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Platelet and leukocyte adhesion to albumin binding self-assembled monolayers

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Abstract This study reports the use of tetraethylene glycol-terminated self-assembled monolayers (EG_4 SAMs) as a background non-fouling surface to study the effect of an 18 carbon ligand (C18) on albumin selective and reversible adsorption and subsequent platelet and leukocyte adhesion. Surface characterization techniques revealed an efficient immobilization of different levels of C18 ligand on EG_4 SAMs and an increase of surface thickness and hydrophobicity with the increase of C18 ligands. Albumin adsorption increased as the percentage of C18 ligands on the surface increased, but only 2.5%C18 SAMs adsorbed albumin in a selective and reversible way. Adherent platelets also increased with the amount of immobilized C18. Pre-immersion of samples in albumin before contact with platelets demonstrated an 80% decrease in platelet adhesion. Pre-immersion in plasma was only relevant for 2.5%C18 SAMs since this was the only surface to have less platelet adhesion compared to buffer pre-immersion. EG_4 SAMs adhered negligible amounts of leukocytes, but surfaces with C18 ligands have some adherent leukocytes. Except for 10%C18 SAMs, which increased leukocyte adhesion after albumin pre-adhesion, protein pre-immersion did not influence leukocyte adhesion. It has been shown that a surface with a specific surface concentration of albumin-binding ligands (2.5%C18 SAMs) can recruit albumin selectively and reversibly and minimize the adhesion of platelets, despite still adhering some leukocytes.

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1 Introduction

Thrombus formation and inflammation are a major problem associated with blood contact medical devices. These two processes are interlinked and involve the interaction between the device surface, proteins, platelets and leukocytes [1].

Upon implantation, biomaterials, like any foreign body, trigger an acute inflammatory response that is characterized by an in situ accumulation of leukocytes. The early inflammatory response involves the activation of humoral defenses such as complement activation, coagulation, fibrinolytic and kinin systems [2, 3]. The formation of a thrombus is dependent upon either or both the behaviors of platelets at or near the surface and on the protein-based coagulation cascade [4].

Platelet and leukocyte adhesion to a biomaterial surface depends on many factors such as material surface chemistry and protein adsorption profiles [5–7]. The overall intensity of the acute inflammatory reaction depends on the chemical composition, wettability, surface free energy, surface charge, porosity and roughness [3, 8]. Since plasma proteins rapidly adsorb to synthetic materials in a biological environment, material surface properties affect cellular interactions via the composition, structure, and

conformation of the adsorbed protein layer. Therefore, protein adsorption onto a substrate is important in controlling cellular interactions with synthetic surfaces in vivo and also in vitro [9].

In order to increase the hemocompatibility of synthetic materials (usually polymers) much effort has been made in developing biologically "inert" materials, i.e. materials that resist adsorption of proteins, cells and bacteria (which are also referred as non-fouling surfaces). The most successful method has been to produce a coating on the surface with a specific hydrogel-like material, poly(ethylene oxide) (PEO) [10]. These non-fouling surfaces minimize adsorption proteins from blood, but are usually only adequate for short term applications or procedures since proteins eventually end up accumulating on the surface after a long period of time.

In the case of blood contacting biomaterials, three host proteins predominate: albumin, IgG and fibrinogen. Platelet adhesion on surfaces is mediated by integrin receptor GPIIb/IIIa and fibrinogen, but interaction with integrin receptor GPIb/IIa and von Willebrand Factor (vWF) can also occur [1]. In this study, research has been conducted to create surfaces that attract and bind albumin from the bloodstream in a selective and reversible way. Albumin was chosen since it is abundantly present in blood and at sites of injury and because it is known to "passivate" biomaterials surfaces, blunting proinflammatory and thrombogenic responses [11, 12].

Several approaches have been attempted to create surfaces that will attract and bind albumin from the bloodstream in a selective way. Albumin is an abundant blood plasma protein which transports many types of compounds including hormones, drugs, toxins, and fatty acids. Albumin coatings can be developed based upon the intrinsic affinity of albumin for these specific biological compounds. This involves the immobilization of ligands (e.g. alkyl chains of 16 and 18 carbon residues [13, 14], Cibacron Blue F3G-A [11, 15, 16], warfarin [17] and linear peptides [18]) or antibodies [19] to albumin, at the surface of the biomaterial. However, these approaches vary in terms of specificity, affinity and stability for albumin, as well as in their functional activity.

Based on the approach of selectively binding albumin to free fatty acids, tetra(ethylene glycol)-terminated self-assembled monolayers (EG_4 SAMs) with different percentages of C18 ligands on the surface were prepared. Selective and reversible adsorption of albumin to these surfaces was studied and related to the adhesion of blood platelets and leukocytes. This investigation using SAMs was performed in order to determine the influence of different amounts of a C18 compound immobilized on a non-fouling surface in the recruitment and exchangeability of albumin from plasma, as well as the influence of the pre-adsorbed protein layer in mediating platelet and leukocyte adhesion.

2 Materials and methods

2.1 Preparation of EG_4-C18 SAMs

Preparation of EG_4-C18 SAMs comprises two steps: (1) preparation of 1-Mercapto-11-undecyl tetra(ethylene glycol) self-assembled monolayers (EG_4 SAMs) on gold; (2) reaction of the OH groups from the EG chains with the isocyanate group of a compound with 18 carbons (C18). This procedure has been described in previous works [20, 21]. Briefly, 1 × 1 cm^2 gold substrates were cleaned and immersed in a 0.1 mM solution of 1-mercapto-11-undecyl tetra(ethylene glycol) (EG_4; Asemblon) in ethanol (99.8%, Merck). After incubation at room temperature for 24 h in a nitrogen environment, the samples were rinsed, dried and maintained in argon until used. For surface derivatization with the C18 ligands, EG_4 SAMs were immersed in solutions with different percentages (0, 2.5, 5 and 10%) of octadecyl isocyanate (C18; 98%, Aldrich). After incubation for 2 h at 40°C in a nitrogen environment, the samples were rinsed, dried and maintained in argon until used. A schematic representation of the structure of EG_4-C18 SAMs is described in Fig. 1.

2.2 Characterization of EG_4-C18 SAMs

The surfaces of EG_4-C18 SAMs were examined by different surface characterization techniques.

X-ray photoelectron spectroscopy (XPS) measurements were carried out on a VG Scientific ESCALAB 200A (UK) spectrometer using magnesium K∝ (1253.6 eV) x-rays as a radiation source. The photoelectrons were analysed at a take off angle of 55°. Survey spectra were collected over a range of 0–1150 eV with an analyser pass energy of 50 eV. High resolution C (1*s*), O (1*s*), S (2*p*), N (1*s*) and Au (4*f*) spectra were collected with an analyser pass energy of 20 eV. The binding energy (BE) scales were calibrated by setting the Au4$f_{7/2}$ BE to 84.0 eV. The spectra were fitted using an XPS peak fitting program (XPSPEAK version 4.1).

The static contact angles of water (with conductivity not higher than 1 μS/cm) and methylene iodide (>99%, Sigma-Aldrich) on the SAMs were measured with a contact angle measuring system from Data Physics, model OCA 15, equipped with a video CCD-camera and SCA 20 software. SAMs were placed in a closed, thermostated chamber at 25°C saturated with the contact angle liquid to prevent evaporation from the drop. 4 μl drops were deposited using

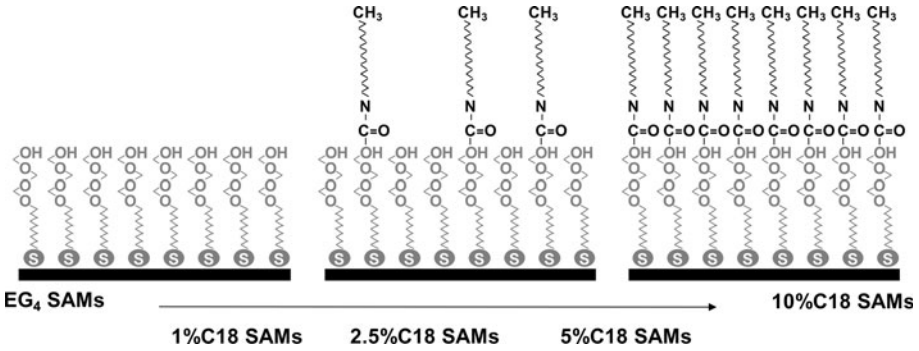

Fig. 1 Structure of EG_4 SAMs with different amounts of immobilized C18 ligand

the normal sessile drop method. The polar component (γ_s^h) and the dispersive component (γ_s^d) of the surface energy of the SAMs were evaluated by the Owens and Wendt Method [22]. Polar (γ_l^h) and dispersive (γ_l^d) components of the surface tension of the liquids that were used are 50.1 and 21.4 mN/m for water and 0 and 50.1 mN/m for methylene iodide, respectively [23].

Fourier transform infrared reflection absorption spectroscopy (FT-IRAS) measurements were performed on a Perkin Elmer FTIR spectrophotometer, model 2000, coupled with a VeeMax II Accessory (PIKE) and a liquid-nitrogen-cooled MTC detector. Dry nitrogen was purged into the instrument for 5 min before and during measurement of each sample, in order to minimize water vapor adsorption. For each monolayer, a similar gold surface was used as a background. Incident light was p-polarised and spectra were collected using the 80° grazing angle reflection mode. For each sample, 1000 scans were collected with 4 cm^{-1} resolution. Spectra were baseline corrected and smoothed.

Ellipsometry measurements were performed using an Imaging Ellipsometer, model EP^3, from Nanofilm Surface Analysis. This equipment was operated in a polarizer-compensator-sample-analyzer (PCSA) mode (null ellipsometry). The light source was a solid-state laser with a wavelength of 532 nm. The gold substrate refractive index (n) and extinction coefficient (k) were determined using a delta and psi spectrum with a variation of angle between 60° and 75°. These measurements were made in four zones to correct for instrument misalignment. To determine the thickness of the SAMs, similar spectra were used and n and k for the organic layer were set as 1.45 [24] and zero, respectively.

2.3 Protein adsorption studies

Protein adsorption to EG_4-C18 SAMs was performed using ^{125}I-albumin. Competition tests were made comparing adsorption from pure albumin solution with mixtures of albumin with fibrinogen. Exchangeability of adsorbed albumin for other albumin or fibrinogen was also evaluated.

Fibrinogen was selected for competition and exchangeability studies with albumin since it is one of the most important competitors for surface adsorption and the main protein responsible for blood clotting, namely by inducing platelet adhesion and activation [25]. The presence of fibrinogen in the layer of adsorbed proteins is considered to attract more inflammatory cells than when fibrinogen is absent [26]. Fibrinogen is therefore of great importance in the initial acute inflammatory response to biomaterials.

Protein solutions were prepared by dissolving human serum albumin (HSA) (Sigma, ref. A1653) or human fibrinogen (HFG) (Sigma, ref. F4129) in phosphate buffered saline (PBS) (Sigma, pH 7.4, lot 082K8215).

Quantification of adsorbed HSA to the different SAMs was performed using radiolabeled albumin. HSA was labeled with ^{125}I according to the iodogen method as described in previous studies [20]. ^{125}I-HSA was added to unlabeled 0.1 mg/ml HSA solution in order to obtain a final activity of 2×10^8 cpm/mg.

For the HSA adsorption measurements, SAMs were placed in a 24-well tissue culture plate (Sarsted) with the SAM surface facing up. A drop of PBS was added to the periphery of each well to maintain moisture and a 7 µl drop of HSA solution was pipetted onto each SAM. Protein adsorption was carried out at 25°C over a 30 min period. After rinsing the samples four times with 2 ml of PBS, the γ-activity was counted. HSA surface concentration was calculated by the following equation:

$$\mathrm{HSA(mg/m^2)} = \frac{\mathrm{counts(cpm)} \cdot C_{\mathrm{solution}}\mathrm{(mg/ml)}}{A_{\mathrm{solution}}\mathrm{(cpm/ml)} \times \mathrm{SA(m^2)}}$$

where the counts are the radioactivity measurements from the SAMs, the C_{solution} and A_{solution} are the concentration and the specific activity of the protein solution, respectively, and SA is the surface area of the drop. SA was calculated using the drop surface contact diameter obtained using the contact angle measuring system software for the

same conditions used during the protein adsorption tests (7 μl of 0.1 mg/ml HSA solution; 25°C; 30 min).

Competition studies with albumin were performed using fibrinogen as competitive protein. ^{125}I-HSA was added to a mixed solution with unlabeled 0.1 mg/ml HSA and 0.01 mg/ml HFG in order to obtain a final activity of 2×10^8 cpm/mg. Adsorption studies were performed as in single ^{125}I-HSA solution.

Exchangeability tests were carried out by immersing the SAMs with adsorbed ^{125}I-HSA in an unlabeled HSA pure solution (1 mg/ml, 25°C) or an HFG pure solution (1 mg/ml, 25°C) over 24 h. Samples were then rinsed four times with 2 ml PBS and residual radioactivity counted.

2.4 Platelet and leukocyte adhesion studies

Whole blood (plasma, platelets, leukocytes and erythrocytes) anticoagulated with citrate phosphate dextrose (MacoPharma) was collected from non-medicated healthy donors and processed at the Portuguese Blood Institute. *Buffy coat* (platelets and leucocytes) was obtained from centrifugation of the total blood unit at $4997 \times g$ (3900 rpm) for 10 min at 22°C.

The platelet concentrate (PC) is the supernatant obtained by centrifugation of the *buffy coat* at $328 \times g$ (1000 rpm) for 10 min at 22°C. The PC rested for 1 h and after was stored at 22°C with constant horizontal agitation. The number of platelets in the PC was counted using a hematology analyzer (Cell Dyn 3700 system from Abbott Diagnostic Division) and concentration adjusted to 3×10^8 platelets/ml.

In order to obtain the leukocyte suspension, some of the platelets had to be removed from the *buffy coat*, and therefore it was rinsed three times with a platelet additive solution (PAS; MacoPharma) by centrifuging at 1000 rpm for 10 min and discarding the supernatant where platelets tend to accumulate. Leukocyte isolation was performed using Histopaque 1077 and 1119 reagents (Sigma) which, by centrifugation, separate leukocytes from other remaining blood cells according to a double density gradient. 3 ml of Histopaque 1077 was smoothly added to 3 ml of Histopaque 1119 and then 6 ml of *buffy coat* was gently layered on the top. The tubes were centrifuged at $700 \times g$ for 30 min at 25°C. After centrifugation, an opaque white ring could be observed, corresponding to leukocytes, whereas undesired cells like erythrocytes are deposited in the bottom of the tube. Leukocytes were then rinsed twice with PBS. Cell counting was performed in a Neubauer chamber and leukocyte concentration was adjusted to 1×10^6 leukocytes/ml.

Platelet and leukocyte adhesion assays were performed in 24 well plates (Sarstedt). In order to avoid activation by the polystyrene of the 24-well plate, all the wells were filled with 1% (w/v) bovine serum albumin (BSA) in PBS, incubated for 1 h at 37°C and rinsed five times with PBS.

Since protein adsorption is known to influence cell adhesion, samples were pre-immersed in different protein solutions so as to investigate the effect of protein adsorption on subsequent adhesion of platelets and leukocytes. EG_4-C18 SAMs were pre-immersed for 30 min at room temperature in PBS, 0.1 mg/ml HSA or 1% human plasma solutions (in PBS). After protein adsorption, samples were rinsed three times with PBS to remove loosely bound proteins.

After pre-immersions, EG_4-C18 SAMs were incubated in the previously blocked 24 well plates with platelet concentrate or leukocyte suspension. In platelet adhesion studies, each sample was immersed in a platelet concentrate (3×10^8 platelets/ml) and incubated for 30 min, at 22.4°C, in a horizontal shaker (platelet incubator from Helmer-model PC 3200 series 300996K) at 70 rpm. In leukocyte adhesion studies, SAMs were immersed in wells containing 1 ml of RPMI 1640 culture medium (Gibco) supplemented with 10% heparinized plasma and 400 μl of leukocyte suspension (1×10^6 leukocytes/ml), being that the final leukocyte concentration in each well was 0.7×10^6 leukocytes/ml. Samples were incubated for 30 min at 37°C. All samples were then rinsed three times with PBS to remove weakly attached platelets or leukocytes.

SAMs with adherent platelets were treated for visualization by scanning electron microscopy (SEM). Adherent platelets were fixed with a freshly prepared solution of 1.5% glutaraldehyde (Merck) in 0.14 M sodium cacodylate (Merck) buffer for 30 min at room temperature. After fixation, SAMs were rinsed twice with PBS and dehydrated in a graded ethanol series (50, 60, 70, 80, 90 and 99%, for 10 min each). Finally, 0.01 ml hexamethyldisilazane (Sigma) was added to each sample and left to dry over night. Platelets were examined with a scanning electron microscope JEOL JSM-6301S at 10 keV and magnifications of 1000× and 5000× were used.

SAMs with adherent leukocytes were treated for visualization by light reflection microscopy. Adherent leukocytes were fixed with Hemacolor® Solution 1 (Sigma). After 2 min, samples were rinsed twice with ddH_2O and allowed to dry at room temperature. Each sample was then glued to microscope slides and stained with Hemacolor dyes for microscopy (Hemacolor® solutions 2 and 3), rinsed again and observed by light microscopy using an Olympus PM3 reflection microscope. A 50× objective was used, and each field corresponded to 8×10^{-3} mm^2.

Adherent platelet and leukocyte densities were determined by photographing and counting 10 different fields per sample (at least three replicates were used for each sample in each experiment) and expressed as mean number of platelets/mm^2 and median number of leukocytes/mm^2.

3 Results

All techniques used for surface characterization showed an efficient immobilization of C18 ligands on EG_4 SAMs.

XPS was used to obtain information on the chemical composition of the surface. This technique revealed an increase of the atomic percentage of nitrogen (N) associated with the increase of C18 on the surface, which was expected since N is present in C18 ligands but not in the EG_4 thiols that constitute EG_4 SAMs. Ellipsometry was used to measure the thickness of the SAMs and showed an increase of thickness as the percentage of C18 increases (Fig. 2). This is also in accordance with what was expected since ellipsometry determines the average thickness of a surface.

FT-IRAS was used to investigate the chain order and crystalline structure of the SAMs. The orientation of the alkyl chains relative to the surface can be deduced from the relative intensities of their CH_2-stretching vibrations. The asymmetric (v_a) and symmetric (v_s) CH_2 stretching modes for crystalline methylene units can be observed at c.a. 2920 and 2850 cm^{-1} (Fig. 3). The increase of intensity of these peaks corresponds to the increase of C18 ligands immobilized on EG_4 SAMs. None of the analysed spectra presented the characteristic peak from isocyanate (–N=C=O) at 2270 cm^{-1} (data not shown), indicating the absence of unreacted octadecyl isocyanate.

Contact angle measurements were performed to study the hydrophilicity and surface energetics of the surfaces. Static water contact angles indicated that SAMs get more hydrophobic with the increase of C18 immobilized, with the contact angles ranging from hydrophilic (37.7°) in EG_4 SAMs to hydrophobic (94.8°) in 10%C18 (Table 1). Contact angle measurements using water and methylene iodide showed that the surface free energy of SAMs decreased with the increase of C18 percentage in solution, meaning that SAMs with –OH functionalities yield higher surface energy than those terminated with methyl groups.

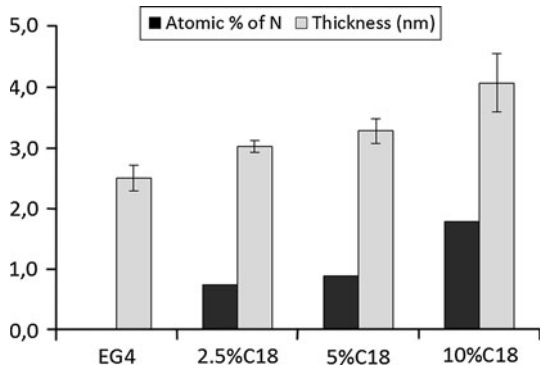

Fig. 2 Atomic percentage of nitrogen (determined by XPS) and thickness (determined by ellipsometry) of EG_4-C18 SAMs

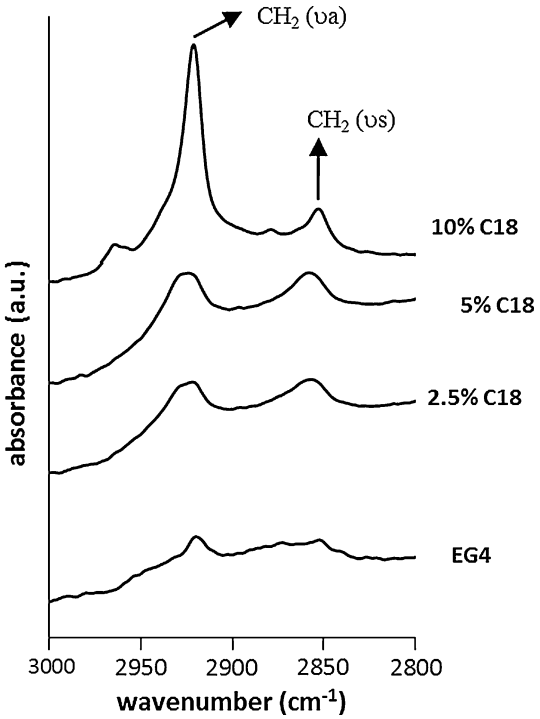

Fig. 3 IRAS spectra for CH-stretching vibrations near 3000–2800 cm^{-1} of EG_4-C18 SAMs

Figure 4 represents HSA adsorption on EG_4-C18 SAMs determined by radiolabeling (^{125}I) from a single albumin solution and from a mixed solution with albumin and fibrinogen in the same proportion as present in blood. Pure albumin adsorption studies showed an increase of HSA adsorption as surfaces get more hydrophobic, going from a low value of 0.14 mg/m^2 in EG_4 SAMs to 1.43 mg/m^2 in 10%C18 SAMs.

Regarding competition studies, EG_4 and 2.5%C18 SAMs maintain the amounts of adsorbed HSA even when exposed to a solution with both HSA and HFG. However, 5%C18 and 10%C18 SAMs decrease by 17 and 21% the adsorption of HSA when both proteins are present, meaning that fibrinogen is also adsorbing to these surfaces.

Table 1 Water and methylene iodine contact angles and surface free energy of EG_4-C18 SAMs

	Contact angle (°)		Total surface free energy (mN/m)		
	Water	Methylene iodide	Dispersive	Polar	Total
EG4	37.7 ± 0.5	36.0 ± 0.8	41.3	23.5	64.8
2.5%C18	55.9 ± 1.3	42.1 ± 0.7	38.3	14.7	53.0
5%C18	69.8 ± 0.8	46.6 ± 1.6	35.9	8.3	44.2
10%C18	94.8 ± 2.6	62.9 ± 0.8	26.7	1.6	28.3

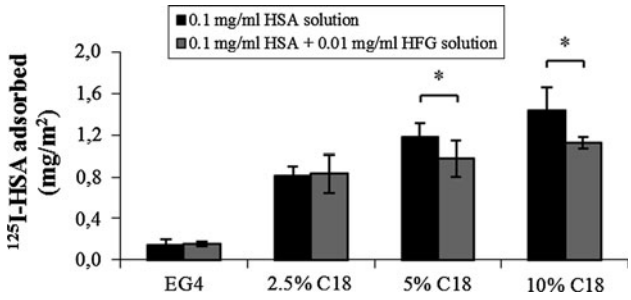

Fig. 4 Competitive adsorption between albumin (HSA) and fibrinogen (HFG) to EG_4-C18 SAMs

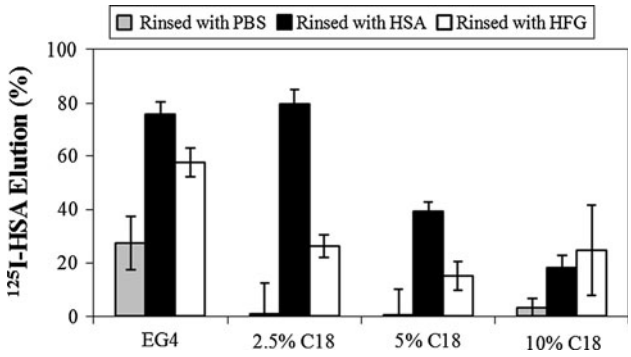

Fig. 5 Exchangeability of ^{125}I-albumin adsorbed to EG_4-C18 SAMs by buffer (PBS), other albumin (HSA) or fibrinogen (HFG)

SAMs with pre-adsorbed ^{125}I-labeled HSA (black bars from Fig. 4) were left for 24 h in unlabelled 1 mg/ml pure protein solutions of HSA or HFG, and PBS. The reversibility of albumin adsorption is presented in Fig. 5.

Background surfaces (EG_4 SAMs) have released approximately 0.035 mg/m^2 albumin from the surface when in contact with PBS over 24 h, meaning that 25% of the pre-adsorbed HSA was loosely bound. Regarding specific exchange, the two blood proteins are similar; EG_4 SAMs exchange $\approx$70% by other HSA and $\approx$60% by HFG. In contrast, 2.5%C18 SAMs presented a very selective exchange, replacing 80% of the pre-adsorbed HSA by other HSA, but only 25% by HFG. Regarding the other surfaces, 5%C18 SAMs retain higher amounts of ^{125}I-HSA, despite still having some selectivity on the exchange, whereas 10%C18 SAMs have 80% of the pre-adsorbed HSA irreversibly bound and do not have a preferred protein to exchange with.

Platelet adhesion to EG_4-C18 SAMs after pre-immersion in buffer (PBS), albumin (HSA) or plasma is represented in Fig. 6. Following the trend observed in protein adsorption, there is an increase of platelet adhesion as the percentage of C18 ligands increases. When a pre-immersion of HSA is performed, there is an 80% decrease in the number of platelets adherent to all surfaces with C18 ligands, stressing the passivant effect of albumin in blood contact surfaces. However, when SAMs are pre-immersed in plasma, the only surface that shows a significant ($P < 0.05$) reduction in the number of adherent platelets comparing to buffer pre-immersion is 2.5%C18 SAMs (from 499 to 236 plt/mm^2).

Regarding leukocyte adhesion to EG_4-C18 SAMs, the effect of protein pre-adsorption to the surfaces was also evaluated (Fig. 7). The background surface (EG_4 SAMs) exhibited no leukocyte adhesion, despite the pre-immersion. Even though SAMs with C18 ligands adhered more

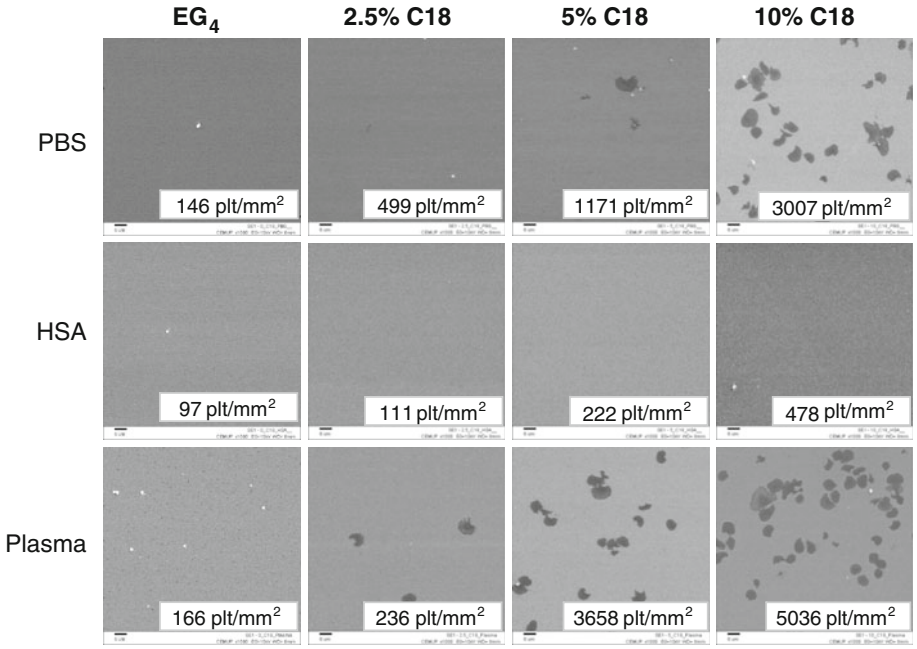

Fig. 6 Representative images of platelet adhesion to EG_4-C18 SAMs after pre-immersion in buffer (PBS), albumin (HSA) or plasma. Images obtained by SEM (magnification: $\times$1000). The mean number of adherent platelets per mm^2 is stated on the *bottom right* corner of each image. Platelet counts were statistically different ($P < 0.05$) among the four surfaces within each pre-immersion; One-Way ANOVA. Comparing each pre-immersion to PBS of the same surface, all values were significantly different ($P < 0.05$), except plasma pre-immersion in EG_4 SAMs; independent-samples T test

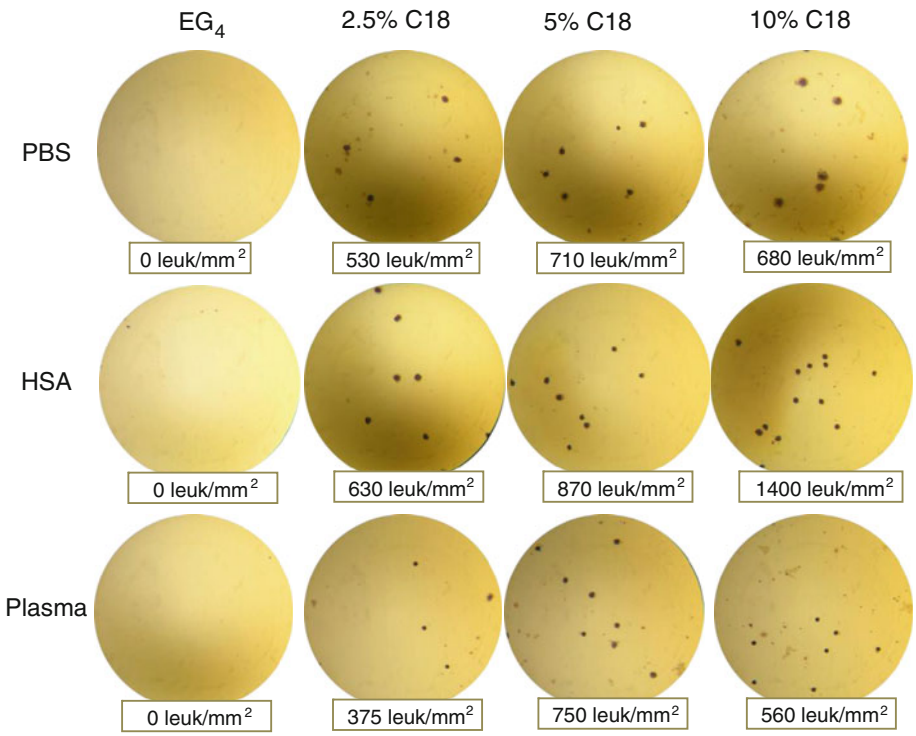

Fig. 7 Representative images of the median of leukocyte adhesion to EG_4-C18 SAMs after pre-immersion in buffer (PBS), albumin (HSA) or plasma. Images obtained by optical reflection microscopy (magnification: ×500). The median number of adherent leukocytes per mm^2 is stated on the *bottom* of each image. Leukocyte counts from all surfaces were statistically different ($P < 0.05$) from EG_4 SAMs within each pre-immersion ($P < 0.05$); Chi-square Test. Comparing the pre-immersions for the same surface, no statistical differences were observed, except for 10%C18 SAMs where HSA pre-immersion counts were significantly higher; Chi-square Test

leukocytes compared to the background surface, the surface coverage is still relatively low. Differing from results with platelets, leukocyte adhesion was not reduced when SAMs were pre-immersed in albumin, comparing to buffer. Pre-adsorbed albumin even potentiated slightly leukocyte adhesion to 10%C18 SAMs. Regarding pre-immersion in plasma, no differences were observed in leukocyte adhesion comparing to buffer pre-immersion.

4 Discussion

This work used oligoethylene glycol-terminated SAMs as background non-fouling surface to study the effect of an 18 carbon (C18) ligand on albumin selective and reversible adsorption and subsequent platelet and leukocyte adhesion.

On the top of the ethylene glycol terminated SAMs (EG_4 SAMs), different percentages of 18 carbon ligands (C18) were immobilized. These ligands were chosen so that they would simulate fatty acids with 18 carbons since albumin is the main transport vehicle for long chain fatty acids in blood. The ligand density on the surface should be appropriate so albumin can recognize the alkyl chains, bind to them by the hydrophobic pockets specific for fatty acids and cover the surface; however, the concentration cannot be too high or the proteins will just see a homogeneous layer of methyl groups on the surface.

The immobilization of C18 ligands in SAMs was studied by contact angle measurements, XPS, FT-IRAS and ellipsometry. Contact angle measurements indicate that there is an increase of hydrophobicity with the increase of the immobilization density of C18 on EG_4 SAMs reaching 95° in 10%C18 SAMs, which was expected since the C18 ligands are CH_3-terminated. Other authors [27, 28] have obtained higher values of water contact angles ($\theta_w = 110°$) for pure, fully covered, CH_3 SAMs, suggesting that 10%C18 SAMs have a high density C18 of ligands but are not completely covering the surface and are poorly organized. Nitrogen is a good marker of the presence of C18, since this element is not present in EG_4 SAMs. The observed increase of atomic percentage of N(1*s*) by XPS was therefore associated with the increase of C18 ligands immobilized. These results were also supported by IRAS, where the peak from the CH_2 (ν_a) vibration at $\approx 2920\ cm^{-1}$ increased as the percentage of octadecyl isocyanate in solution increased. Regarding ellipsometry, assuming as did other authors [29, 30] that $d(CH_2) = 1.1$ Å and $d(OCH_2CH_2) = 2.7$ Å, the theoretical thickness of a EG_4 SAMs is approximately 25.3 Å. Therefore, the thickness of 25 Å obtained for EG_4 SAMs is in accordance with the theoretical values and similar to the obtained by Palegrosdemange et al. and Zhu et al. [29, 31]. If the EG_4 SAMs were completely covered with C18 ligands, the expected thickness would be approximately 48 Å,

suggesting that 10%C18 SAMs, with a measured thickness of 40.7 Å, are not fully covered.

This work intended to create a surface that would adsorb albumin in a selective and reversible way, providing a dynamic, renewable and natural coat of this protein on the surface so as to improve its hemocompatibility. The rationale behind this approach is that albumin forms a platelet compatible surface, but is probably degraded with time in vivo. If the albumin could be renewed with fresh material, the surface might maintain its platelet-compatible properties.

Protein adsorption results presented in this paper are in accordance with a previous work [20], where there is an increase of albumin adsorption with increase of surface hydrophobicity due to the increase of C18 ligands.

Competition studies between albumin and fibrinogen showed that EG_4 SAMs and 2.5%C18 SAMs adsorb albumin preferentially when both proteins are in solution, whereas 5%C18 and 10%C18 SAMs adsorb some fibrinogen. These results are in accordance with observations that hydrophobic surfaces adsorb more fibrinogen than hydrophilic surfaces [25].

Concerning exchangeability of adsorbed ^{125}I-HSA, results showed that 2.5%C18 SAMs replace 80% of the adsorbed HSA by HSA in solution, but not by HFG (only 25% of exchangeability). EG_4 SAMs worked well as non-fouling surface, inhibiting non-specific interactions. These surfaces have an exchangeability of ca. 75% with other albumin but, in contrast to 2.5%C18 SAMs, EG_4 SAMs exchange the pre-adsorbed HSA with either HSA or HFG in solution, showing high reversibility, but a lack of selectivity.

Platelet and leukocyte adhesion were evaluated on EG_4-C18 SAMs with and without prior immersion in buffer, HSA and plasma to better understand the effect of the adsorbed layer of proteins in the behavior of platelets and leukocytes.

Following the trend observed in the protein adsorption studies, there is an increase of platelet adhesion as the percentage of C18 ligands increases (in buffer pre-immersion). However, when a pre-immersion of HSA is performed, there is an 80% decrease in the number of platelets, stressing the passivant effect of albumin for blood contact surfaces. When SAMs are pre-immersed in plasma, and the whole cocktail of proteins is competing for the surface, EG_4 SAMs have approximately the same adhesion of platelets as in buffer, but SAMs with 5%C18 and 10%C18 demonstrated higher adhesion and activation than in buffer. These results can be justified by the competitive studies, where EG_4 and 2.5%C18 SAMs maintain their preference for HSA when both HSA and HFG were present in solution, but 5%C18 and 10%C18 SAMs show some affinity to HFG, and therefore are likely to trigger the adhesion and activation of platelets through GPIIb/IIIa receptors. The only surface that shows a reduction in the number of adherent platelets after plasma pre-immersion is 2.5%C18 SAMs, suggesting that the selective adsorption of albumin from plasma is taking place in the manner hypothesized. However, this reduction is smaller than after pre-immersion in a pure albumin solution, implying that other plasma proteins are also adsorbing to 2.5%C18 SAMs, though probably at low levels. Experiments from Jenney and Anderson have shown that PEO-coupled glass and octadecyl modified glass adsorb some vWF ($\sim$0.5 and $\sim$1.5 ng/cm^2, respectively), despite in much smaller amounts than HSA ($\sim$40 and $\sim$100 ng/cm^2, respectively) [7]. Therefore, a potential competitive protein (other than fibrinogen) that may be acting as the bridge between 2.5%C18 SAMs and platelets is vWF, since it is known to interact with GPIb/IIa receptors from platelets.

Regarding leukocyte adhesion, results have demonstrated that the background EG_4 SAMs induce minimum or no adhesion of leukocytes, regardless of the pre-immersion. Decreased adhesion on the ethylene glycol surfaces (EG_4 SAMs) was expected based on previous work by Tegoulia et al. [32] in which SAMs with EG_3 headgroups exhibited very low PMN leukocytes attachment. It has been suggested that the ethylene oxide units present on the surface of the EG_4 SAMs provide a template for water nucleation and a stable interfacial water layer that prevents direct contact between the surface, proteins and the cells [33]. These surfaces are hypothesized to reduce protein adsorption and therefore lack the specific ligands for the cell-surface receptors that promote cell adhesion and survival. It has been reported that hydrophilic surfaces limit macrophage adhesion and fusion and inhibit cytokine secretion by adherent cells, possibly resulting in a reduced inflammatory reaction [34].

Results with EG_4-C18 SAMs showed that the presence of C18 ligands increased the adhesion of leukocytes. Importantly, C18 ligands are methyl terminated and wettability studies confirmed that EG_4-C18 SAMs get more hydrophobic as the amount of C18 ligands on the surface increase. Previous experimental observations by several authors are in accordance with our results since they revealed that hydrophobic surfaces adsorb higher amounts of PMN leukocytes [32, 35, 36].

In contrast however, a higher number of adherent leukocytes on hydrophilic OH-terminated SAMs and a lower number on hydrophobic CH_3-terminated SAMs have been reported in in vitro studies by Sperling et al. [37]. A similar observation was made in vitro and in vivo by Barbosa et al. [5, 38] and Lindblad et al. [2]. Sperling et al. [37] believe that the complement activating potential of the OH-group surfaces is the most important influence since complement activation has a strong chemotactic effect on leukocytes.

Another factor that may influence leukocyte adhesion to solid surfaces is surface free energy [39]. Neumann and co-workers showed enhanced leukocyte adhesion to surfaces with higher surface free energy or increased wettability [40]. These findings are also not in accordance with our results since EG_4 SAMs, that are hydrophilic and have higher surface free energy (64.8 mN/m), showed lower leukocyte adhesion than 10%C18 SAMs, that are hydrophobic and have lower surface free energy (28.3 mN/m).

However, as mentioned before, leukocyte adhesion to a biomaterial surface does not depend only on the material surface properties. The amount of protein pre-adsorbed on a surface can also affect cell adhesion by generating new surface properties [32]. Since protein adsorption from plasma-containing medium occurs rapidly, rendering direct recognition of pre-adsorption surface functional groups by the leukocytes virtually impossible, functional groups are believed to affect cell adhesion indirectly via their effect on the adsorbed protein layer. Therefore, differences observed in cell adhesion on different surfaces are most likely due to differences in the adsorption of proteins by the surface [41].

The formation of the protein layer on the surface is a complex process in which proteins competitively adsorb to the material based on protein concentration in the media and protein binding affinity [7, 42]. During this process, the time and plasma concentration dependent Vroman effect is observed [43]. The first proteins to reach the surface are those present in plasma at higher concentration and with the lower molecular weight, which are subsequently replaced by other proteins with higher affinity to the surface [44]. Once the protein layer is formed, the adsorbed proteins can expose ligands for leukocyte adhesion receptors, and leukocytes then interact with the adsorbed protein layer adherent to the materials surface. It has been shown that the receptors of monocytes are capable of binding extracellular matrix and blood proteins such as complement factor C3, immunoglobulin G, fibrinogen, fibronectin and vitronectin adsorbed onto natural or synthetic surfaces [7, 45].

Elucidating the role of adsorbed and denatured protein in leukocyte adhesion and activation based upon published literature was complicated by the great variation in substrate materials, type of proteins, protein concentrations and culture medium supplements used in the experiments, because protein adsorption and denaturation processes depend on these parameters.

Jenney and Anderson [7] have reported that in vitro macrophage culture on protein preadsorbed surfaces confirmed the inhibitory effect of vWF and the promoting effect of IgG on long-term macrophage adhesion. This effect of IgG in leukocyte adhesion has also been observed in studies by Collier et al. [6] where the depletion of IgG from serum caused a significant decrease in initial adherent cell density. McNally and Anderson have demonstrated that C3 is potentially a major mediator of monocyte adhesion to a variety of chemically different surfaces. Adhesion was prevented and reduced when C3-depleted serum was used and completely restored when C3-depleted serum was replenished with purified C3 [46]. Wattero et al. [36] believe that an enhanced adsorption of IgG to hydrophobic surfaces and synergistic effects between adsorbed IgG and the complement system (involving C1q, C3 and C5a) are responsible for increased adhesion of leukocytes on hydrophobic compared to hydrophilic surfaces. Fibrinogen has been shown to mediate a pro-inflammatory effect at implant surfaces, mainly by causing an increased recruitment and adhesion of leukocytes in inflammation and tissue repair at implant surfaces [26, 45]. Adhesion of leukocytes to adsorbed fibrinogen is mediated by the β_2-integrin Mac-1 [26, 47] and intercellular adhesion molecule-1 (ICAM-1) [48], whereas fibrinogen-induced activation of these cells is mediated primarily by toll-like receptor-4 [49]. In agreement with this, it has been previously reported that proteins recognized only by β_2-integrins can serve as adhesion substrates, but do not result in activation of the adherent leukocytes [50].

According to Jones et al. [51] the adhesion of PMN leukocytes to immobilized fibrinogen is mediated by β_2-integrins and their binding to the Arg-Gly-Asp (RGD)-containing peptide sequence present in the fibrinogen molecule. However, Tang et al. [26], reported that the proinflammatory activity of fibrinogen resides within the D fragment, that contains neither the fibrin cross-linking sites nor RGD sequences. The major (and perhaps, exclusive) proinflammatory sequence appears to be fibrinogen γ190-202. Altieri et al. [47] have previously shown that this sequence mediates the binding of leukocytes via the Mac-1 (CD11b/CD18) β_2-integrin.

Remembering competition studies between albumin and fibrinogen, where EG_4 and 2.5%C18 SAMs adsorb mainly albumin, and 5%C18 and 10%C18 adsorb some fibrinogen, and observations that hydrophobic surfaces adsorb more fibrinogen than hydrophilic surfaces [25], the initial inflammatory activation by fibrinogen bound to the surface was expected to be low on hydroxylated and hydrophilic surfaces and high on hydrophobic ones. Given this, after pre-immersion in plasma (which has fibrinogen), the lower leukocyte adhesion in EG_4 and 2.5%C18 SAMs, and higher in 5%C18 and 10%C18 SAMs were predictable.

The extent to which a protein unfolds during and after the adsorption process depends on the surface properties of the protein and the substrate. Albumin and other proteins soluble in aqueous solutions fold spontaneously so that hydrophobic amino-acid side chains are internalized, and polar residues are exposed at the surface. For adsorption to

hydrophilic substrates, water-soluble proteins can interact via their polar (surface) residues. Adsorption of proteins to hydrophobic surfaces, however, results in protein unfolding and binding via their non-polar (interior) residues [50]. Results obtained with EG_4-C18 SAMs are consistent with this, and revealed that around 80% of the albumin adsorbed to EG_4 SAMs can be exchanged by other albumin molecules (i.e., adsorption in a reversible way) whereas in hydrophobic 10%C18 SAMs only 20% of the adsorbed albumin is exchangeable. The 80% that is irreversibly bound is probably denatured on the surface.

Even though albumin is not known to contain specific ligands for leukocyte adhesion [7, 50], Brevig et al. [50] stated that several studies have shown that neutrophils can migrate in vitro on adsorbed albumin, suggesting that denatured protein might be involved in leukocyte invasion into inflamed tissues. Also, the latter group has performed studies showing that pre-denatured albumin was consistently more potent as a cell-adhesion substrate when adsorbed to a hydrophobic surface in contrast to a hydrophilic surface. The high degree of albumin denaturation on 10%C18 SAMs is probably responsible for the higher leukocyte adhesion to these surfaces comparing to all others. Another important finding by Brevig et al. [50] that can be of importance to our work is that although macrophages may recognize and adhere to albumin adsorbed to hydrophobic as well as hydrophilic implant surfaces, such interactions fail to induce macrophage secretion of proinflamatory TNF-α (by contrast, macrophage adhesion to fibrinogen induces secretion of TNF-α, MIP-1α and MIP-1β). Therefore, 2.5%C18 SAMs, that preferentially adsorb albumin and reduce platelet adhesion and activation but presented some leukocyte adhesion, should still be considered as potential surfaces to improve the hemocompatibility of implants.

5 Conclusions

EG_4 SAMs with different percentages of C18 on the surface were obtained and an increase of albumin adsorption was observed as the surface became more hydrophobic due to the increased concentration of immobilized C18 ligands.

EG_4 SAMs proved to be a good background non-fouling surface for this study since low amounts of proteins adsorbed and very few platelets and no leukocytes adhered to these surfaces.

The importance of the amount of C18 ligands was stressed in the reversibility studies since 2.5%C18 SAMs are the only surfaces to exchange almost all the pre-adsorbed HSA by HSA in solution, but not by HFG. This investigation has therefore shown that, not only the ligand (C18 ligand), but also its concentration on the surface, greatly influences albumin adsorption. A surface with specific percentage of albumin-binding ligands (2.5%C18) on EG4 SAMs can recruit albumin selectively from plasma and therefore minimize the adhesion of platelets, despite promoting adhesion of small amounts of leukocytes.

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