface, is a material widely used in bone-replacing implants and was used as reference in the blood plasma experiments. Surface mass densities on a-C, CN_x films and Ti references resulting from exposure to human blood plasma and subsequent antibody incubations are plotted in Fig. 8 for antibodies of factor C3c,

HMWK and fibrinogen. Some variations of the adsorbed amounts of plasma (lower part of bars in Fig. 8) among the films can be observed. Slightly higher amounts are found on a-C and CN_x as compared to the Ti reference and slightly higher amounts are obtained on the amorphous films following the trend from SE measurements of HSA adsorption. A plasma deposition of ~400 ng/cm² on the Ti surface is a reasonable value compared to earlier work [34-36].

The results from exposure to antibody solutions probes the composition of the layer adsorbed from plasma. The levels of adsorbed antibodies found for Ti, i.e. low for a-fib and high for a-HMWK, are in good agreement with other references [35,36].

Fig. 8. Adsorbed surface mass density after plasma incubation (green/bright bars) and antibody incubation (blue/dark bars) during 20 min and 30 min, respectively. The left, middle, and right bar in each group corresponds to the response of a-C3c, a-HMWK and a-fib, respectively, as shown for the Ti sample.

The high amounts of a-HMWK adsorbed on the a-C and CN_x films indicate that the intrinsic pathway of coagulation is activated [37] on all samples, most probably due to that the surfaces are negatively charged [38]. The a-C as well as the FL-CN_x film showed relatively low levels of adsorbed a-C3c, indicating a low immune activation. Similar to Ti, the g-CN_x film adsorbs low levels of a-Fib indicating lower binding and activation of platelets and

thereby less clotting [39]. When fibrinogen adsorbs to a surface it becomes partially denatured and then binds platelets, if they are present. The other samples all show high levels of a-Fib which implies a down-regulation of the complement system and thus low a-C3c levels [40]. This agrees well with the a-C and quite well for the FL-CN_x but not for the a-CN_x. The a-CN_x film shows high levels of both a-C3c and a-Fib indicating that the complement is deposited despite the high levels of adsorbed a-Fib, which might be explained by protein-surface charge interactions, similar to what is observed on gold [41]. The high levels of adsorbed a-Fib on top of blood plasma on a-C and FL-CN_x surfaces indicate hydrophobic surface-protein interactions [42] and agree well with contact angle measurements.

In addition, smooth and inert surfaces are likely to induce complement activation, and thus deposition from blood plasma, to a higher extent compared to surfaces with nano-morphology in the range 2-3 nm. The latter present less binding sites for proteins, and therefore may influence the number of bound proteins. The smoothest film (a-CN_x) shows the highest a-C3c level of the three films within this topography range, which is in agreement with the hypothesis that smooth surfaces denature proteins to a higher extent than nano-rough surfaces of the same material. The g-CN_x film with a RMS value of 13 nm is outside this morphology range and follows probably another adsorption scenario.

3.4 Morphology and adsorption

It is known from literature that samples with larger surface area adsorb more protein and e.g. Mora et al. [43] have shown that pore size and surface area has a great impact on the behavior of adsorption on carbon materials. A large surface area is also correlated with an increased surface roughness and/or a decreased grain size of the material. A smooth surface with low surface roughness has room for a less number of protein molecules per macroscopic area compared to a surface with higher surface roughness above the nano level as illustrated by a) and b) in Fig. 9. When the grain size and, in this case also, the surface roughness increase, as suggested in Fig 9 c) and d), the amount of adsorbed proteins increases if normalised to the macroscopic area, i.e. the sample area measured with a ruler. However, if normalised to the microscopic area, the adsorbed amount would be the same, but can of course change due to changes in local conditions. The two films with a smoother surface in the study, the a-C and a-CN_x films might correlate to a schematic morphology between a) and b) whereas FL-CN_x and g-CN_x can be represented by Fig 9 c) and d), respectively. According to the first sentence in this section, that a larger surface area adsorbs more protein, the g-CN_x and FL-CN_x films,

which have the largest RMS values, should adsorb larger amounts of protein per macroscopic area compared to the two smoother films of a-C and a-CN_x. This would only be true if identical surfaces are compared, since only then, one may expect the adsorption per microscopic area to be the same. In fact, the SE results in Fig. 6 show that the samples with larger surface roughness have *less* microscopic adsorption. Thus, if we consider the SE results in Fig. 6, we can from Fig. 9 going from b) to d) imagine that an increased surface area not necessarily leads to an increased macroscopically observed adsorbed amount. Other factors like differences in surface chemistry among the four materials play important roles. In summary we find that the RIA results in Fig. 6, i.e. the amount adsorbed per macroscopic area, tell us that from a macroscopic point of view, e.g. as seen by a cell, the four films have similar amount of bound HSA around 500 ng/cm². (It is to remember though that this show probably not only adsorbed protein but also absorbed free iodide.) However, the SE results represent amount adsorbed per microscopic area and from a microscopic point of view, as seen by proteins and other macromolecules, the g-CN_x and FL-CN_x films have less surface mass density of HSA.

The state of hydrophobicity influences the adsorption process as well, and hydrophobic surfaces have been found to adsorb more proteins than hydrophilic surfaces [28,42]. Looking at our results this is true for the a-C film with a contact angle of 79° but not for the FL-CN_x film having a similar contact angle of 76°. But, since a variation in surface topography and hydrophobicity at the same time occurs for our films and the fact that a change in surface morphology also changes many other parameters, such as the electrical double layer, charge, electrical field divergence, accessible area, water structuring and ion uptake, etc., it is not possible to attribute a change in the adsorption behavior to one specific parameter.

Some authors mean that the wetting behavior has a greater impact on adsorption than has the chemical microstructure, such as the varying sp³ content in a-C [44]. It is somewhat difficult in this case, though, to separate the influence of wettability from the influence of surface roughness as well as microstructure, since all parameters differ among the four films.

Furthermore, it has been suggested that surfaces with stronger water-binding adsorb less amount of protein. The water uptake of carbon and CN_x was investigated by Broitman et al. using quartz crystal microbalance (QCM) measurements which showed that amorphous structures of carbon and CN_x adsorbed 10 times more water compared to the nano-structured films, and rough surfaces adsorbed more than smooth films [45,46]. Those results, regarding the nano-structure, are in apparent contradiction with the present work since the a-C and a-CN_x

films showed larger protein adsorption compared to the nano-structured films. On the other hand the nanostructured films showed larger RMS values compared to those in the reference. It is important though to take into account that the films in this study were exposed to air prior to adsorption experiments. The films in the QCM study were not exposed to air. This means probably that the film surfaces in our study were saturated with water and oxygen before the adsorption experiments started.

Fig. 9. Schematic surface morphologies as seen by HSA molecules (the ellipsoids). a)-d) represents surfaces of increasing roughness where a) is a perfectly smooth surface and d) represents the g-CN_x film. The other films in the study would fall somewhere among the morphologies represented by a), b) and c). Diameter values represent average grain sizes. Notice the different scale in d) including the HSA ellipsoid.

3.5 Calcium phosphate formation in SBF

For the testing of bone bonding potential of the carbon and carbon nitride thin films, they were exposed to SBF in 3, 14 and 21 days. The precipitation of calcium phosphate on the

samples after exposure was examined in SEM. No calcium phosphate formation was observed on any of the surfaces after three days in SBF, neither on Ti and Si references nor on carbon-based surfaces. In Fig. 10, Ti and FL-CN_x represent all the different films, since there was no detectable difference among them. After 14 days the Ti reference and the a-C film still did not show any calcium phosphate crystals. The other films showed calcium phosphate formation to various degrees. A few spots were found on the FL-CN_x film, whereas the g-CN_x, a-CN_x and Si surfaces showed an increased formation, with Si having the largest amount.

After 21 days all surfaces except Ti showed some calcium phosphate formation. From Fig. 11 one can estimate that a-C and FL-CN_x films had similar but low crystal density, whereas g-CN_x, Si and a-CN_x, in this order, displayed increasing calcium phosphate densities. A calcium phosphate crystal on the a-CN_x sample is shown in Fig. 12 at an increased magnification.

4. CONCLUSIONS

Carbon and CN_x films were exposed to HSA, human blood plasma and antibody solutions of complement factor C3c, HMWK, and fibrinogen. The adsorbed amounts were measured by SE, radioactivity measurements and NE. According to the SE measurements the carbon and CN_x films adsorbed different amounts of HSA, with larger surface mass densities observed on amorphous structures. The different adsorption results for the different films can not be correlated to one specific parameter, but it is clear that the roughness has a great impact on the adsorption. Results from parallel radioactivity measurements, indicate a significantly higher protein uptake compared to the SE method, most likely due to free iodide uptake by the carbon materials. Spectroscopic ellipsometry proves thus in this case to be a highly suitable calibration method for the revealing of radio-labeling method bugs.

Indications of surface response to blood coagulation, complement activation and clotting were observed after incubations in human blood plasma. All carbon and CN_x films activated the intrinsic pathway of coagulation, indicating a negative surface charge at physiological conditions. The amorphous carbon and graphitic films are more activating than the others, especially compared to the a-CN_x film that show the weighted best results in this study. Low fibrinogen binding was indicated by g-CN_x, and low complement deposition by a-C films.

The a-C and FL-CN_x films may have a future in soft tissue applications due to their low immune complement deposition, whereas the g-CN_x is a possible bone replacement candidate.

Fig. 10. SEM images of Ti and FL- CN_x after three days in SBF.

Fig. 11. SEM images of Ti, Si, a-C and CN_x films after 21 days in SBF.

Fig. 12. Magnified view of a calcium phosphate crystal on a-CN_x.

The a-CN_x film shows a remarkably complex and high bioactivity and the g-CN_x film shows a high bioactivity as compared to Ti.

The bone bonding ability of the a-C and CN_x films was investigated and the highest density of calcium phosphate crystals after 21 days of incubation in SBF was observed for the a-CN_x film.

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