INÊS DE CASTRO GONÇALVES DE ALMADA LOBO

PhD Thesis

MOLECULARLY ENGINEERED MATERIALS FOR SELECTIVE ALBUMIN BINDING TO REDUCE THE RISK OF THROMBUS FORMATION

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This is not the end.

It's not even the beginning of the end.

But it is, perhaps, the end of the beginning.

Sir Winston Churchill

To my Bernardos…

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ABSTRACT

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

When a biomaterial contacts blood, the first event to occur is an almost immediate adsorption of proteins onto its surface. The composition, structure and conformation of the adsorbed protein layer determine all subsequent biological responses. Albumin-coated surfaces have been found to reduce the adhesion of thrombogenic proteins, like fibrinogen, and subsequent platelet and cell adhesion to the underlying material, thereby reducing surface-induced thrombosis. However, these surfaces have associated problems such as denaturation of albumin over time or exchange of the albumin with other proteins in solution. Therefore, research has been conducted to create surfaces that attract and bind albumin from the bloodstream 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. Since albumin is a carrier of long chain (16 to 18 carbons) free fatty acids in the circulatory system, in this work we have adopted the strategy of immobilizing 18 carbon alkyl chains (C18 ligands) on different surfaces, expecting albumin to be capable of recognizing and binding them selectively from plasma.

For a better understanding of the material-protein-cell interactions, hydrophilic self-assembled monolayers (SAMs) were first used as model surfaces. These surfaces were chemically modified with different amounts of C18 ligands, and their effect on protein adsorption and subsequent platelet and leukocyte adhesion was analyzed. Competition and exchangeability studies with albumin and other plasma proteins revealed that SAMs with a specific amount of immobilized C18 ligands (2.5%C18 SAMs) induced albumin adsorption in a selective and reversible way and reduced platelet adhesion and activation. Given this, knowledge obtained using SAMs was subsequently transposed to a biomedical polymer in order to test the applicability of the concept developed with SAMs. Different percentages of C18 ligands were immobilized on poly(2-hydroxyethyl methacrylate) – pHEMA discs, and protein adsorption studies and clotting time assays were performed. Also with pHEMA, samples

prepared from a solution with 2.5%C18 adsorbed more albumin selectively and reversibly. Furthermore, these samples did not accelerate coagulation of recalcified plasma compared to the negative control (polystyrene).

The work presented in this thesis demonstrates that not only the presence, but also the amount of C18 ligands on the surface greatly influence albumin adsorption. A specific percentage of C18 ligands (2.5%C18 in solution) induces the best performance in binding albumin in a selective and reversible way and may be beneficial for blood contact applications.

RESUMO

A formação de trombos e a inflamação são dos principais problemas associados à utilização de dispositivos médicos para contacto com o sangue. Estes dois processos estão relacionados e envolvem a interacção entre a superfície do dispositivo, proteínas, plaquetas e leucócitos.

Quando um biomaterial contacta o sangue, ocorre em primeiro lugar a adsorção quase imediata de proteínas do plasma à sua superfície. A composição, estrutura e conformação das proteínas que aderem determinam todas as respostas biológicas seguintes. Estudos anteriores demonstraram que superfícies revestidas com albumina reduzem a adesão de proteínas trombogénicas, como o fibrinogénio, e a subsequente adesão de plaquetas e células aos materiais, reduzindo por isso a formação de trombos induzidos pela superfície. No entanto, estas superfícies trazem problemas associados, tais como a desnaturação da albumina ao longo do tempo ou a troca da albumina por outras proteínas do plasma. Assim sendo, realizaram-se estudos de forma a criar superfícies que atraem e ligam a albumina da corrente sanguínea de forma selectiva e reversível, originando um revestimento dinâmico, renovável e natural desta proteína na superfície, de modo a melhorar a sua hemocompatibilidade. Uma vez que a albumina é uma proteína transportadora de ácidos gordos no sistema circulatório, cadeias com 18 carbonos (ligandos C18) foram imobilizadas em diferentes superfícies, esperando que a albumina reconheça os ligandos e se ligue de forma selectiva na presença de outras proteínas do plasma.

Para uma melhor compreensão das interacções material-proteína-célula, monocamadas auto-estruturadas (*self-assembled monolayers*, SAMs) hidrofílicas foram inicialmente usadas como superfícies modelo. Estas superfícies foram quimicamente modificadas com diferentes quantidades de ligandos C18, tendo-se estudado o seu efeito na adsorção de proteínas e subsequente adesão de plaquetas e leucócitos. Estudos de competição e permuta entre albumina e outras proteínas do plasma revelaram que SAMs com uma quantidade específica de ligandos C18 imobilizados (2.5%C18 SAMs) induzem a adsorção de albumina de uma forma selectiva e reversível e reduzem a adesão e activação de plaquetas. Posteriormente, os conhecimentos adquiridos aquando da utilização das SAMs foram transferidos para um polímero biomédico de forma a testar a aplicabilidade do conceito desenvolvido com as superfícies modelo. Foram imobilizadas diferentes percentagens de ligandos

C18 em discos de poli(2-hydroxietilmetacrilato) – pHEMA, e realizados ensaios de adsorção de proteínas e tempo de coagulação. Também com pHEMA, amostras preparadas em soluções com 2.5%C18 adsorvem mais albumina de forma selectiva e reversível. Para além disto, estas amostras não aceleram a coagulação de plasma recalcificado, comparando com o controlo negativo (poliestireno).

O trabalho apresentado nesta tese demonstra que não só a presença, mas também a quantidade de ligandos C18 na superfície têm uma grande influência na adsorção de albumina. Concluiu-se que uma percentagem específica de ligandos C18 (2.5%C18 em solução) induz um melhor desempenho na ligação de albumina de forma selectiva e reversível e pode ser benéfica no desenvolvimento de biomateriais para contacto com o sangue.

RÉSUMÉ

La formation de caillots sanguins et l'inflammation sont les principaux problèmes associés à l'utilisation des dispositifs médicaux destinés à être en contact avec le sang. Ces deux processus sont étroitement liés et impliquent des interactions entre la surface du dispositif, les protéines, les plaquettes et les leucocytes.

Le premier évènement ayant lieu lorsqu'un biomatériau entre en contact avec le sang est l'adsorption immédiate des protéines à sa surface. La composition, la structure et la conformation de la couche des protéines adsorbées conditionnent toutes les réponses biologiques qui ensuivent.

Par ailleurs, il a été démontré que le revêtement de surface avec de l'albumine entraîne une diminution de l'adsorption des protéines thrombogéniques telles que le fibrinogène, réduisant ainsi l'adhésion des plaquettes et des cellules au matériau. En conséquence, la coagulation induite par ses surfaces se trouve minimisée. Néanmoins, la dénaturation de l'albumine au cours du temps ou son échange par d'autres protéines en solution est source de problèmes.

Face à cet inconvénient, l'objectif de ce travail a été la mise au point d'une surface capable de capter de manière sélective et réversible l'albumine circulante dans le sang, formant ainsi un revêtement protéique dynamique, renouvelable et naturel favorisant son hémocompatibilité. Il est connu que l'albumine est capable de transporter les acides gras à longue chaîne (16 à 18 carbones) dans le système circulatoire. Ainsi, notre stratégie a consisté à greffer de manière covalente des chaînes alkyles comprenant 18 carbones (C18 ligands) sur différentes surfaces afin de promouvoir l'adsorption de l'albumine de façon spécifique en présence d'autres protéines plasmatiques.

Afin de mieux comprendre les interactions matériau-protéine-cellule, des monocouches auto-agrégantes (SAMs) hydrophiles ont été utilisées dans un premier temps comme surfaces modèles. Elles ont été chimiquement modifiées par différents pourcentages du ligand C18. L'effet de ces modifications sur l'adsorption des protéines et sa portée sur l'adhésion des plaquettes et des leucocytes a été réalisé. Les études de compétition et de permutation entre l'albumine et d'autres protéines plasmatiques ont montré que les SAMs ayant une quantité définie de ligand C18 immobilisé (2.5%C18 SAMs) induisent une adsorption d'albumine de façon ciblée et réversible et

réduisent l'adhésion et l'activation des plaquettes. Dans un second temps, l'ensemble de ces résultats a été par la suite exploré dans le contexte d'un polymère biomédical afin de valider le concept élaboré avec les SAMs. En effet, différents pourcentages de ligand C18 ont été immobilisés sur le poli(2-hydroxietilmetacrilato) – pHEMA et des essais d'adsorption de protéines et de temps de coagulation ont été effectués. Ici aussi, les échantillons de pHEMA préparés à partir de solutions de 2.5%C18 adsorbent plus d'albumine de manière sélective et réversible. De plus, ces échantillons ne modifient pas la vitesse de coagulation du plasma recalcifié, en comparaison avec le contrôle (le polystyrène).

Ce travail de thèse démontre que la présence du ligand C18 sur une surface ainsi que sa concentration conditionnent l'adsorption de l'albumine. Un pourcentage déterminé, 2.5%C18, aboutit à une meilleure sélectivité et réversibilité de la liaison d'albumine offrant ainsi des nouvelles possibilités pour l'optimisation des biomatériaux destinés à être en contact avec le sang.

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Chapter VII – General discussion, concluding remarks and future perspecives

CHAPTER I

INTRODUCTION

1. MOTIVATION

Hemocompatibility is one aspect of biocompatibility related to the specific interactions between biomaterials and circulating blood.[1] A biomaterial is defined as a material intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body.[1] Some examples of cardiovascular applications include catheters for blood access and blood vessel manipulation, heart assist devices, stents and permanently implanted devices to replace diseased heart valves and arteries.[2]

Thrombosis and embolism remain major challenges for medical devices that are used in contact with blood. Therefore, anticoagulant therapy is usually given to patients with cardiovascular implants, despite the high costs and considerable risks to the patients (e.g. bleeding episodes).[3,4]

Thus, improved compatibility with blood is a desired feature for a variety of medical devices that contact blood during clinical use. The materials used for the manufacture of medical devices generally are not inherently compatible with blood and its components. Most medical device materials (e.g. Dacron® polyester, Nylon polyamide, Gore-Tex® polytetrafluoroethylene) were initially selected primarily based upon their use and ready availability for other applications, and continue to be selected because they have already gone through the regulatory approval process for a variety of products. While it is sometimes possible to improve device hemocompatibility by altering bulk materials used in fabrication, it is often easier and more effective to apply a thin film coating to the surface of an existing device.

There are three main strategies which are typically used to reduce device thrombosis: (1) reduction of non-specific protein adsorption; (2) enhancement of specific adsorption of non-thrombogenic blood proteins; and (3) promotion of active thrombin consumption. The approach proposed in this thesis regards mainly to strategy (2) since we intend to immobilize a ligand on a surface to specifically adsorb a protein (albumin), even though strategy (1) is also used since the background surface that will be used is supposed to avoid protein adsorption.

When a biomaterial contacts blood, the first event to occur is an almost immediate adsorption of proteins onto its surface. The performance of the biomaterial is influenced by this film of adsorbed proteins once it can elicit adverse host responses, such as activation of plasma enzyme cascades

(coagulation, fibrinolytic, kinin and complement systems) and the adhesion and activation of platelets and leukocytes.[5,6] For this reason, the control of the amount and composition of the adsorbed protein is a good strategy to improve blood compatibility.

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).[7] 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.

Another strategy that has been mentioned to increase hemocompatibility of a surface consists of saturating it with a protein layer, usually albumin, which resists adsorption of other blood proteins. Basically, albumin-treated surfaces are resistant to platelet adhesion,[8] thrombus formation[9] and bacterial adhesion,[10] while other proteins, especially fibrinogen, promote these actions.[11] 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[12,13], Cibacron Blue F3G-A[14-16] and warfarin[17]) or antibodies[18] 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.

2. OBJECTIVES

The aim of this work was to create a surface that binds albumin in a selective and reversible way in order to reduce the risk of thrombus formation.

The strategy that was followed was the immobilization of a ligand (resembling a long-chain fatty acid) on a surface that could act as a cluster to bind the hydrophobic pockets of albumin. The surface is supposed to adsorb albumin selectively from blood plasma, preventing other proteins, like fibrinogen, from adsorbing and therefore preventing subsequent adhesion and activation of platelets and white blood cells.

For a better understanding of the interaction between material and blood proteins and cells, self-assembled monolayers (SAMs) were first used as model surfaces. SAMs with non-fouling end-groups were created in order to prevent non-specific adsorption of blood proteins. These monolayers were chemically modified by immobilization of different percentages of albumin receptor: a methyl-terminated alkyl chain with 18 carbons (C18 ligand). The amount of C18 ligands on the surface is a critical parameter. If the surface is completely covered by C18 ligands, an homogeneous carpet of methyl groups will be formed, resulting in non-specific protein adsorption and subsequent protein denaturation on the surface. If an intermediate concentration on C18 ligands exists on the surface, albumin should recognize it as a “fatty acid like” structure and bind to it by its hydrophobic pockets specifically and reversibly, without denaturing on the surface.

In order to verify which concentration of immobilized C18 ligands allowed an optimized adsorption of albumin in a selective and reversible way, studies of competitive protein adsorption (e.g. albumin vs fibrinogen, and albumin vs plasma) and reversible adsorption (exchange of pre-adsorbed albumin for other albumin, fibrinogen or plasma proteins) were conducted.

After studying protein adsorption to these SAMs, the subsequent aim was to investigate the influence of pre-adsorbed proteins in platelet adhesion and activation and leukocyte adhesion, so as to confirm the efficiency of the modified surfaces.

The final goal of the work was to transpose the knowledge obtained using the above model surfaces to the modification of a polymer. A currently used synthetic polymer, poly(2-hydroxyethyl

methacrylate) (pHEMA) was thus prepared, and the same C18 ligands were introduced on its surface using the same chemistry employed for preparing the SAMs.

3. THESIS SYNOPSIS

The work is divided into seven chapters. The reminder of the thesis is as follows:

Chapter II provides a general introduction to blood contact biomaterials. It is the aim of the chapter to present the reader a brief overview of some issues related to biomaterials for blood contact.

Chapter III describes the preparation and characterization of hydroxyl-terminated self-assembled monolayers (OH SAMs) with different percentages of C18 ligands immobilized. Protein adsorption studies to these model surfaces reveal that albumin adsorption increases with the increase of C18 ligands and that SAMs obtained from solutions with 2.5% of C18 present considerable albumin adsorption in a selective and reversible way, although 50% of albumin remains irreversibly adsorbed.

Chapter IV reports the preparation and characterization of tetra (ethylene glycol)-terminated SAMs (EG_4 SAMs) with different percentages of C18 ligands immobilized. The aim of using EG_4 SAMs as background surface (instead of OH SAMs referred in Chapter III) is to decrease non-specific adsorption and increase reversibility of bound albumin. EG_4 SAMs present very low albumin adsorption that increases with the percentage of C18 ligands immobilized. Protein competition studies reveal that all the surfaces have more affinity to albumin than to fibrinogen. Protein reversibility experiments show that SAMs with 2.5%C18 exchange almost all the pre-adsorbed albumin by other albumin in solution (only 15% remains irreversibly bound), but not by fibrinogen. Finally, platelet studies after pre-immersion in protein solutions indicate that 2.5%C18 SAMs recruit albumin selectively from plasma and minimize the adhesion and activation of platelets.

Chapter V addresses leukocyte adhesion to EG_4-C18 SAMs after pre-immersion in buffer, albumin, fibrinogen or plasma solutions. Leukocyte adhesion to EG_4 SAMs is minimum or negligible, and increases with the amount of C18 ligands until 5%C18, being maintained for 10%C18 SAMs. Pre-adsorption of albumin to EG_4-C18 SAMs does not have a passivating effect on leukocyte adhesion, and even potentiates the adhesion of leukocytes to 10%C18 SAMs. No differences are observed between buffer, fibrinogen and plasma pre-immersions, indicating that the main parameter influencing leukocyte adhesion to EG_4-C18 SAMs is the amount of C18 ligands on the surface.

In Chapter VI, the knowledge obtained using SAMs as model surfaces is transposed to a synthetic polymer. Poly(2-hydroxyethyl methacrylate) (pHEMA) is used as background polymer and different percentages of C18 ligands are immobilized using the same isocyanate chemistry described for SAMs. Protein adsorption studies reveal that even though 2.5%C18 samples present lower albumin adsorption than the pHEMA background surface, samples with this specific amount of ligands appear to be the best for inducing albumin adsorption in a selective and reversible way. PHEMA with 2.5%C18 adsorbs albumin preferentially in the presence of fibrinogen and other plasma proteins and exchanges 58% of the bound albumin when in contact with pure albumin or plasma. Furthermore, samples with this specific percentage of C18 ligands are not prone to activate coagulation, since recalcified plasma clotting times are similar to the negative control used.

Finally, Chapter VII provides the concluding remarks, correlating results from Chapters III, IV, V and VI and suggesting directions for future research.

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CHAPTER II

BIOMATERIALS FOR BLOOD CONTACT: AN OVERVIEW

1. HEMOCOMPATIBILITY

Thrombosis and embolism remain major challenges for medical devices that are used in contact with blood.

Blood compatibility (hemocompatibility) can be defined as the property of a material or device that allows it to function in contact with blood without inducing adverse reactions.[1] The response of blood to an artificial biomaterial surface can be considered as a series of complex events starting with rapid blood protein adsorption, followed by the activation of the intrinsic coagulation cascade, platelet adhesion and activation, activation of the fibrinolytic and complement systems, and the adhesion and activation of white blood cells.[2]

In recent years, various biomaterials that are natural or synthetic polymeric materials have been widely used for manufacturing biomedical applications including artificial organs, medical devices and disposable clinical apparatus, such as vascular prostheses, blood pumps, artificial kidney, heart valves, catheters, pacemaker lead wire insulation, intra-aortic balloon, artificial hearts and dialyzers, which could be used in contact with blood. However, the polymers presently used, such as cellulose, chitosan, poly (tetrafluoroethylene) (PTFE), poly (vinyl chloride) (PVC), segmented polyetherurethane (SPU), polyethylene (PE), silicone rubber (SR), nylon, and polysulfone (PSf), when in direct contact with blood, are still prone to initiate the formation of clots, as platelets and other components of the blood coagulation system are activated. It is well known that 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.[3]

Synthetic vascular grafts, for example, have been successfully used in the treatment of the pathology of large arteries (internal diameter >6 mm). However, the replacement of smaller sized arteries has often been unsuccessful since synthetic vascular grafts with diameter less than 6 mm are known to be highly thrombogenic and need special treatments to improve their patency after implantation.[4]

All implantable biomaterials, especially blood contacting medical devices which are used for long periods of time, such as the artificial heart valves and vessel prostheses, and also those used outside the organism for short periods of time, such as in blood purification devices and blood catheters, must completely prevent the activation of the coagulation system and clot formation. Because

adsorbed proteins may trigger the coagulation sequence, the material's biocompatibility is generally considered to have close relation with the rapid adsorption of plasma proteins that occurs when it is brought into contact with blood.

1.1 The role of platelets and adhesion proteins

Platelets are the smallest human blood cellular constituents with a size of ca. 3-4 μm diameter in a disc shape at the resting state. Platelets are megakaryocyte fragments without cell nuclei produced in the bone marrow and circulating at an average concentration of $2.5x10^5$ platelets/μl. The external surface coat of the platelet contains membrane-bound receptors (e.g., glycoproteins (GP) Ib (CD42) and IIb/IIIa (CD41/CD61)) that mediate the contact reactions of adhesion (platelet-surface) and aggregation (platelet-platelet).[1]

There are many kinds of proteins in human blood with concentrations ranging from a few μg/ml to mg/ml. Among them, some proteins are known to be involved in hemostasis, including a variety of clotting factors and some adhesive proteins, such as fibrinogen (HFG), von Willebrand's factor (vWF), fibronectin (Fn) and vitronectin (Vn). All these plasma adhesion proteins contain the cell-binding sequence RGD (Arg-Gly-Asp) and can bind platelets via specific cell membrane receptors. However, fibrinogen and vWF are considered to be the two most important adhesion proteins in mediating platelet adhesion and activation on biomaterials surfaces.[5]

The concentration of fibrinogen in normal human plasma is ca. 3 mg/ml. This large dimeric molecule with a molecular weight of 340 kD has an isoelectric point of 5.5 and its dimensions are 6 x 9 x 45 nm^3.[6] Fibrinogen is composed of three types of disulphide-linked polypeptide chain subunits: Aα, Bβ and γ (with two copies each).[7] Three distinct sites have been suggested to be involved in platelet binding to fibrinogen: two RGD sequences in the Aα chain (RGDF in Aα95-98 and RGDS in Aα572-575)[8] and a dodecapeptide at the carboxyl terminus of the γ chain (HHLGGAKQAGDV in γ400-411) (Figure 1).[9]

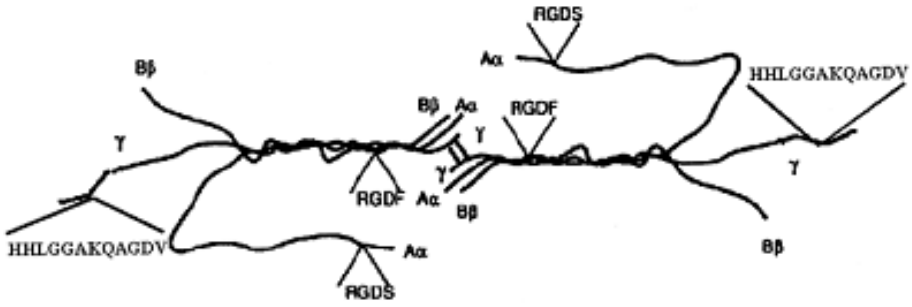

Figure 1. Schematic representation of fibrinogen molecule with the three pairs of assumed platelet binding sites: the RGDS and RGDF of the Aα chain and the 12-amino acid peptide of the γ-chain. Adapted from Tsai et al.[10]

The RGD sequences and the dodecapeptide bind to the platelet integrin GPIIb/IIIa. Although the RGDs are common to other proteins, the dodecapeptide is unique to fibrinogen. This γ chain C-terminal dodecapeptide was shown to be the most important site in mediating platelet adhesion and aggregation.[10] Recent studies suggested that there is a synergistic role of the dodecapeptide sequence in the activation process of the integrin GPIIb/IIIa necessary for fibrinogen RGD recognition.[11] The interaction between γ400-411 and GPIIb integrin, will induce conformal changes within the GPIIIa integrin subunit, allowing its affinity interaction with the RGD site of fibrinogen.[10]

vWF is present in plasma as a multimer with MW ranging from 500-20000 kD. Its concentration in normal human blood is ca. 10-50 μg/ml. vWF binds to the GPIb-IX complex or GPIb as well as GPIIb/IIIa on the platelet membrane. The RGD sequence at the carboxyl terminus is thought to be responsible for vWF binding to platelet receptors GPIIb/IIIa, whereas residues 474-488 and 694-708 are involved in the vWF binding to GPIb.[12,13]

Platelets in the resting state will not bind to soluble fibrinogen molecules in the circulating blood (unless exposed to an agonist such as ADP or thrombin). However, they will recognize and bind to surface adsorbed fibrinogen. This is related with the availability of the dodecapeptide sequence of fibrinogen after adsorption, which is dependent of the conformation/orientation of adsorbed fibrinogen. Activated platelets expressing activated GPIIb/IIIa receptors can bind to soluble fibrinogen molecules and also use them as bridges to form platelet aggregates. Similar functions can be performed by vWF interacting with platelets through the GPIIb/IIIa as well as GPIb.[7] Tsai et al. reported that a low level of surface-bound fibrinogen (c.a. 0.34 mg/m^2) is sufficient to mediate

platelet adhesion. However, platelet adhesion and activation by a surface depends more on the conformation and/or orientation than the amount of adsorbed protein.[14]

Platelets are extremely sensitive and may respond to minimal stimulation. When platelets are activated, the GPIIb/IIIa molecules undergo a conformational change leading to a higher binding affinity to fibrinogen. Two mechanisms, "inside out" and "outside in", have been suggested for platelet integrins. "Inside out" signaling refers to the mechanism in which platelets binding to an agonist such as thrombin gives rise to the intracellular signal that is propagated to the intracellular part of the GPIIb/IIIa. This signal transduction leads to a conformational change in GPIIb/IIIa and allows it to become activated and be able to bind soluble fibrinogen molecules. In contrast, "outside in" signaling refers to the fact that when integrins like GPIIb/IIIa bind to adsorbed proteins such as fibrinogen, the binding event will generate intracellular signals that will induce platelet activation.[2] Activation causes platelets to become sticky and change in shape to irregular spheres with spiny pseudopods, accompanied by internal contraction and extrusion of the storage granule contents into the extracellular environment (Figure 2). These secreted platelet products stimulate other platelets, cause irreversible platelet aggregation, and lead to the formation of a fused platelet thrombus.[12]

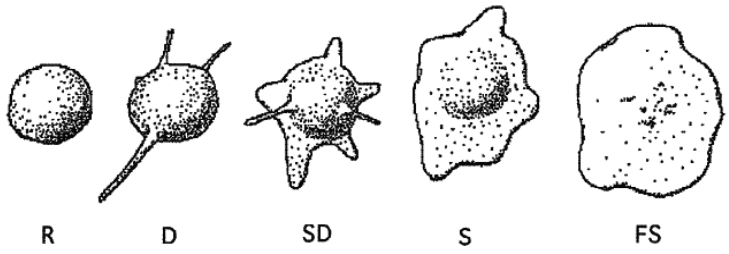

Figure 2. Diagrammatic representation of platelet spreading divided into five shape categories. From left to right, the stages of spreading are defined as follows: round (R) or discoid: no pseudopodia present; dendritic (D) or early pseudopodial: one or more pseudopodia with no evident flattening; spread dendritic (SD) or intermediate pseudopodial: one or more pseudopodia flattened, hyaloplasm not spread between pseudopodia; spreading (S): hyaloplasm spread between pseudopodia; and fully spread (FS): hyaloplasm extensively spread, no distinct pseudopodia. Adapted from Goodman et al.[15]

1.2 The role of leukocytes

Circulating leukocytes comprise neutrophils, monocytes, lymphocytes, basophils and eosinophils. Neutrophils and monocytes are the major players in the inflammatory response with cardiovascular devices. Neutrophils are the most abundant white blood cells, representing 40-60% of the leukocyte

population (3-5x10^6 neutrophils/ml), while monocytes represent 5% with a concentration of 0.2-1x10^6 monocytes/ml.[13]

The inflammatory response of the host is a key factor in determining the outcome of biomaterials implanted into the human body. The inflammation that usually protects the body from invasion by foreign organisms can also produce unwanted effects that may frustrate therapeutical attempts to improve human health. For example, the implantation of biomaterials may lead to an inflammatory response that can go as far as destroying the foreign material.[16]

Adhesion of leukocytes to solid surfaces depends on many factors, including surface free energy, surface hydrophilicity, surface chemistry and charge, protein adsorption, complement activation, and adhesion of other cells, such as platelets and bacteria.[17]

Since protein adsorption from biological fluids occurs rapidly rendering direct recognition of surface functional groups by the cells virtually impossible, functional groups are believed to affect cell adhesion indirectly via 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.[18]

Once the protein layer is formed, the adsorbed proteins can expose ligands for leukocyte adhesion receptors. It has been shown that the receptors of monocytes are capable of binding extracellular matrix and blood proteins such as complement factor C3, immunoglobumin G, fibrinogen, fibronectin and vitronectin adsorbed onto natural or synthetic surfaces.[19,20]

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.[20,21] Adhesion of leukocytes to adsorbed fibrinogen is mediated by the β_2-integrin Mac-1[21,22] and intercellular adhesion molecule-1 (ICAM-1)[23], whereas fibrinogen-induced activation of these cells is mediated primarily by toll-like receptor-4.[24] In agreement with this, proteins recognized only by β_2-integrins can serve as adhesion substrates, but do not result in activation of the adherent leukocytes.[25]

Leukocyte adhesion to biomaterials is known to occur during many types of processes involving blood-material interactions. As a result of leukocyte adhesion, several reactions may be initiated, such as leukocyte spreading, formation of microthrombi through platelet-leukocyte interactions, detachment of thrombi by action of leukocyte proteases, detachment of adherent platelets and

adsorption of proteins by leukocytes, release of leukocyte products that may give rise both to local and systemic vascular reactions, inflammatory responses that are leukocyte-dependent, and promotion of fibroblast proliferation.[16,26]

1.3 Thrombus formation mechanism

It has been reported that fibrinogen is a major part of the adsorbed protein layer which is partially replaced by kininogen to provide the medium needed for the attachment and, thus, the activation of Factor XII (Hageman Factor). Factor XII is an intermediate of the intrinsic coagulation pathway and its activation can be initiated by contact with the foreign material or by contact with aggregated platelets. Other pathways involve platelet activation, the complement system and tissue factors. All these aspects of coagulation are inter-related and the blood compatibility assessment of a biomaterial must consider not only the ways in which platelet behavior is affected by contact with the material, but also other blood factors, which might be more subtly influenced by the foreign material.[27] Certainly, the main parameter of blood compatibility commonly investigated is platelet response to the foreign material. The process of coagulation starts on the surface where platelets aggregate with the formation of a fibrin network. A thrombus is formed from the combination of mutually fused platelets plus the insoluble fibrin and the cells that it has trapped from the blood.[3]

1.3.1 Complement and coagulation cascades

Complement system and hemostasis both can be viewed as partners in an inflammation that is aimed at stabilizing a living system that has encountered various disturbances to its homeostasis. The complement and coagulation systems are organized into the proteolytic cascades composed of serine proteases (Figure 3).

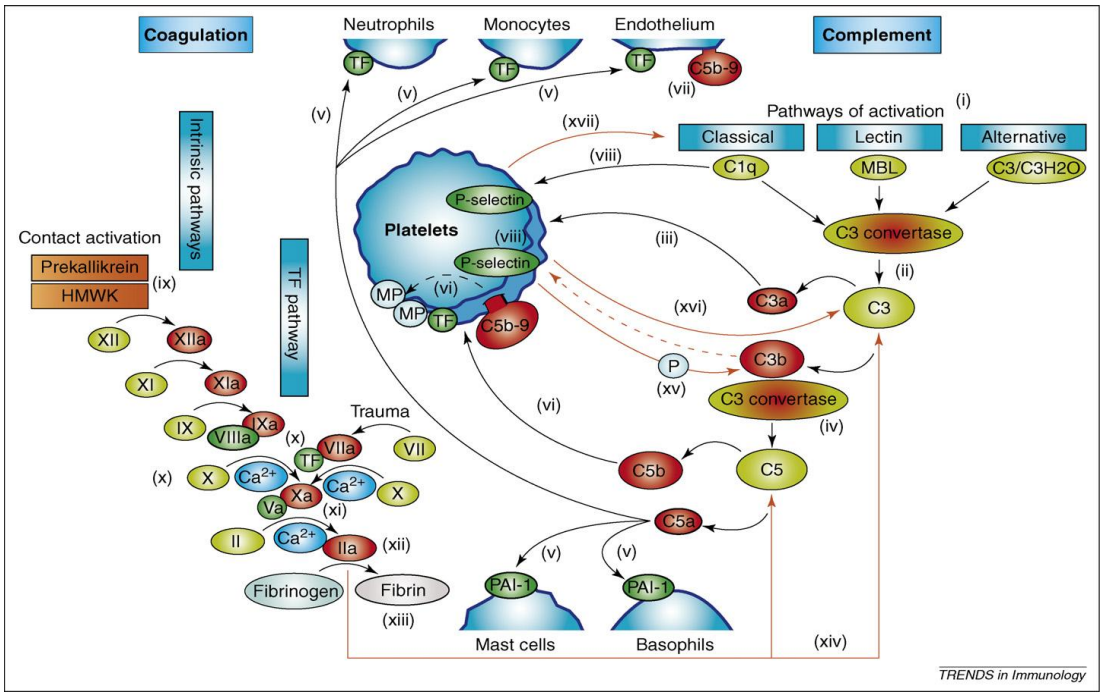

Figure 3. Complement–coagulation reciprocal interactions. Complement and coagulation cascades are composed of serine proteases that are activated through partial cleavage by an upstream enzyme. Zymogens are marked in light green, and active components are shown in red. Complement is activated through the classical, lectin or alternative pathways (i) that converge at the central molecule of the complement system, C3 (ii). The C3 convertases, generated through various pathways, cleave C3 to C3a and C3b (ii). C3a anaphylatoxin activates platelets, enhancing their aggregation and adhesion (iii). C3b contributes to the formation of C5 convertase, which cleaves C5 to C5a and C5b (iv). In addition to its well-established role in inflammation, C5a enhances blood thrombogenicity, mainly through the upregulation of tissue factor (TF) and plasminogen activator inhibitor-1 (PAI-1) expression on various cell types (v). C5b contributes to the formation of the terminal complement complex (TCC) (also known as C5b-9), which is incorporated into the cellular membrane of platelets, inducing an alteration in membrane polarization and, thus, increasing the surface area on which clotting can occur (vi). TCC also induces the release of microparticles (MPs) bearing TF on their surface (vi) and affects procoagulant properties of endothelium (vii). C3b binds to P-selectin, the expression of which is induced on platelets by C1q (viii), an initiator of the classical pathway of complement activation. Black arrows illustrate the interactions of complement with coagulation, increasing the propensity of blood to clot. Coagulation launched through the contact (intrinsic) pathway begins with contact activation of HMWK, prekallikrein and factor XII (ix). TF expressed on various cells or released from injured cells initiates the physiologically more important TF (extrinsic) pathway (x). Both pathways merge at the level of factor X (xi), which, following activation, converts prothrombin (II) to thrombin (IIa) (xii). The final step of the coagulation process, catalyzed by thrombin, requires partial cleavage of soluble fibrinogen and polymerization to insoluble fibrin (xiii). Thrombin cleaves C3 to C3a and C3b, and C5 to C5a and C5b, thus amplifying the activation of complement (xiv). Platelets, the central cells in hemostasis, also contribute to the amplification of complement through the phosphorylation of C3b (xv), which prolongs the life span of this molecule. Activated platelets are also involved in C3 cleavage (xvi) and initiation of the classical pathway of complement activation (xvii). The amplification of complement activation exerted through the components of the coagulation system is shown as red arrows. Abbreviation: P, P-selectin. Copyright Elsevier Ltd. Adapted from Mrkiewski et al.[28]

1.3.1.1 Pathways of coagulation

The coagulation system is a component of the homeostatic process and a major contributor to thrombosis (the pathological formation of thrombi within the blood vessels). The coagulation sequence comprises a series of transformations of proenzymes to activated enzymes, culminating in the formation of thrombin (IIa), which converts soluble fibrinogen into insoluble fibrin. With a few exceptions, these enzymatic reactions occur on the phospholipid surface of activated endothelial cells and platelets in the presence of calcium ions. The process of coagulation is activated through the contact (intrinsic) and tissue factor (TF) (extrinsic) pathways, both of which converge at the point of activation of factor X. The intrinsic pathway is initiated in vitro by contact activation of factor XII, closely linked to the kinin–kallikrein system. The extrinsic pathway, responsible for a rapid and efficient in vivo coagulation, is triggered by TF, a cellular lipoprotein released from damaged cells or expressed on the surface of activated monocytes, endothelial cells, and other nonvascular cells. TF converts factor VII to factor VIIa. The process of coagulation is controlled and restricted to the site of vascular injury by natural anticoagulants. These inhibitors of coagulation belong to one of three categories: antithrombins, the protein C and protein S group, or the TF pathway inhibitor system. Dissolution of blood clots (fibrinolysis) is regulated by the plasminogen–plasmin system. This system, activated by plasminogen activators (Pas), breaks down fibrin and controls fibrin polymerization. Plasminogen activator inhibitor-1 (PAI-1), produced by various cells including the endothelium, mast cells and basophils, inhibits the activation of this system. Members of the coagulation cascade cooperate closely with platelets, which have a central role in hemostasis. Activated platelets provide negatively charged phospholipid surfaces, where the process of coagulation can occur, and release microparticles (MPs) bearing TF and various mediators, contributing to hemostasis and thrombosis.

1.3.1.2 Complement activation and functions

Complement is not only a part of the innate immune system but also an effector of antibody-mediated immunity. The major biological functions of complement include the defense against infections, bridging innate and adaptive immunity, and the clearance of immune complexes and apoptotic cells. The system is composed of ~30 proteins circulating in plasma and expressed on

cellular surfaces. Circulating components of complement are activated through three pathways: classical, lectin and alternative. The classical pathway is initiated by the binding of C1q to antigen–antibody complexes, whereas the lectin pathway begins with the binding of mannose-binding lectin (MBL) or ficolins to sugars presented at the bacterial cell wall. Both pathways lead to the formation of a C3 convertase. The alternative pathway can be triggered directly by foreign surfaces, such as biomaterials, through the adsorption of C3 on their surface, or triggered by spontaneous hydrolysis of the internal thioester bond within C3 in the fluid phase, leading to the formation of $C3{\bullet}H_2O$. Hydrolyzed C3, through the binding and subsequent activation of factor B, contributes to the formation of the alternative pathway C3 convertase. C3 convertases cleave C3 to C3a and C3b. C3b participates in the formation of the C5 convertase and functions as a bacterial opsonin, facilitating the phagocytosis of opsonized pathogens by macrophages and neutrophils. C5 convertase cleaves C5 to C5a and C5b. C3a and C5a, termed anaphylatoxins, are pleiotropic inflammatory mediators. C5b initiates the formation of the terminal C5b-9 complement complex (TCC), called the membrane attack complex (MAC), which, when incorporated into the membranes of pathogens or cells, induces their lysis, or, when incorporated into cells in sublethal doses, induces their activation. Several surface-expressed complement inhibitors that limit the activation of this system to the site of infection and protect host cells from complement attack and lysis tightly regulate the activation of complement.[28]

2. PROTEIN ADSORPTION ON SOLID SURFACES

The interaction of proteins with solid surfaces is not only a fundamental phenomenon with implications for nanotechnology, biomaterials and biotechnological processes, but also key to several novel applications. In the biomaterials field, protein adsorption is the first step in the integration of an implanted device or material with tissue.[29]

2.1 Adsorbed protein films

Proteins are high-molecular-weight molecules consisting of chains of amino acids. As there are polar, non-polar and ionic amino acids, proteins contain a mixture of hydrophilic and hydrophobic groups.[30]

Protein chains are normally highly folded in solution with the hydrophobic amino acids tending to reside in the centre of the globule—away from water—while the hydrophilic amino acids tend to be at the globule surface. When a protein adsorbate reaches an oil–water or air–water interface, it partially unfolds exposing its hydrophobic groups to the non-aqueous phase. In that way the protein reduces the surface free energy of the system, and the binding energy per molecule is much higher than for surfactant adsorbates. Therefore, proteins are also surface-active molecules. [30]

The adsorption of proteins onto a biomaterial surface from the surrounding fluid phase is rapid, with the surface properties of the biomaterial determining the type, amount, and conformation of the adsorbed proteins. The composition of the adsorbed protein layer (i.e., the type and concentration of the proteins present in the adsorbed film) can differ from the fluid phase composition and can change with time adsorbed. Upon adsorption, a protein can retain the conformation or structure it has in the biological environment or it may conformationally change in response to local environments. The nature of the surface strongly influences the composition and recognizability of the adsorbed protein layer, which in turn affects the subsequent cellular interactions. [31]

Many globular proteins can also form chemical and physical linkages with other protein molecules. When proteins adsorb, they reach higher local concentrations than in the bulk, and so the protein–protein interactions play a more important role. Moreover, when they unfold, a large number of chemical groups on the protein molecule are exposed to other neighbouring molecules. As a

consequence, the formation of a protein–protein linkage is a very common event in globular protein films. Indeed, many of these films behave like two-dimensional gels, held together by a cross-linked network of bonds, some strong (permanent) and some weak (transient). [30]

Adsorption can result either from the universal van der Waals interactions (physical adsorption or physisorption) or it can have the character of a chemical process (chemical adsorption or chemisorption). Contrary to physisorption, chemisorption occurs only as a monolayer. Physical adsorption can be compared to the condensation process of the adsorptive. As a rule, it is a reversible process that occurs at a temperature lower or close to the critical temperature of an adsorbed substance. Chemisorption occurs usually at temperatures much higher than the critical temperature and — by contrast to physisorption — is a specific process which can only take place on some solid surfaces. Under favourable conditions, both processes can occur simultaneously or alternately. Physical adsorption is accompanied by a decrease in free energy and entropy of the adsorption system and, thereby, this process is exothermic.[31]

2.2 Protein-surface interactions

The adsorption of plasma proteins, such as fibrinogen, fibronectin or vitronectin, can influence the adhesion of platelets, leukocytes and macrophages, ultimately leading to fibrous encapsulation. A detailed understanding of the protein-surface interaction would be of value to the biomaterials field, and the availability to tailor specific protein-surface interactions would benefit the development of nanoscale materials.[1]

Despite several decades of study of protein adsorption, the ability to control the protein surface interactions is still imperfect. This is in part due to the large number of complex, interdependent, and dynamic interactions between proteins and a surface.

The primary forces that drive protein adsorption to a solid surface are hydrophobic dehydration resulting from the interaction between hydrophobic patches on a protein and a hydrophobic surface and electrostatic interactions between solvent-accessible charged groups on a protein and the surface. [32]

Apart from proteins, a large number of substances compete for adsorption on a biomaterial surface. Water molecules are major competitors, and due to the strength of their intermolecular bonds proteins have to overcome a layer of water molecules before they successfully adsorb.[33]

A general trend is that protein adsorption increases with increasing hydrophobicity of the surface. Since rupturing the water structure in the vicinity of hydrophilic surfaces is more difficult than on hydrophobic surfaces, protein adsorption is less favorable on the former.

As previously mentioned, electrostatic interactions are also an important factor in protein adsorption, but their contribution depends not only on the electrostatic properties of the protein and the surface, but also upon the solution pH and ionic strength. Protein adsorption due to electrostatic interactions is minimal at a solution pH close to the isoelectric point of the protein, and scales inversely with ionic strength, because an increase in the solution ionic strength shields the charges on the protein and surface more efficiently, and consequently reduces protein adsorption. [32]

Proteins are often thought to denature at both solid-liquid and air-liquid interfaces, although they retain more their original structure on electrostatically neutral hydrophilic surfaces than on hydrophobic or charged surfaces.[29] As a rule, proteins with high conformational stability (hard proteins), like RNAse and lysozyme, tend to adsorb readily on a hydrophobic surface whereas proteins with low conformational stability (soft proteins), such as albumin and IgG, adsorb to a great variety of surfaces (hydrophilic and hydrophobic). Upon adsorption to hydrophobic surfaces, soft proteins change their conformation to a greater extent than hard proteins.[34]

In addition, protein adsorption also depends upon protein concentration. At low solution concentration, a protein can maximize interactions with the surface both by its orientation as well as by unfolding that leads to denaturation and irreversible adsorption of the protein at the surface. At high protein concentration, however, proteins undergo fewer interactions with the surface, and hence retain their stable conformation and are desorbed more easily.[32]

Finally, the interfacial properties of an adsorbed protein layer alter with time due to two effects: the first—termed the Vroman effect[35]—occurs in the adsorption of proteins on a surface from multicomponent mixtures, and is manifested as a time-dependent change in the composition of the adsorbed layer of proteins, as proteins with high solution concentration but low surface activity are replaced by other proteins that are present at lower concentration in solution but which have a

higher surface activity. Second, adsorbed proteins can also undergo time-dependent unfolding at the surface, thereby changing their conformation at the solid–liquid interface.[32,36]

2.2.1 Vroman effect –importance of fibrinogen

Vroman et al. have advocated a prominent role for fibrinogen in adsorption. They believe that fibrinogen dominates the protein layer deposited in the first 1 or 2 seconds and then, on "activating" surfaces (those which ultimately provoke coagulation), the fibrinogen is replaced by high molecular weight kininogen (HMWK). It has been suggested that HMWK is the relevant species based on the observation that fibrinogen adsorbed from HMWK-deficient plasma is not displaced or is displaced more slowly. However, when diluting the plasma sufficiently, the concentration of HMWK decreases to the point where it is not sufficient to replace fibrinogen. [35,37]

Brash et al. have also observed that below a "critical" plasma concentration (between 0.5 and 0.25%, for adsorption to a glass surface), competition between fibrinogen and other plasma proteins becomes negligible, given that the critical plasma concentration varies from surface to surface. These critical plasma concentrations may be interpreted as indicating the affinity of the various surfaces for fibrinogen relative to the competing species: the higher the critical concentration, the greater the affinity for fibrinogen relative to the competing species since the plasma does not have to be diluted as much to make fibrinogen competition effective.[38]

Donaldson et al. has reported that HMWK exists in normal plasma as a complex with prekallikrein. They suggest that the complex is the active species for replacement of fibrinogen and that at the critical concentration it is too extensively dissociated to have measurable fibrinogen-replacement activity. The existence of such a complex would thus be consistent with the notion of a critical concentration.[39]

2.3 Nonspecific protein adsorption

Control over the nonspecific adsorption of proteins to surfaces is fundamentally important in technologies that involve the contact of synthetic surfaces with biological fluids. It is generally

believed that surfaces that strongly resist protein adsorption will also resist cell adhesion, as the adhesion receptors on the cells would lack specific adsorbed ligands.

Surfaces that resist the nonspecific adsorption of proteins and attachment of cells commonly are called “inert” or “non-fouling” surfaces. Even though superhydrophobic surfaces (with contact angles higher than 150°) are also known to be non-fouling, the most common approaches to reducing protein and cell binding to biomaterial surfaces have been to make them more hydrophilic. Some synthetic hydrophilic non-fouling surfaces include PEO polymers and surfactants, neutral polymers (e.g., poly(2-hydroxyethyl methacrylate), polyacrilamide, poly(N-vinyl-2-pirrolidone),…), anionic polymers, phosphorylcholine polymers, glow discharge deposited coatings, self-assembled n-alkyl molecules with oligo-EG head groups or other polar head groups and zwitterionic materials (with two oppositely charged groups in the same side chain). Natural non-fouling hydrophilic surfaces include passivating proteins (e.g., albumin and casein), polysaccharides (e.g., hyaluronic acid), liposaccharides, phospholipid bilayers and glycoproteins (mucins).[12]

The most successful method to obtain non-fouling hydrophilic surfaces has been to modify various polymer materials with a water-soluble polymer for biomedical use such as poly(ethylene glycol) (PEG) or poly(ethylene oxide) (PEO) that can prevent plasma protein adsorption, platelet adhesion, and thrombus formation by the steric repulsion mechanism.[40-42] Steric repulsion by surface-bound water-soluble polymer chains occurs as a result of overlapping polymer layers that could lead to loss in configurational entropy because of volume restriction and/or osmotic repulsion between interdigitated polymer chains. The accepted mechanism for preventing protein adsorption by the grafted PEO chains is that such technique decreased interfacial free energy of the surface and increase the steric repulsion force between PEO chains and the proteins.[43]

Even though many strategies to inhibit non-specific protein adsorption (non-fouling surfaces) have been developed, some questions still hang around and keep driving research in this area. How resistant to protein pick-up can such surfaces be made? Why are they resistant to protein adsorption? How long can they remain resistant to protein fouling?

3. SURFACE MODIFICATION TO IMPROVE HEMOCOMPATIBILITY

Interactions between the biological environment and artificial materials are most likely dominated by the materials' surface properties,[44] meaning that the blood compatibility of a material is determined primarily by its surface, rather than by its bulk properties and can be improved by surface modification, whereas the physical properties of the material remain essentially unchanged. Surface modification is therefore an important technique for improving the blood compatibility of biomedical devices which do not meet the requirement of hemocompatibility.[45]

The application of a thin-film coating modifies the surface characteristics either by passivation (i.e., nonspecific protein and cell repulsion) to prevent undesired biological responses, and/or by activation to incorporate a specific functionality or functionalities at the blood-device interface. There are three main strategies that are typically used to reduce device thrombosis: (1) reduction of non-specific protein adsorption; (2) enhancement of specific adsorption of non-thrombogenic blood proteins; and (3) promotion of active thrombin consumption.

In the line of strategy (1) several polymers such as poly(vinylpyrrolidone) (PVP)[46-48], polyacrylic acid (PAA)[49-51], poly(2-hydroxyethylmethacrylate) (pHEMA)[52,53] and phosphorylcholine (PC)[54,55] based materials have been explored as non-fouling materials. However, and as previously described in section 2.3 of this chapter, poly(ethylene glycol) (PEG) or poly(ethylene oxide) (PEO) has been the most extensively studied polymer for this purpose. The use of PEO in forming a biomaterial is attractive for many reasons: it is only slightly immunogenic, compatible with living cells, and due to its weakly basic ether linkages, there are few possibilities for PEO binding with proteins. Numerous publications have clearly shown decreased protein adsorption to PEO-modified surfaces.[40-42] The biocompatibility of various PEO-modified polymer surfaces examined using different bioassays (mainly anticoagulant activity, platelet adhesion and protein adsorption) are summarized in a review by Chen et al.[56] More recently, sulfobetaine/carboxybetaine polymers have been considered excellent candidates for nonfouling materials. These polyampholyte materials with a uniform distribution of mixed charged groups in different side chains are similar to zwitterionic materials with two oppositely charged groups in the same side chain.[57] Even though passivating a surface is a very useful and widely used strategy to improve the biocompatibility of a surface, achieving extremely low

protein adsorption is not easy in non-uniform polymer surfaces and maintaining resistance to fouling has proven difficult in long-term applications.

In contrast to hydrophilic passivating coatings that minimize nonspecific protein adhesion, protein binding coatings enhance the adsorption of specific blood proteins that do not promote thrombosis (strategy (2)). A promising way for preparing materials compatible with blood is to provide a dynamically renewable, natural albumin coat between the surface of the device and the blood.[58] Albumin acts as a bystander molecule in the case of many surface contact-activated biological reactions because it contains none of the peptide sequences known to interact with either adhesion receptors on cell membranes or enzymes in the coagulation and complement cascades.[59] Furthermore, albumin-coated surfaces have been found to reduce platelet reaction to synthetic materials.[60] Since this strategy was the one followed in this study, it will be discussed in more detail in section 3.1 of this chapter.

Strategy (3) regards the inhibition of thrombin, a key thrombosis-promoting enzime. This strategy used to improve hemocompatibility is based on the incorporation of specific bioactive molecules on a polymer surface so as to promote or support a favorable reaction. For instance, in order to produce thrombin-resistant surfaces, direct thrombin inhibitors such as hirudin,[61,62] Cys-*D*-Phe-Pro-Arg (C(D)FPR) [63], *D*-Phe-Pro-Arg-CH_2Cl (PPACK)[64], and thrombomodulin[65] have been used to modify various biomaterials. In addition to these direct thrombin inhibitors, immobilization of heparin, an indirect inhibitor of thrombin, on a biomaterial surface has been found to efficiently prevent thrombin formation.[66] Heparin inhibits primarily thrombin, as well as other coagulation factors through the binding of anti-thrombin III (ATIII). Once bound to heparin, the catalytic activity of ATIII is increased 1000- to 10000-fold. Because heparin inactivates thrombin catalytically, i.e., it is not consumed in the process, heparin immobilization provides localized thrombin activity to a medical surface without appreciably affecting systemic blood clotting mechanisms.

An ongoing goal in this area of blood contact biomaterials is to produce a more effective biocompatible antifouling surface coupled with the use of specific ligands to induce anticipated protein and cell responses.

3.1 The role of albumin

Human serum albumin (HSA) accounts for about 60% of the total protein in blood with a concentration of ~40 mg/ml and has a heart shape with dimensions of 8 x 8.7 x 6 nm^{3}.[6]

This globular protein of approximately 65 kD in size, consists of 585 amino acids. Its amino acid sequence contains a total of 17 disulphide bridges, one free thiol (Cys 34), and a single tryptophan (Trp 214). The disulphides are positioned in a repeated series of nine loop-link-loop structures centered around eight sequential Cys-Cys pairs.[67] The isoelectric point of HSA is 4.7, indicating that in physiological conditions this protein has a negative charge.

The serum albumins belong to a multigene family of proteins that includes α-fetoprotein and human group-specific component or vitamin D-binding protein. They are relatively large multi-domain proteins which, as the major soluble protein constituents of the circulatory system, have many physiological functions. The albumins contribute significantly to colloid osmotic blood pressure and aid in the transport, distribution and metabolism of many endogenous and exogenous ligands. These ligands represent a spectrum of chemically diverse molecules, including fatty acids, amino acids (notably tryptophan and cysteine), steroids, metals such as calcium, cooper and zinc, and numerous pharmaceuticals.

The compounds that albumin binds most strongly are hydrophobic organic anions of medium size, 100 to 600 Dalton – long chain fatty acids (Association constant, K_A = (1-69 x 10^7 M^{-1}), hematin (K_A = 1.1 x 10^8 M^{-1}) and bilirubin (K_A = 9.5 x 10^7 M^{-1}). Smaller and less hydrophobic compounds, such as tryptophan (K_A = 1.0 x 10^4 M^{-1}) and ascorbic acid (K_A = 3.5 x 10^4 M^{-1}), are held less strongly, but their binding can still be highly specific.[68]

The plasma free fatty acids (FFA) are composed primarily of 16 to 18 carbon atom fatty acids.[69] These long chain fatty acids are poorly soluble in aqueous solutions. The formation of a noncovalent complex with albumin, however, enables appreciable quantities of FFA to be dissolved in the plasma.[70] The FFA concentration in the blood plasma is relatively low, ordinarily between 0.3 and 1.2 mM.

The binding of free fatty acids (FFA) by albumin has been extensively studied and can serve as a model for alkyl chain binding. HSA has seven strong binding sites and several weak binding sites (Figure 4).[70-72] Each binding site pocket has a unique binding constant, and as binding occurs, the conformation of the protein changes to create new binding sites.[70] These binding sites are flexible

and conform to the size and shape of the alkyl chain. The strength of the binding interaction increases as the carbon chain length increases from 6 to 18, with oleate being the most tightly bound. For chains of greater length, the binding strength decreases, perhaps because the chain cannot fit completely into the binding site. As more FFA is bound, the protein expands slightly in size, and becomes less susceptible to denaturation by heat, urea, or guanidine hydrochloride.[73]

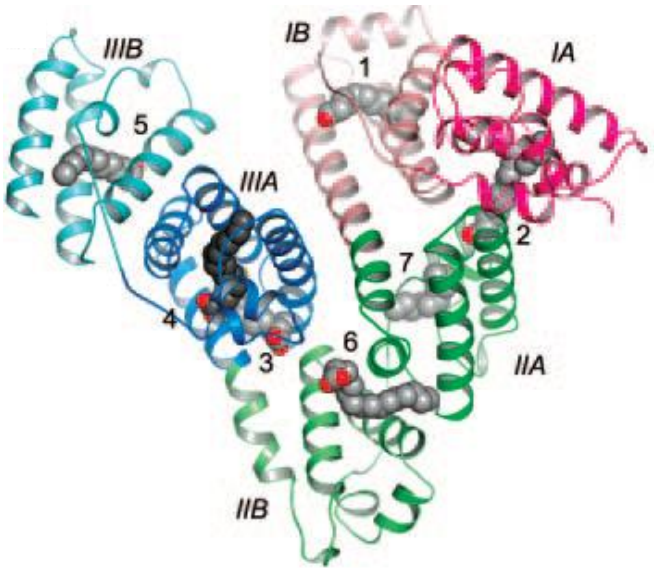

Figure 4. Structure of human serum albumin revealing seven long-chain fatty acid binding sites. Albumin contains three domains (I, red; II, green; III, blue), each containing A and B subdomains (dark- and light-coloured shades, respectively). Adapted from Simard et al.[72]

3.1.1 Alkylation of surfaces for albumin selective adsorption

Eberhart et al. have studied the passivating tendencies of spontaneously adsorbed albumin *in vitro*, and have found adsorption from solution to provide only sparse coverage.[74] The preadsorbed albumin layer was, however, easily desorbed in a fluid shear field.

To avoid this problem, it is necessary to create surfaces that bind albumin from the bloodstream in a selective way. This involves the immobilization of ligands (e.g. alkyl chains of 16 and 18 carbon residues[58,75], Cibacron Blue F3G-A[76-78] and warfarin[79]) or antibodies[80] to albumin at the surface of the biomaterial.

The alkylation of blood contacting polymers has been suggested as a means of reducing thrombus deposition at the blood/polymer interface. Munro et al. have shown that covalent bonding of C18 and C16 linear alkyl chains to the surfaces of polyurethanes and other polymers increased the amount of albumin adsorbed at short times from single and binary protein solutions. [58,81] Platé[82] and Munro[58] hypothesized that albumin, which has binding sites for free fatty acids, employs these binding sites to attach to the alkyl chains of the derivatized polymers. They also suggested that the increased concentration of albumin on the surface would decrease the number of platelets available to aggregate into a thrombus.

3.1.2 Drawbacks of alkylation for albumin adsorption

Despite the promising observations of improved blood compatibility, the molecular mechanisms promoting increased human serum albumin (HSA) adsorption to alkylated polymers have not been thoroughly investigated. In general HSA binds any organic compound with at least five or six CH_2 groups whether or not there is an accompanying hydrophilic group such as a carboxylate, sulfonate, sulfate or hydroxyl group.[83] The binding energy is derived primarily from hydrophobic interactions between the alkyl chain and the hydrophobic side chains of the amino acids forming the binding site "pockets". There are also cationic residues near the mouth of the pockets, which can interact with an anionic group such as a carboxylate or sulfate, although the contribution of this electrostatic or hydrogen bonding interaction is small compared to the hydrophobic interactions.[70]

Although the binding of the alkyl chains of a polymer to the albumin binding sites is an obvious mechanism for increasing the albumin's affinity for a surface, other less obvious mechanisms may also be important. It is generally observed that the adsorption of relatively hydrophobic proteins (such as albumin) increases as the polymer substrate becomes more hydrophobic. This increase in adsorption is attributed to a general increase in the hydrophobic interactions between the protein and the polymer. The entropy created by these hydrophobic interactions is one of the dominant driving forces in protein adsorption, and addition of alkyl chains to a surface could make that surface more hydrophobic, and thus increase the amount of albumin adsorbed.[73]

There are other considerations which make the hypothesis of specific binding of albumin to the alkyl chains less plausible. For example, in an aqueous environment, entropic forces would tend to reduce

the area of a hydrophobic surface. This would force the hydrophobic alkyl chains flat against the polymer surface, although transient extensions into the aqueous solution could occur due to thermal vibrations. However, an HSA molecule closely contacting such a surface may push enough water molecules away that the flattened alkyl chains would become free to diffuse into the protein and become bound.

4. FROM REAL WORLD POLYMERS TO MODEL SURFACES

Elucidating relationships between surface properties and cellular responses is often difficult since the polymer systems typically used in biointeraction studies do not allow for systematic, controlled variations in material surface properties. Furthermore, for many biomaterials the surface chemistry is quite complex and it is difficult to be sure of the chemical composition at the blood-biomaterial interface. Also, many polymer surfaces are dynamic and can undergo conformational rearrangements in response to environmental changes. When exposed to biological media, buried polar groups in a mobile polymer may reemerge at the surface. As a result the adsorbing protein layer may adapt to the existing polymer surface which may differ compositionally from the composition of the polymer in air.

To effectively determine the chemical functionalities and/or composition responsible for influencing protein adsorption and cell growth, a set of surfaces exhibiting systematic variations in surface chemistry which exhibit conformational stability is required.

Molecular self-assembly techniques provide an effective means of fabricating organic surfaces with well-defined structure and chemistry.[84] Self-assembled monolayers (SAMs) with different terminal groups have stimulated interest in several areas of bioengineering, including biosensors, biomaterials and biomimetics. Their well-defined structures and intrinsic stability are conducive to precision molecular engineering and quantitative analysis.

With this technique, the surface properties can be controlled at the molecule-level and a well defined, highly ordered and oriented surface can be prepared with the goal of developing a more hemocompatible surface.[3]

4.1 Self-Assembled Monolayers – SAMs

Self-assembled monolayers (SAMs) provide a convenient, flexible, and simple system with which to tailor the interfacial properties of metals, metal oxides, and semiconductors. SAMs can be defined as "molecular assemblies that are formed spontaneously by the immersion of an appropriate substrate into a solution of an active surfactant in an organic solvent"[85]; the adsorbates organize spontaneously

into crystalline (or semicrystalline) structures. The driving force for self-assembly is usually the specific interactions between the surface active head group of the surfactant and the surface of the substrate. Most surfactants used in monolayer studies consist of three distinctive parts: the surface active head group which binds strongly to the surface, the terminal group which is located at the monolayer surface and normally determines the interfacial properties of the assembly, and the alkyl chain which facilitates the packing of the molecules in the monolayer and serves as a linker between the head and the end groups.[86]

Many examples of surface-surfactant interactions which promote self-assembly are known. Apart from gold-thiol monolayers which are formed because of the creation of the strong S-Au bond, other commonly studied monolayers include alkyltrichlorosilane layers on hydroxylated surfaces (such as SiO_2), long chain carboxylic acids on metal oxide surfaces and alkyl phosphonate salts on zirconium.[86]

There are five characteristics of gold that make it a good choice as a substrate for studying SAMs. First, gold is easy to obtain, both as a thin film and as a colloid. The preparation of thin films of gold by physical vapor deposition, sputtering, or electrodeposition is straightforward. Second, gold is exceptionally easy to pattern by a combination of lithographic tools (photolithography, micromachining, others) and chemical etchants. Third, gold is a reasonably inert metal: it does not oxidize at temperatures below its melting point; does not react with atmospheric O_2, and does not react with most chemicals. These properties make it possible to handle and manipulate samples under atmospheric conditions instead of under ultra high vacuum (UHV) – a great practical advantage for conducting experiments that require “dirty” conditions, e.g., microfabrication (outside a clean room environment) and cell biology. Gold binds thiols with a high affinity, and does not undergo any unusual reactions with them, e.g., the formation of a substitutional sulfide interphase. Fourth, thin films of gold are common substrates used for a number of existing spectroscopies and analytical techniques, including surface plasmon resonance (SPR) spectroscopy, quartz crystal microbalances (QCM), infrared reflection absorption spectroscopy (IRAS), and ellipsometry. This characteristic is particularly useful for applications of SAMs as interfaces for studies in biology. Fifth, gold is compatible with cells, that is, cells can adhere and function on gold surfaces without evidence of toxicity.[87]

Despite the many advantages, gold-thiol monolayers also have some drawbaks comparing to other model surfaces. When compared to glass, for example, gold substrates are more expensive, are not

transparent (difficulting visualization by optical microscopy when used in cell biology) and have higher surface roughness (despite depending on the gold deposition method). Their stability under physiological conditions is also a controversial subject and will be discussed further on in section 4.1.3Behavior of SAMs under Physiological Conditions.

4.1.1 Designing SAMs to be model biological surfaces

To test a range of experimental conditions and facilitate the interpretation of the interactions observed, SAMs should have, at least, three characteristics:

- they should be able to prevent nonspecific adsorption of proteins or other biomolecules on the surface and only allow interactions between the molecules and ligands of interest;
- they should allow modifications to the composition and density of the immobilized ligands or biomolecules (proteins, sugars, antigens);
- they should present the ligands of interest in a structurally well-defined manner that minimizes the influences of the surface, e.g., limited mass transport, blocked binding sites, or induced conformational changes. It is also helpful if the model surfaces can be used easily with common analytical methods without modifying the existing instrumentation or without subjecting the samples to unnatural (for biology) conditions, e.g., in dehydrated form in ultra high vaccum.[87]

4.1.2 Protein-Resistant SAMs

Alkanethiols terminated with ethylene glycol groups are a standard component of SAMs used to study biology and biochemistry.[88] On gold, the alkane chains form a dense, ordered monolayer with the same molecular conformation found for n-alkanethiols, i.e., all-trans chains with a 30° tilt; the terminal ethylene glycol end-group adopts either a helical conformation aligned perpendicular to the surface or an amorphous conformation (when examined spectroscopically in dehydrated form in air or vacuum).[89] The helical structures yield a quasi-crystalline surface phase, but the amorphous chains produce a liquid-like phase.

The non-fouling properties of ethylene glycol terminated SAMs are attributed to a combination of qualities: the packing density of the alkane chains, the hydrophilicity, steric repulsion and detention

of water molecules by the mobile strands (ethylene oxide repeat unit), the absence of sites that can participate in long-range attraction of charged groups or short-range attraction of hydrophobic groups, and the inability of proteins to "melt" structured water on ethylene oxide.[90]

4.1.3 Behavior of SAMs under Physiological Conditions

Knowledge of the structure and properties of SAMs immersed in solvents is substantially less than that for SAMs of alkanethiolates in air or in vacuum. The use of SAMs as substrates for studies in biology requires, however, extended contact between SAMs and an aqueous environment containing a high concentration of salts and biomolecules (enzymes, extracellular matrix proteins, plasma components, sugars). The structure and dynamics of the exposed surface of a SAM under these conditions have not been characterized completely but are critical for understanding the origin of certain properties (especially resistance to adsorption of proteins). Grunze and co-workers have shown the conformational changes at the exposed surface of SAMs terminated with PEG (45 EG subunits) upon exposure to water.[91] Each PEG at the surface adopts a helical structure in air to form a quasi-crystalline phase with the rods oriented nearly perpendicular to the surface. The structure of the SAM changes when immersed in water: the ends of the helical EG units transition to an amorphous state, and the amorphous interfacial region is solvated in a manner equivalent to dissolved PEG chains. For SAMs terminated with short oligomers of ethylene glycol (3-6 units), measurements suggest that the entire oligomer becomes amorphous in water (Figure 5).[92]

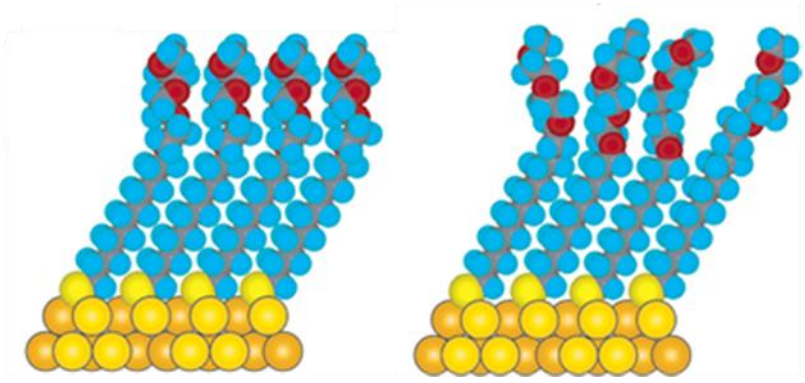

Figure 5. Schematic illustration of the order-disorder transition evidenced by SAMs of alkanethiols terminated with triethylene glycol. Adapted from Love et al.[87]

Another poorly understood parameter is the effect of physiological conditions on the long-term stability of SAMs of alkanethiols. Contact angle analysis performed by Jones et al. revealed instabilities in CH_3 and COOH terminated SAMs upon incubation in serum-free media at 37°C or under dry, room temperature conditions, but not in OH terminated SAMs.[93] Langer and co-workers have shown that SAMs terminated with EG develop substantial defects after immersion in phosphate buffer solution or in calf serum for 4-5 weeks.[94] The presence of cells at the surfaces accelerates the process: the ability of EG-terminated SAMs to prevent the adhesion of cells is compromised in ~7-14 days.[95] One probable mechanism for the loss of resistance in these systems is oxidation of bound thiolates and subsequent desorption.[96]

4.1.4 Characterization of SAMs

There are many techniques available for characterization of SAMs. These techniques are necessarily very surface-sensitive since the amounts of material are very small. Some of them are: X-ray photoelectron spectroscopy (XPS), near edge X-ray absorption fine structure (NEXAFS), static time-of-flight secondary ion mass spectrometry (Tof SIMS), surface probe microscopies (such as SPM or AFM), Fourier transform infrared (FTIR), Raman spectroscopy, sum frequency generation (SFG), high resolution electron energy loss (HREELS), contact angle goniometry, ellipsometry and cyclic voltametry.

The techniques that have been used for characterization of SAMs during the work presented in this thesis, as well as their general analytical capabilities, are presented in Table 1 and briefly described afterwards.

Table 1. Analytical capabilities of commonly used techniques for SAM characterization. Adapted from Liedberg and Cooper[97]

Experimental technique	**Analytical capability**						
	Thickness of SAM	Interfacial tension	Coverage	Chemical composition	Orientation of molecule or group	Alkyl chain density	Roughness, chemical homogeneity
Ellipsometry	++	--	0	--	--	0	0
Contact angle goniometry	--	++	-	0	+	0	+
Infrared spectroscopy	+	--	+	+	++	++	--
X-ray photoelectron spectroscopy	0	--	++	++	+	0	--
Analytical capability: ++ excellent, + good, 0 fair, - poor, -- none.							

Ellipsometry is an optical measurement method based on changes in the polarization state of a light beam after reflection on a substrate and has for a long time been employed for the determination of material optical properties, and for measurements of thicknesses of thin films on reflecting surfaces.[98] This technique is most often used for determining the thickness of SAMs. The ellipsometric readings delta and psi can be correlated to the thickness and the optical constants of the SAM, e.g. the complex refractive index $N=n+ik$, where n is the refractive index, and k the extinction coefficient. For alkanethiolates, the absorption is normally very weak, meaning that k is set to zero and N=n. Thus, for a thiol SAM where n=1.5 (a typical value for an alkyl assembly) and by assuming a three-layer parallel slab model for the gold/SAM/air interface, it is possible to determine the thickness of the film.[86,97,99,100]

Regarding contact angle goniometry, the sessile drop method is perhaps the simplest, and at the same time, the most elegant method for characterizing SAMs. It is a technique where the contact angle (α) of a probing liquid is measured and correlated to the interfacial tensions of the constituting phases. This technique can provide information about the chemical nature of the surface, as well about its morphological properties through the hysteresis, $\Delta\alpha = \alpha_a - \alpha_r$, where α_a and α_r are the advancing and receding contact angles, respectively.[86,97,101]

Infrared spectroscopy provides information about chemical bonds present in a sample and the conformation of the constituent molecules. Due to the dipolar nature of the excitations in the infrared, the technique can also provide information about orientation of molecules and molecular

entities using polarized light. This feature is of particular importance for SAMs since it can be employed to determine chain and functional group orientation on the surface, an information which can be of considerable importance considering biological recognition processes.[97,100]

X-ray photoelectron spectroscopy provides information about the core electronic levels in atoms and molecules. It is the most quantitative method of those described above and has therefore been frequently used to find correlations between the solution and the surface (SAM) composition. It can also provide data of the depth of the SAM, by studying the attenuation of the photo-emitted electrons, at different take-off angles relative to the substrate surface.[97,99]

5. FROM SAMS BACK TO REAL WORD POLYMERS

Though SAMs are the best model system to study the interfacial properties of surface, proteins and cells, they have several limitations that restrict their use in real-world applications. SAMs are fragile owing to their molecular scale thickness, defects in the SAM and the propensity of the chemisorbed thiolate to oxidize. Furthermore, the employment of this technique is limited by the synthesis of terminally functionalized alkanethiols or silanes, as well as the use of restricted substrates such as gold, silver or silicon surfaces. Since a large proportion of medical devices are made from polymeric materials, successful transition of SAM structures to real-world polymers is desirable.

The number of publications addressing the construction of SAMs on polymers is very limited. According to Kwok et al [102], Savig[103] first described the covalent binding of octadecyl trichlorosilane onto spin-cast poly(vinyl alcohol) and later reported on synthesizing correspondent multilayers based on the previous successful results. Bohme et al[104] used bola-amphiphile molecules for the preparation of Langmuir-Blodgett monolayers on the surface of spin-cast poly(allylamine hydrochloride) film. Whitesides and co-workers systematically attempted to form SAMs on oxidized poly(dimethyl siloxane) and polyethylene slab surfaces pretreated with an oxygen plasma[105]. Comparing data from X-ray photoelectron spectroscopy (XPS) and contact angle measurements with the data from a model gold surface, they concluded that dense and ordered monolayers were formed with alkyl chains in an all-trans configuration on these oxidized surfaces. However, their strategy to modify these surfaces involved multistep reactions.

Kwok et al. [102] have expanded the repertoire of methods for preparing SAMs on polymeric biomaterials used in medical devices by constructing SAM structures on poly(2-hydroxyethyl methacrylate) (pHEMA). The chemical structure of pHEMA is represented in Figure 6. Because of the presence of a hydroxyl group in the side chain of the polymer, various modifications of pHEMA using its primary alcohol are possible and provide a wide range of pHEMA derivatives for various biomedical applications. They have derivatized the polymer surface using isocyanate chemistry to form urethane linkages catalyzed by dibutyltin dilaurate.

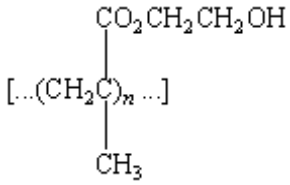

Figure 6 – Chemical structure of pHEMA.

Using this study from Kwok et al. as inspiration, and since the same isocyanate chemistry was used in the studies with SAMs with C18 ligands, pHEMA was chosen in this work as the polymer to which the knowledge obtained using model surfaces could be transposed.

As referred in the beginning of section 3, pHEMA is used as non-fouling polymer. PHEMA is one of the most frequently used synthetic polymers, finding an ever increasing number of biomedical and pharmaceutical applications. The history of pHEMA dates back to 1934, when Woodhouse filed a patent without judging the remarkable water-sorption property.[106] Only two decades after the discovery of Woddhouse were polymers of 2-hydroxyethyl methacrylate (HEMA) recognized as promising biomaterials. The literature is richly documented with extensive studies on pHEMA because of the high water content, non-toxicity, and favorable tissue compatibility, which leads to many biomedical applications.[107,108] These applications include soft contact lenses, drug delivery systems, and kidney dialysis membranes. The presence of a hydroxyl and a carboxyl group on each repeat unit makes this polymer compatible with water, and the hydrophobic methyl groups and backbone impart hydrolytic stability to the polymer and support the mechanical strength of the polymer matrix.[109]

As a hydrogel, pHEMA is a water-swollen macromolecular matrix consisting of polymeric chains insoluble in water at physiological temperature, pH, and ionic strength. The water-absorbing capacity of hydrogels, that swell to an equilibrium value of 10-98% H_2O, is not only responsive to the pH, temperature, and ionic strength of the swelling reservoir, but also to the chemical architecture of the network, such as chain flexibility, crosslinking density, hydrophilicity, osmotic potential, and free volume. The high water content of the hydrogel not only imparts a soft and rubbery texture to the material, but also develops anti-thrombogenicity in the polymer matrix because of the lowered free

energy of the hydrated interface. These physicochemical properties of hydrogels enable them to serve as potential biomaterials in biomedical engineering and related fields.[110]

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CHAPTER III

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Protein adsorption on 18-alkyl chains immobilized on hydroxyl-terminated self-assembled monolayers

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1. ABSTRACT

Surfaces of devices that contact blood accumulate adsorbed and denatured proteins perhaps triggering activation of the coagulation system. A renewable layer of albumin would biologically "passivate" the surface and prevent thrombus formation. Based on the approach of selectively binding albumin to fatty acids, different percentages of a compound with 18 carbons (C18) were immobilized on OH-terminated self-assembled monolayers (SAMs). Fourier transform infrared reflection absorption spectroscopy (IRAS), ellipsometry, contact angle (and surface free energy) and X-ray photoelectron spectroscopy (XPS) measurements were used to characterize these surfaces and proved that there is an efficient immobilization of C18. There is an increase of the thickness and hydrophobicity of SAMs with an increasing percentage of C18. Adsorption of human serum albumin (HSA) was evaluated using radiolabeled ^{125}I-HSA and IRAS. This study showed a gradual increase of HSA adsorption with the increase of surface hydrophobicity. Regarding competitive binding and exchangeability of albumin towards fibrinogen, it was proved, by radiolabelling, that SAMs prepared from solutions with 2.5% C18 presented considerable adsorption in a selective and reversible way.

KEYWORDS: Albumin, competitive protein adsorption, self-assembled monolayers, surface modification, fatty acids, selective adsorption.

2. INTRODUCTION

The interaction of proteins with solid surfaces is a fundamental phenomenon with significance for nanotechnology, biomaterials and biotechnological processes. In the biomaterials field, protein adsorption is the first step in the integration of an implanted device or material with tissue.[1]

Indwelling, blood contacting medical devices should ideally be constructed of materials that do not cause activation of coagulation and immune defence systems. One of the promising ways for preparing materials compatible with blood is to provide a dynamically renewable natural albumin coat between the surface of the device and blood.[2] A thin layer of albumin prevents other blood proteins from adsorbing to the surface and appears to minimise adhesion and aggregation of platelets avoiding subsequent thrombus formation.[3,4]

Human serum albumin (HSA) accounts for about 60% of the total protein in blood serum with a concentration of approximately 40 mg/ml and is the main transport vehicle for long chain free fatty acids (FFA) in the circulation.[3,5] The plasma FFA is composed primarily of 16 and 18 carbon atom fatty acids.[6] Covalently binding 16-18 carbon aliphatic chains to polymer surfaces, has been proposed as a method capable of providing high affinity, reversible, binding sites for albumin.[2] However, this hypothesis has to be investigated further, particularly if long-term protection against adsorption of other proteins, namely fibrinogen, is envisaged.

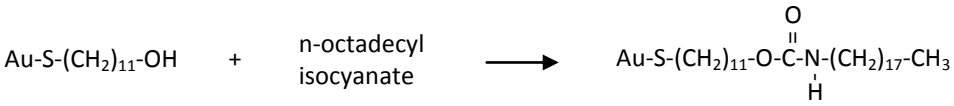
Mechanistic aspects of albumin adsorption to surfaces can be studied at atomic level using self-assembled monolayers (SAMs). SAMs of long alkanethiols on gold have been used in many studies with proteins and cells because they form highly ordered systems, are easy to prepare and enable a wide range of functional groups to be explored.[7] In long-chain alkanethiols, $HS(CH_2)_{n>10}X$, the sulphur moiety of the alkanethiol binds strongly to gold substrata, exposing a functional group (X) to the surface of the monolayer.

The aim of this work is to study the effect of the immobilization of different concentrations of n-alkyl chains with 18 carbons (C18) on the selectivity and reversibility of HSA adsorption.

The strategy of using methyl terminated C18 chains was performed so that albumin could recognize these “fatty acid like” compounds and bind them by its hydrophobic pockets. If there was a

homogeneous layer of methyl groups, albumin would bind to it but would denature and change its conformation. For this not to happen, we spaced the methyl terminated chains so that albumin could identify a "fatty acid like" structure and bind to it in a selective and reversible way, keeping its native shape. However, by doing so, the surface beneath the C18 chains also becomes exposed to proteins. Since gold surfaces adsorb proteins in a non-specific way, we used SH-$(CH_2)_{11}$OH to cover the gold, creating a surface with low affinity to proteins,[8-10] and only then we immobilized some methyl terminated chains with 18 carbons.

Initially, creating a mixed SAM in a single step that would expose 18 carbons to the surface was envisaged. However, it would be necessary to make a thiol solution with SH-$(CH_2)_{11}$OH and a methyl-terminated thiol with 30 carbons (SH-$(CH_2)_{29}CH_3$). Since this last compound is not commercially available, we used a reaction where we introduce the desired functionality into a SAM after its assembly. This method has two steps: (1) preparation of a well-ordered homogeneous SAM of 11-Mercapto-1-undecanol (C11OH) on gold, which has low binding affinity for all proteins; (2) reaction of the hydroxyl groups with the isocyanate group of a compound with 18 carbons (C18), as described in the equation bellow:



SAMs were characterized in terms of molecular structure, thickness, surface energy and elemental composition by Fourier transform infrared reflection absorption spectroscopy (IRAS), ellipsometry, contact angle measurements and x-ray photoelectron spectroscopy (XPS), respectively.

HSA adsorption, competitive adsorption of HSA and human fibrinogen (HFG), and exchangeability of adsorbed HSA for HSA or HFG were studied using radioiodine-labeled albumin. HSA adsorption on C18 SAMs was also studied by IRAS to confirm the results obtained by radiolabelling.

3. MATERIALS AND METHODS

3.1 Gold substrates

To prepare the gold substrates, chromium (5nm) and gold (25nm) films were deposited onto silicon wafers. The thin layer of chromium was used to improve adhesion of gold to silicon. Further details can be found in Martins et al..[10] The wafers were coated with 1.5 μm of photoresist, which is soluble in acetone, to protect the film surface. Gold substrates with 1x1 cm^2 were used for the preparation of all SAMs except for the ones to be used in radiolabelling that had 0.5x0.5 cm^2.

3.2 Monolayer formation

3.2.1 C11OH SAMs

A pure 11-mercapto-1-undecanol ($SH-(CH_2)_{11}OH$; 97%, Aldrich) solution was prepared in ethanol (99,8%, Merck) with a final concentration of 1 mM in a dry nitrogen environment.

Just before being used, gold substrates were cleaned twice in acetone and immersed in "piranha" solution (7 parts concentrated H_2SO_4 and 3 parts 30% H_2O_2) for 5 minutes. Substrates were rinsed sequentially with ethanol, water (distilled and deionised) and ethanol for 2 minutes in an ultrasonic bath. The gold slides were blown dry with a stream of argon, and then immersed in the alkanethiol solution. After incubation at room temperature over 24 hours in a nitrogen environment, the monolayers were washed three times in fresh ethanol in an ultrasonic bath for 2 minutes. Monolayers were blown dry with a stream of argon and maintained in argon until used.[10]

3.2.2 Reaction with C18 compound

Different concentrations (0%, 1%, 2.5%, 5%, 10% and 20%) of pure octadecyl isocyanate ($CH_3(CH_2)_{16}CH_2–N=C=O$; 98%, Aldrich) solutions were prepared in tetrahydrofuran (THF; 99.9%, Aldrich) with 0.1% dibutyltin dilaurate (95%, Aldrich).

The derivatization of the C11OH SAMs using isocyanate chemistry to form urethane linkages was catalysed by dibutyltin dilaurate to avoid the undesirable allophanate side reaction.[11]

The C11OH SAMs were immersed in the octadecyl isocyanate solutions and incubated at 40°C for 2 hours. The best reaction time was previously evaluated using 10% C18 SAMs. Results obtained for 1, 2 and 4 hours of reaction showed that after 2 hours of reaction maximum thickness and

hydrophobicity can be achieved. A 10% C18 SAM without catalyst (dibutyltin dilaurate) was used as a control to ensure that catalyst was needed to enable the reaction between the hydroxyl and the isocyanate.

After incubation, the monolayers were washed twice with THF and once in ethanol for 2 minutes in an ultrasonic bath. SAMs were then blown dry with a stream of argon and maintained in argon until analysed.

This reaction is very sensitive to water and small variations can occur from batch to batch, despite results having always the same trend. Therefore, surface characterization and protein adsorption studies were made with C18 SAMs from the same batch.

3.3 Surface characterization

3.3.1 XPS

Measurements were carried out on a VG Scientific ESCALAB 200A (UK) spectrometer using magnesium Kα (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 (1s), O (1s), S (2p), N (1s) and Au (4f) spectra were collected with an analyser pass energy of 20 eV. The spectra were fitted using an XPS peak fitting program (XPSPEAK version 4.1).

3.3.2 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. In order to ensure that there was no water vapour adsorption, dry nitrogen was purged into the instrument for 5 minutes before and during measurement of each sample. 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, 100 scans were collected with 4 cm^{-1} resolution.

3.3.3 Ellipsometry

Measurements were performed using an Imaging Ellipsometer, model EP^3, from Nanofilm Surface Analysis. This ellipsometer 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 any instrument misalignment. To determine the thickness of the SAMs, the same kind of spectrum was used and *n* and *k* for the organic layer were set as 1.45 [12] and zero, respectively. Results are the average of 3 measurements on each of the 3 samples for each type of SAM.

3.3.4 Contact angle and surface energy

In order to ensure the presence of well-formed SAMs and to evaluate their surface energy, the contact angles of water (with conductivity not higher than 1 μS/cm) and methylene iodide (>99%, Sigma-Aldrich) droplets formed on the SAMs were measured by 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 be used in order to prevent evaporation from the drop. Deposition of 4μl drops was made using the sessile drop method. Droplet profiles were fitted using different mathematical functions in order to calculate the contact angle. The Young-Laplace fitting method was used to calculate contact angles higher or equal to 90°, the ellipse fitting method for contact angles between 90 and 30° and the tangent fitting method for contact angles lower than 30°. The contact angle of each SAM was calculated by extrapolating the time dependent curve to zero. Results are the average of 3 samples for each type of SAM. The polar component (γ_s^h) and the dispersive component (γ_s^d) of the surface energy of the SAMs were evaluated by the application of the Owens and Wendt Method [13]. 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.[14]

3.4 Protein adsorption

3.4.1 Radiolabelling with ^{125}I

A 0.1 mg/ml HSA solution was prepared by dissolving HSA (Sigma, ref. A1653) in degassed phosphate buffered saline (PBS) (Sigma, pH 7.4, lot 082K8215) with 0.01 M of NaI – iodinated PBS (PBSI). Buffer with iodide (PBSI) was used to inhibit adsorption of free radioactive iodide based on the Horbett et

al.[15] observation that the addition of small amounts of non-radioactive iodide to the protein solution effectively suppresses the binding of $^{125}I^-$ ion to surfaces. This fact was also observed by us on gold surfaces (data not shown).

Quantification of adsorbed HSA to different monolayers was performed using ^{125}I-HSA. HSA was labeled with ^{125}I according to the iodogen method where iodination was performed at 0°C in an Eppendorf tube coated with Iodo-gen (Sigma) and evaporated with a gentle flow of nitrogen.[16,17] Purification of ^{125}I-HSA to remove free iodine was performed using Sephadex G-25 M columns (PD-10, Amersham Pharmacia biotech). The yield of the iodination reaction was approximately 93%, determined by precipitating the ^{125}I-HSA with 20% trichloroacetic acid (TCA) (Merck). ^{125}I-HSA was added to unlabeled HSA solution in order to obtain a final activity of $2x10^8$ 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 PBSI 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 minutes period. Sigal et al. and Silin et al. have carried out albumin kinetics studies on SAMs with different functional groups, namely OH- and CH3-terminated SAMs, and have demonstrated that an adsorption time of 30 min is enough to reach an adsorption plateau, even when concentrations of proteins < 0.1 mg/ml are used.[8,18] After rinsing the samples four times with 2 ml of PBS, the γ-activity was counted. Results are the average of three samples. HSA surface concentration was calculated by the following equation:

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

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).

Competitive adsorption of HSA and HFG onto the different SAMs was performed using different ratios of ^{125}I-HSA and unlabeled HFG. The concentration of ^{125}I-HSA was kept at 0.1 mg/ml and the concentration of HFG was 0.01 or 0.1 mg/ml. Results are the average of three samples.

Exchangeability tests were carried out by immersing the SAMs with adsorbed labeled HSA in an unlabeled HSA pure solution (1 mg/ml, 25°C) or an HFG (Sigma, ref. F4129) pure solution (1 mg/ml, 25°C) over 24 hours. Samples were then washed four times with 2 ml PBS and residual radioactivity counted. Results are the average of three samples.

3.4.2 IRAS

A 0.1 mg/ml HSA solution was prepared by dissolving HSA in PBS.

For the HSA adsorption tests, SAMs were placed in a 24-well tissue culture plate (Sarsted) with the surface facing up. 500 μl of HSA solution were added to each well and protein was left to adsorb for 30 minutes at 25°C. SAMs were then rinsed four times with 2 ml of PBS, dried with a stream of argon and kept in a nitrogen environment until analysed.

IRAS measurements were made as in the surface characterization section, above, except for the removal of water vapour where, instead of being purged with dry nitrogen for 5 minutes, SAMs were placed in a vacuum chamber overnight.

4. RESULTS

4.1 Surface characterization

By XPS, the relative atomic composition (atomic %) of SAMs was calculated. Survey spectra (data not shown) suggested the absence of contaminants since no other elements than the expected ones were found. No detectable intensity was present in the binding energy region above 164 eV of any SAMs studied, indicating that no unbound thiol molecules are present (data not shown). Figure 1 shows the results obtained by XPS. 0% C18 SAMs (C11OH SAMs) indicate that only C, O and S are part of their chemical configuration. The presence of N (1s) on SAMs prepared with solutions of 1-20% of C18 indicates the immobilization of the isocyanate on C11OH SAMs. The atomic percentage of N (1s) gets higher as we increase the percentage of C18 reaching a plateau at 5% C18. The atomic percentage of C (1s) decreased in the following order: 20% ≅ 10% > 5% > 2.5% > 1% ≅ 0% C18 SAMs.

XPS high resolution spectra of sulphur region demonstrated that S (2p) spectra were well fitted with a doublet structure centered at 162.1 and 163.3 eV and a peak-to-area ratio of 2:1, as described by Castner et al..[19] S (2p) percentage should be roughly the same for all SAMs since octadecyl isocyanate does not have this element in its constitution. However, there is a small decrease of the relative percentage of S (2p) with the increase of C18 related to signal attenuation of the S at the base of the film by the increased chain length. The percentage of O (1s) is higher in 0% and 1% C18 SAMs than in the others. This was somewhat unexpected since there is an incorporation of this element during C18 immobilization. This is believed to occur because in 0% and 1% C18 SAMs, O (1s) was situated on the outermost side of the monolayer and its signal is unattenuated by a C18 overlayer.[20]

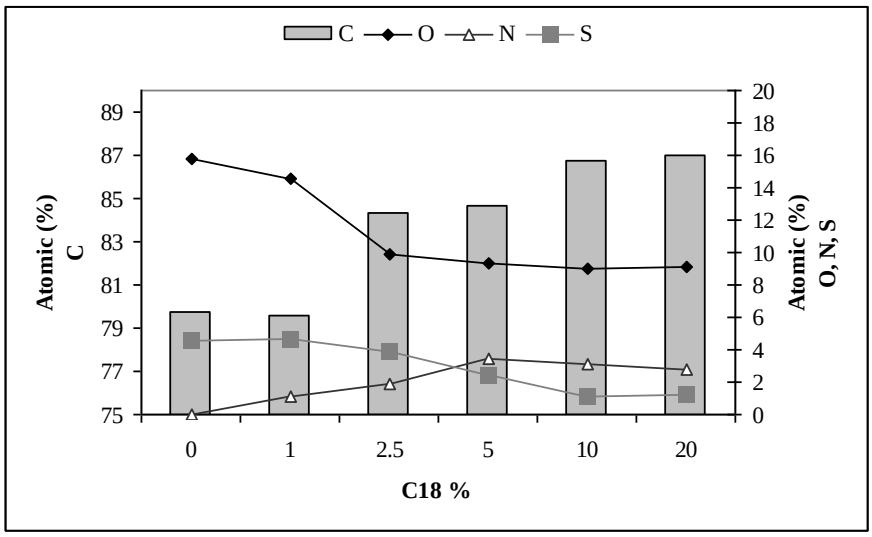


Figure 1 - Relative atomic composition (%) of C18 SAMs with 2 hours of reaction without gold percentages.

Figure 2 shows the C-H stretching vibrations from 3000 to 2800 cm^{-1} for SAMs with different percentages of C18. At 2920 cm^{-1} and 2850 cm^{-1} one can observe the asymmetric (ν_a) and symmetric (ν_s) CH_2 stretching modes, respectively. The peak at 2965 cm^{-1} corresponds to the CH_3 asymmetric stretching mode (ν_a). CH_3 symmetric mode (ν_s) has one peak at 2938 cm^{-1} and should have another at 2878 cm^{-1}. The peak at 2888 cm^{-1} might correspond, with a slight deviation, to this second symmetric CH_3. None of the analysed spectra presented the characteristic peak from isocyanate group (-N=C=O) at 2270 cm^{-1} (data not shown), indicating the absence of unbound octadecyl isocyanate.

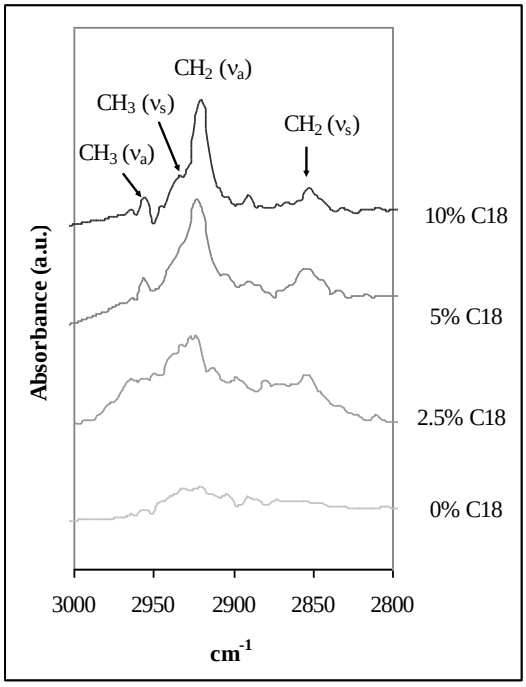


Figure 2 - IRAS spectra for C-H stretching vibrations near 3000-2800 cm^{-1} of 0-10% C18 SAMs with 2 hours of reaction.

Ellipsometry measurements were made using gold substrates with a refractive index of 0.4632 and an extinction coefficient of 2.3171. These values were used to calculate the thickness of each monolayer. Figure 3 shows the thickness of SAMs with and without octadecyl isocyanate on them. One can observe that 0% and 1% C18 SAMs have a thickness of approximately 1.4 nm, but as we increase the percentage of C18, SAMs get thicker to a maximum of 3.01 nm in 20% C18. Comparing 10% C18 SAMs with and without catalyst, the first one is two times thicker, showing the importance of the catalyst.

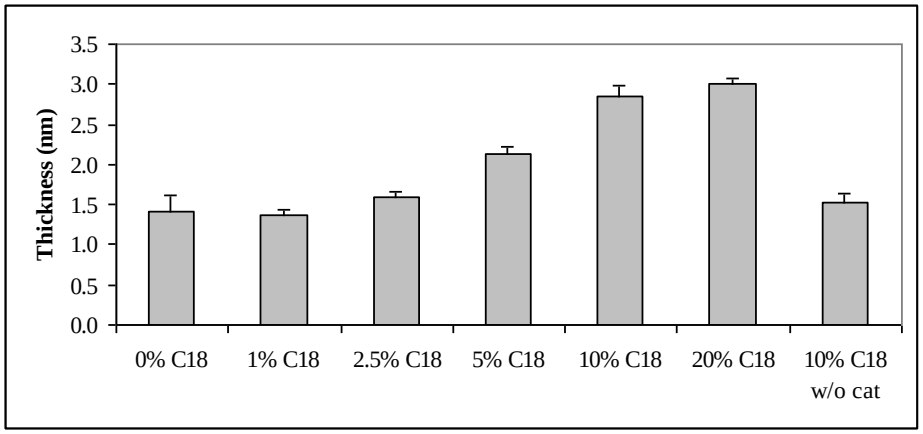


Figure 3 - Thickness of C18 SAMs with 2 hours of reaction determined by ellipsometry.

Table 1 shows the results of water and methylene iodide contact angles of SAMs. The total surface free energy (more accurately, an approximation of the total surface free energy) and its components (polar and dispersive) are also presented in this table. SAMs range from hydrophilic (θ_w = 25.1°) in 0% C18 SAMs to hydrophobic (θ_w = 99.6°) in 20% C18 SAMs. The 10% C18 SAMs without catalyst have a contact angle similar to 1% C18 SAMs. The total surface free energy values decrease with the increase of the percentage of C18 indicating that SAMs with –OH functionalities yield much higher surface energy than those terminated with methyl groups. For 20% C18 SAMs, the dispersive component reaches 94% of the total surface energy, whereas for 0% C18 SAMs the polar component is about 57% of the total surface energy.

Table 1 - Contact angles and surface energy of 0-20% C18 SAMs with 2 hours of reaction

	Contact angle (°)		**Surface free energy (mN/m)**		
	Water	**Methylene iodide**	**Dispersive**	**Polar**	**Total**
0% C18	25.1 ± 1.5	38.6 ± 2.1	40.0	30.2	70.2
1% C18	36.8 ± 0.8	39.6 ± 1.5	39.5	24.9	64.4
2.5% C18	53.1 ± 0.4	45.4 ± 2.7	36.6	17.1	53.6
5% C18	83.1 ± 1.3	52.1 ± 2.6	32.9	3.7	36.5
10% C18	91.3 ± 1.6	73.2 ± 0.6	21.0	3.8	24.7
20% C18	99.6 ± 1.7	72.0 ± 1.1	21.6	1.4	23.0
10% C18 w/o cat	36.1 ± 1.4	39.3 ± 0.3	39.7	25.2	64.9

4.2 Selective binding of albumin

Albumin adsorption on SAMs was tested by radiolabeling and IRAS.

The amide group of proteins and polypeptides shows characteristic vibrational absorption modes in the infrared. Amide I (1690 ± 45 cm^{-1}) primarily represents the C=O stretching vibrations and amide II (1540 ± 60 cm^{-1}) represents the N-H in-plane deformation coupled with C-N stretching modes.[21] IRAS spectra (amide I bands) of surface adsorbed HSA were correlated with concentration measurements determined by ^{125}I-HSA, as shown in Figure 4. With the increase of C18 ligands immobilized on SAMs, there is a linear increase of the absorbance of amide I band and of ^{125}I-HSA adsorption. In radiolabelling results, the contact drop diameter used in the calculations of the surface area range from 3.2 to 4.5 mm, depending on the C18 percentage on the SAM surface. Figure 4 also showed that SAMs with 0% C18 presented the lowest HSA adsorption with a concentration of 1.3 ± 0.1 mg/m^2. In SAMs with octadecyl isocyanate, HSA adsorption increased from 1.6 ± 0.1 mg/m^2 (1% C18) to 3.5 ± 0.2 mg/m^2 (10% C18).

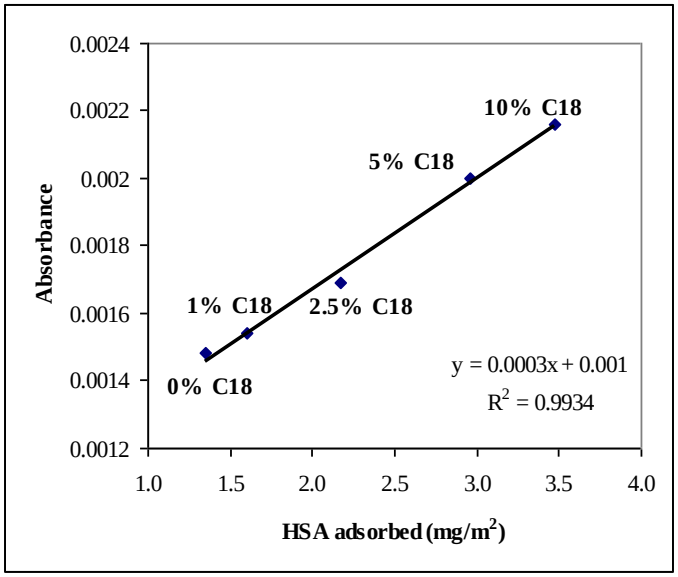


Figure 4 - Correlation between amide I (1664 cm-1) band intensities, measured using IRAS, and the amount of HSA adsorbed on C18 SAMs, measured using 125I-HSA.

Table 2 shows the competitive adsorption of HSA and HFG on the different C18 SAMs. The concentration of ^{125}I-HSA was kept at 0.1 mg/ml and two different concentrations of HFG were tested: 0.01 and 0.1 mg/ml. Calculations were performed considering that 100% adsorption corresponds to the concentration of HSA adsorbed from HFG-free solution (presented in Figure 4). Fibrinogen was used as a competitive protein because it is known to readily adsorb to implant surfaces and because surfaces that avidly adsorb fibrinogen may be thrombogenic. Table 2 demonstrates that when HFG is present in 0.01 mg/ml, the approximate proportion relative to albumin that is present in blood, ^{125}I-HSA adsorption on 2.5% and 5% C18 SAMs is practically as high as when only HSA is present, specially on 2.5% C18 SAMs, suggesting that these surfaces have low binding affinity to HFG. When HSA and HFG are present in the same ratio, the competitive effect of HFG is lower on 2.5% and 5% C18 SAMs since the HSA adsorption only decreases ~40%, and higher on 10% C18 SAMs, that proved to have higher affinity for fibrinogen since a decrease of ~65% on albumin adsorption is observed.

Table 2 - Competitive adsorption of HSA and HFG to SAMs with different percentages of C18 (25°C; 30 min)

	C18 SAMs				
HFG (mg/ml)	**0%**	**1%**	**2.5%**	**5%**	**10%**
0	100 ± 16	100 ± 12	100 ± 6	100 ± 12	100 ± 10
0.01	82 ± 13	87 ± 10	97 ± 6	91 ± 9	78 ± 10
0.1	39 ± 5	49 ± 5	63 ± 3	61 ± 12	34 ± 6

The concentration of ^{125}I-labeled HSA was kept at 0.1 mg/ml and two different amounts of HFG were tested: 0.01 and 0.1 mg/ml. Calculations were performed considering as 100% adsorption the concentration of HSA adsorbed using a pure albumin solution.

4.3 Reversible binding of albumin

Exchangeability of bound albumin was evaluated by the exchange of the pre-adsorbed ^{125}I-HSA for unlabeled HSA or HFG. Figure 5 shows the percentage of HSA retention onto the different SAMs after soaking in HSA or HFG solutions (0.1 mg/ml) for 24 hours. Although retention of ^{125}I-HSA gets higher as we increase the amount of C18 in SAMs, retention values are always lower when SAMs are washed with HSA then when they are washed with HFG. These results indicate that until 2.5% of C18, HSA is reversibly bound to SAMs and is easily exchanged for HSA but also almost as easily exchanged for HFG.

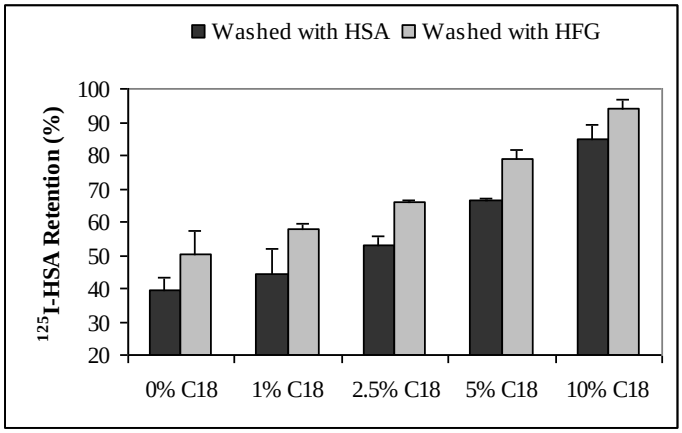


Figure 5 - Retention of ^{125}I-HSA previously adsorbed to C18 SAMs, after washing with unlabeled HSA or HFG solution (1 mg/ml; 25°C; 24 h).

5. DISCUSSION

This work used SAMs of alkanethiols on gold as model surfaces to study the effect of different percentages of an n-alkyl compound with 18 carbons (C18) on albumin adsorption.

Regarding surface characterization, XPS results confirm the immobilization of octadecyl isocyanate (C18) on C11OH SAMs. Nitrogen is a good marker of the presence of C18, since this element is not present on the C11OH SAMs. The observed increase of atomic percentage of nitrogen and carbon is associated with the increase of C18 on the surface. Results obtained for 10 and 20% C18 SAMs are similar to the expected composition of SAMs completely covered by C18 (88% C, 6% O, 3% S and 3% N) calculated from the stoichiometry of the thiols used. The decrease of sulphur can be explained because its elemental concentration in the SAMs is low, and photoelectrons originating in S (2p) are inelastically scattered by interaction with the alkyl chains.[22] For 0% C18 SAMs (C11OH SAMs), the O (1s) atomic percentage (15.7%) was higher than the theoretical value (7.7%). This is believed to occur because O (1s) was situated on the outermost side of the monolayer.[20]

Results from IRAS measurements showed that there is a noteworthy increase of the intensity of the peaks that correspond to CH_2 groups as the percentage of C18 increases. This is indicative of the immobilization of octadecyl isocyanate on C11OH SAMs. The absence of the characteristic peak from isocyanate group (-N=C=O) indicated that there is no unbound C18 compound.

IRAS is a convenient method of checking a SAM for well-ordered and crystalline structure. The CH stretching vibrations of the alkyl chain are very sensitive to packing density and to the presence of gauche defects, which makes them ideally suited as probes to determine SAM quality. In particular, the asymmetric CH_2 stretching vibration (ν_a) at ~2918 cm^{-1} is a useful indicator; its position varies from 2916 or 2917 cm^{-1} for SAMs of exceptional quality, to 2918 cm^{-1} which is the normal value for a high-quality SAM, to ~2926 cm^{-1} which is indicative of a heavily disordered, "spaghetti-like" SAM.[23] In the results obtained, 10% C18 SAMs presented the CH_2 (ν_a) vibration at 2919 cm^{-1}, suggesting that for this SAMs the immobilized C18 alkyl chains are well ordered and densely packed, confirming the results obtained by Kwok et al.[11] As expected, SAMs with 5% and 2.5% C18 have the CH_2 (ν_a) vibration at 2920 and 2921 cm^{-1}, respectively, indicating a small disorder of the C18 chains due to the lower degree of immobilization. CH_2 (ν_a) vibration was not well detected on 0% C18 SAMs since there is not a good definition between CH_2 (ν_a) and CH_3 (ν_s) vibrations for the shorter monolayers. However, these results also demonstrate the success of the immobilization once the observed decrease of definition with decrease of C18 can be attributed to the decrease of the thickness of the monolayer due to the lack of C18 groups as shown by ellipsometry (Figure 3).

Porter et al. [24] related the thickness of SAMs with different number of carbons of the alkyl chain (n). They showed that long chain thiols ($CH_3(CH_2)_{n>9}SH$) assume a tilt of 20-30° from the surface normal and that by using an average tilt of 25°, ellipsometric measurements for the region between n=9 and n=21 are linear and give a slope of 0.11 nm per CH_2 group and an intercept of 0.51 nm. When this equation is extrapolated to our layers, 0% C18 SAMs, which have 11 CH_2 groups, should have, in theory, a thickness of 1.7 nm. The thickness of 1.5 nm that we obtained is therefore close to the theoretical values. Regarding 10% and 20% C18 SAMs, which have a homogeneous layer of 28 CH_2 groups, their theoretical thickness is 3.6 nm plus the thickness of the urethane group (-O-CO-NH-). The results obtained are therefore smaller than expected since these types of SAMs only presented a thickness of ~3 nm.

Contact angle measurements indicate that there is an increase of hydrophobicity with increase of the immobilization of C18 on C11OH SAMs. The water contact angle of 25.1° obtained for 0% C18 SAMs (C11OH SAMs) is similar to 26.1° obtained by Ahn et al..[25] Other authors have reported a water contact angle of 110° for CH3-terminated SAMs [20,25] but our values are lower (99°).

Surface energy results are in agreement with those obtained by Ahn et al. [25] where their C11OH and our 0% C18 SAMs both presented a surface energy of 70.2 mN/m. Comparing their 1-octadecanethiol SAMs (CH_3 terminated SAMs) with our 20% C18 SAMs, results were of 22.0 and 23.0 mN/m, respectively.

All the techniques used for surface characterization showed that there is not a significant difference between 10% and 20% C18 SAMs. Therefore, 20% C18 SAMs were not used to study protein adsorption.

Ellipsometry, contact angle measurements and IRAS studies showed that 10% C18 SAMs have lower thickness and contact angle but are as well packed as CH3-terminated SAMs described in literature. This suggests that, for this surface, some C18 chains may be folded.

Regarding protein adsorption, both radiolabeling and IRAS, have shown a higher HSA adsorption on more hydrophobic surfaces, as described by other authors.[8,10] As shown in Figure 4, intensities of amide I bands from albumin increase gradually with increasing C18 on SAM surfaces. The amide I increase is linearly correlated with the increase of the concentration of HSA adsorbed detected using ^{125}I-HSA. Values of 1.3 ± 0.1 mg/m^2 and 3.5 ± 0.2 mg/m^2 for HSA adsorption on 0% C18 (C11OH) and 10% C18 SAMs are in accordance with results described elsewhere for OH- and CH3-terminated SAMs.[10]

To improve hemocompatibility of the surface, a selective but reversible adsorption may provide a dynamic, renewable, natural albumin coat on the surface.

Competitive adsorption studies in Table 2 show that when HSA and HFG are together in solution, 2.5% and 5% C18 SAMs are the surfaces with higher selectivity for HSA, despite HFG concentration. In accordance with our results, Sigal et al. [8] have demonstrated that although HFG adsorption is higher on pure CH3-terminated SAMs, it also adsorbs to pure OH-terminated SAMs once the hydroxyl group is able to form hydrogen bonds with side-end groups of polar protein amino acids.

Regarding protein exchangeability, after washing with solutions of HSA and HFG, retention is higher on the most hydrophobic surfaces (5% and 10% C18 SAMs), as shown in Figure 5. The loss of exchangeability of the protein after adsorption is in accordance with other authors who describe an irreversible adsorption of HSA on hydrophobic surfaces, possibly from surface-induced conformational changes.[26] Retention of ^{125}I-HSA on more hydrophilic surfaces (0-2.5% C18 SAMs) was

low but not 0%, suggesting that some protein may adsorb randomly and not to the hydrophobic pockets. Although the competitive studies demonstrated that 5% C18 SAMs have similar selectivity to albumin as 2.5% C18 SAMs, this surface (5% C18) presents higher retention of HSA after adsorption.

Exchangeability with HFG is similar but always smaller than exchangeability with HSA. This can be due to SAMs having more affinity for albumin than for fibrinogen or because of the difference of sizes of these two proteins. HSA is a globular protein with a molecular weight of 66438 daltons whereas HFG is a much bigger protein of 340000 daltons.[27] This difference of sizes may be sufficient for albumin but not fibrinogen to fit in the spaces that ^{125}I-HSA was occupying before being desorbed.

The composition of the adsorbed protein layer from blood or plasma changes with time (usually in the first minutes of adsorption). The high concentration proteins take over the surface at short times due to the higher collision rates. As time passes, several exchange processes may occur and proteins with higher surface affinity dominate the surface, even if their bulk solution concentration is very low.[28] This sequential displacement of proteins adsorbing from plasma (or a mixture of proteins in solution) is referred by Brash as the Vroman effect.[29]

In the present work, we verified that SAMs with 2.5% of C18 increase albumin adsorption without increasing fibrinogen adsorption. Furthermore, this density of grafted 18-alkyl chains also allowed reversibility of albumin binding, and will possibly improve the long time compatibility between the material and blood. For these reasons, we suggest that the Vroman effect can, to some degree, be controlled by careful surface modifications.

The preparation of these SAMs with oligo(ethylene glycol) instead of C11OH is now being tested since OH-terminated SAMs are not 100% resistant to non-specific protein adsorption.

6. CONCLUSIONS

All the techniques used for surface characterization demonstrated that there is an efficient immobilization of C18 on OH-terminated SAMs. There is an increase of the thickness and hydrophobicity of the SAMs with the increasing percentage of C18. SAMs with different percentages of C18 on the surface were obtained.

The surface free energy of the SAMs decreased with the increase of C18 percentage in solution, therefore SAMs with –OH functionalities yield much higher surface energy than those terminated with methyl groups.

Concerning protein adsorption, there is an increase of HSA adsorption as the surface becomes more hydrophobic due to the increase of immobilized C18 ligands.

Within all surfaces prepared, SAMs obtained from solutions with 2.5% of C18 presented considerable albumin adsorption in a selective and reversible way. This demonstrates the relevance of the concentration of the immobilized compound for the selective capacity of the surface.

7. ACKNOWLEDGEMENTS

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CHAPTER IV

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Selective protein adsorption modulates platelet adhesion and activation to oligo(ethylene glycol)-terminated self-assembled monolayers with C18 ligands

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1. ABSTRACT

This study focuses on the selective binding of albumin to a surface in order to inhibit other blood proteins from adsorbing thereby reducing platelet adhesion and activation. Tetra (ethylene-glycol)-terminated self-assembled monolayers (EG_4 SAMs) with different percentages of C18 ligands on the surface were characterized by contact angle measurements, X-ray photoelectron microscopy, infrared reflection-absorption spectroscopy and ellipsometry. A specific surface (2.5%C18 SAM) was found to be selective for human serum albumin (HSA) in the presence of both albumin and fibrinogen (HFG). The importance of this concentration of C18 ligands was stressed in reversibility studies since that surface exchanged almost all the pre-adsorbed HSA by HSA in solution, but not by HFG. The effect of protein adsorption in the subsequent adhesion and activation of platelets was studied by pre-immersing the surfaces in albumin and plasma before contact with platelets. Scanning electron microscopy and glutaraldehyde induced fluorescence technique images showed that as surfaces got more hydrophobic due to the immobilization of C18 ligands, the number of adherent platelets increased and their morphology changed from round to fully spread. Pre-immersion in HSA led to an 80% decrease in platelet adhesion and reduction of activation. Pre-immersion in 1% plasma was only relevant in 2.5%C18 SAMs since this was the only surface that demonstrated less adhesion of platelets comparing with buffer pre-immersion. However, they still adsorb more platelets then when HSA was pre-adsorbed. This was confirmed in competition studies between HSA and plasma that suggested that other plasma proteins were also adsorbing to this surface.

KEYWORDS: self-assembled monolayers, selective protein adsorption, albumin, platelets, biomaterials.

2. INTRODUCTION

Surface molecular engineering approaches are being increasingly deployed as tools to understand and control protein adsorption and cell-surface interactions with the aim of rational design of surfaces for biological applications.[1]

Blood contacting medical devices are used for applications where good performance over extended periods of time is required, but also outside of living organisms for short periods of time. Examples where long term contact is required include left ventricular assist devices, heart valves and vessel prostheses. A few short duration applications are blood purification devices and blood catheters. In all cases, the devices must meet the requirement of low or negligible activation of the coagulation system and no clot formation.[2]

When in direct contact with blood, presently used blood contacting polymers initiate the formation of clots, as platelets and other components of the blood coagulation system are activated. It is well known that 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.[2] 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.[3]

Since albumin-coated surfaces have been found to reduce platelet adhesion to synthetic materials,[4] research has been conducted to create surfaces that attract and bind albumin from the bloodstream in a selective way.[5] Based on the approach of selectively binding albumin to long chain fatty acids, 16 or C18 carbon alkyl groups have been immobilized onto different polymer surfaces.[5-8] However, these early works have not considered the influence of the concentration of C18 ligands nor studied the reversibility of albumin binding, and in some cases protein denaturation was verified.

In order to prepare surfaces with specificity to one protein, non-specific interactions should be inhibited. One strategy to avoid protein denaturation while promoting reversible binding, is to bind the specific attractant onto non-fouling polymers, such as poly(ethylene glycol) (PEG), also called poly(ethylene oxide) (PEO). PEO can prevent plasma protein adsorption, platelet adhesion, and thrombus formation and therefore has been widely used in biomaterials science.[2] The non-fouling properties of PEO are attributed to a combination of qualities: steric repulsion by the mobile strands,

caging (detention) of water molecules by the ethylene oxide repeat unit, the absence of sites that can participate in long-range attraction of charged groups or short-range attraction of hydrophobic groups, and the inability of proteins to 'melt' structured water on PEO.[9]

To promote albumin adsorption in a reversible way and avoid denaturation of bound proteins, Ji et al. have used a sandwich surface architecture, which is composed by a hydrophobic matrix, covered by a layer of flexible hydrophilic PEO and then by hydrophobic 18 alkyl chains.[10] However, when copolymers are used with low concentrations of C18 ligands and/or under physiological environment, the PEO-C18 chains may rearrange, moving the C18 ligands into the interior of the hydrophobic matrix, away from the albumin, misleading the effect of C18 alkylation.

Self-assembled monolayers (SAMs) of alkanethiolates on gold represent a class of substrates that allow precise control of the structure, density and pattern of immobilized ligands.[11] SAMs prepared from alkanethiols terminated in short ethylene glycol oligomers ($HS(CH_2)_{11}(OCH_2CH_2)_{2<n>7}OH$) are resistant to protein adsorption and can be easily derivatized through their hydroxyl terminal groups.[12] The $HS(CH_2)_{11}$– moiety provides a foundation for stable SAMs, and the oligo(ethylene glycol) headgroups should be substantially disordered in order to have non-fouling properties.[13] The advantage of using SAMs instead of polymers to study the effect of the concentration of C18 alkylation on albumin selective and reversible adsorption would be that, even though the short EG moiety is loosely packed, the C18 ligands cannot rearrange away from the adsorbing protein.

Previous studies using different percentages of 18 carbon alkyl chains (C18) as ligands to albumin have already been performed in SAMs. Results using 11-mercapo-1-undecanol (C11OH) SAMs as the background demonstrated that not only the ligand, but also its concentration is important to adsorb albumin in a selective and reversible way.[14] In the present study, oligo (ethylene glycol)-terminated SAMs (EG_4 SAMs) were used as the background in order to increase the reversibility (displaceability) of HSA adsorption noted in the previous research. Selectivity and reversibility of HSA adsorption were studied in the presence of human fibrinogen (HFG), used as a competitive protein, and plasma. Furthermore, the selective and reversible adsorption of HSA was correlated to the adhesion and activation of blood platelets.

3. MATERIALS AND METHODS

3.1 Preparation of gold substrates

Gold substrates were prepared, protected and cleaned as described in Martins et al..[15] Samples with dimensions 0.5x0.5cm^2 were used in protein adsorption studies, 2.3x5.4cm^2 samples were used for IRAS measurements and 1x1cm^2 samples were used in all other surface characterization techniques and in platelet studies.

3.2 Preparation of EG_4-C18 SAMs

1-Mercapto-11-undecyl tetra(ethylene glycol) thiol (EG_4 thiol) was synthesized and purified as previously described in Martins et al.[11]

A 0.1 mM EG_4 solution was prepared in a dry nitrogen environment using ethanol (99,8%, Merck) as solvent. Just before being used, gold substrates were cleaned twice in acetone and immersed in "piranha" solution (7 parts concentrated H_2SO_4 and 3 parts 30% H_2O_2) for 5 minutes. They were then rinsed sequentially with ethanol, water (distilled and deionised) and ethanol for 2 minutes in an ultrasonic bath. The gold slides were blown dry with a stream of argon, and then immersed in the EG_4-thiol solution. After incubation at room temperature over 24 hours in a nitrogen environment, the monolayers were rinsed three times in fresh ethanol in an ultrasonic bath for 2 minutes. Monolayers were blown dry with a stream of argon and maintained in argon until used.

EG_4 SAMs were immersed in solutions with different percentages (0%, 2.5%, 5% and 10%) of octadecyl isocyanate (C19H37NO; 98%, Aldrich) as described in a previous work.[14] The chemical composition of the SAMs is presented in Table 1.

Table 1 - Composition of the self-assembled monolayers

Abbr. of the SAM	Chemical formula
EG_4 SAMs	Au-HS$(CH_2)_{11}(OCH_2CH_2)_4OH$
EG_4-C18 SAMs	Au-HS$(CH_2)_{11}(OCH_2CH_2)_4O$-(C=O)-N-$(CH_2)_{17}$-$CH_3$

3.3 Surface Characterization

The surface of EG_4-C18 SAMs was examined by different surface characterization techniques. X-ray photoelectron spectroscopy (XPS) was used to obtain information on the chemical composition of the surface. Contact angle measurements were performed to study the hydrophilicity, hysteresis and surface energetics. Fourier transform infrared reflection absorption spectroscopy (IRAS) was used to investigate the chain order and crystalline structure. Finally, the thickness of the surface was measured by ellipsometry.

3.3.1 Contact angle, surface energy and hysteresis

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. 4μl drops were deposited using 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.[16] 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.[17]

The contact angle hysteresis is the difference between the advancing and the receding contact angle. The needle-in sessile drop type method used to measure the hysteresis consisted of increasing the volume of the drop (up to 6μl in 1 minute) by use of a monitored syringe, forcing the drop to advance on the solid surface, and by decreasing the volume of the drop, forcing the drop to recede, and making the appropriate measurement.

Droplet profiles were fitted using different mathematical functions in order to calculate the contact angle. The Young-Laplace fitting method was used to calculate contact angles higher or equal to 90°, the ellipse fitting method for contact angles between 90 and 30° and the tangent fitting method for contact angles lower than 30°. The contact angle of each SAM was calculated by extrapolating the time dependent curve to zero. Results are the average of at least 3 samples for each type of SAM.

3.3.2 XPS

Measurements were carried out on a VG Scientific ESCALAB 200A (UK) spectrometer using magnesium Kα (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 (1s), O (1s), S (2p), N (1s) and Au (4f) spectra were collected with an analyser pass energy of 20 eV. The binding energy (BE) scales were calibrated by setting the $Au4f_{7/2}$ BE to 84.0 eV. The spectra were fitted using an XPS peak fitting program (XPSPEAK version 4.1).

3.3.3 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. In order to minimize water vapor adsorption, dry nitrogen was purged into the instrument for 5 minutes before and during measurement of each sample. 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.

3.3.4 Ellipsometry

Measurements were performed using an Imaging Ellipsometer, model EP^3, from Nanofilm Surface Analysis. This ellipsometer 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 [18] and zero, respectively. Results are the average of 3 measurements on each of the 3 samples for each type of SAM.

3.4 Protein adsorption

3.4.1 Selective and competitive adsorption

To evaluate the selectivity of EG_4-C18 SAMs towards human serum albumin (HSA – Sigma, ref. A1653), two competitive studies were performed:

- between HSA and HFG: HSA (0.1mg/ml) and HSA (0.1mg/ml) + HFG (0.01mg/ml)
- between HSA and plasma: HSA (0.3mg/ml) and 1% plasma (that has ≈0.3mg/ml HSA)

Quantification of adsorbed HSA was made using the radiolabeling technique with ^{125}I as described in previous studies [14], and 10% of ^{125}I-HSA was added to each of the four final protein solutions. Protein solutions were prepared by dissolving HSA (Sigma, ref. A1653), HFG (Sigma, ref. F4129) or human plasma (kindly provided by the Portuguese Blood Institute) in phosphate buffered saline (PBS) (Sigma, pH7.4).

A 7 μl drop of protein solution was pipetted onto each SAM and left to adsorb at room temperature over a 30 minute period. Three drops of PBSI were added to the periphery of each well to maintain moisture so that the 7μl drop of protein solution would not dry. After rinsing the samples four times with 2 ml of PBS, the radioactivity was measured in a γ-counter.

HSA surface concentration was calculated by the following equation:

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

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).

3.4.2 Reversibility of adsorption

Albumin that is reversibly bound to a surface by association with a fatty acid should be displaceable upon the addition of exogenous albumin, whereas irreversibly bound albumin should not be displaced. We therefore used the SAMs which had been pre-incubated for 30 minutes with ^{125}I-HSA

(0.1mg/ml) and incubated them at room temperature for 24 hours in PBS, HSA (1mg/ml) or HFG (1mg/ml) solutions to study the exchangeability of the pre-adsorbed HSA. Samples were then rinsed four times with 2 ml PBS and residual radioactivity counted.

3.5 Platelet adhesion and activation

Whole blood (plasma, platelets, leucocytes and erythrocytes) obtained from non-medicated healthy donors was collected to a bag with CPDA1 anti-coagulant (Macopharma) and processed at the Portuguese Blood Institute. Buffy coat (platelets and leucocytes) was obtained from centrifugation of the total blood unit at 4997 g (3900 rpm) for 10 minutes at 22°C. The platelet concentrate (PC) is the supernatant obtained by centrifugation of the buffy coat at 328 g (1000 rpm) for 10 minutes at 22°C. The PC rested for 1 hour 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 a concentration of $3x10^8$ platelets/ml was used.

In order to avoid platelet activation by the polystyrene of a 24-well plate (Sarstedt), all the wells were filled with 1% (w/v) BSA in PBS, incubated for 1 hour at 37°C and rinsed 5 times with PBS. SAMs were then pre-immersed in PBS, HSA (0.1mg/ml) or plasma (1% in PBS) for 30 minutes at room temperature and rinsed 3 times with PBS. Next, PC was added to each sample and was incubated for 30 minutes at 22.4°C in a horizontal shaker (platelet incubator from Helmer – model PC 3200 series 300996K) at 70 rpm. Samples were then rinsed 3 times with PBS to remove weakly attached platelets.

Platelet adhesion and activation was observed by scanning electron microscopy (SEM) and by the glutaraldehyde induced fluorescence technique (GIFT, see below). Adherent platelets were counted on photographs in 10 different sections per sample and expressed as number of platelets/mm^2, and a statistical analysis was performed with SPSS software. One-Way ANOVA was used to compare the effect of different surfaces under the same pre-immersion and Independent-samples T Test was used to compare the effect between two pre-immersions for the same surface. Values of $p<0.05$ were considered statistically significant. Platelet morphology was studied by categorizing platelets into five types, according to shape and spread area, as round or discoid (R); dendritic or early pseudopodial (D); spread dendritic or intermediate pseudopodial (SD), spread or late pseudopodial (S); and fully spread (FS).[19]

3.5.1 SEM

Adherent platelets were fixed with a freshly prepared solution of 1.5% glutaraldehyde (Merck) in 0.14M sodium cacodylate (Merck) buffer for 30 min at room temperature. After fixation, SAMs were rinsed twice with PBS. Adherent platelets were dehydrated in a graded ethanol series (50%, 60%, 70%, 80%, 90% and 99%, for 10 minutes 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 5000x.

3.5.2 Glutaraldehyde induced fluorescence technique (GIFT)

Adherent platelets on SAMs were fixed with a freshly prepared solution of 1.5% glutaraldehyde (Merck) in PBS buffer for 30 min at room temperature. After fixation, SAMs were rinsed three times with PBS and once with distilled/deionized water and placed on regular microscope slides. One drop of fluorescent mounting medium (Vectashield – H-1000; Vector Laboratories, Inc) was added and a coverslip was placed on the top of each sample.[20] Platelets were visualized with an inverted fluorescence microscope (Axiovert 200M, Zeiss, Germany). Samples were excited using the fluorescence filter Cy 3.5 from Zeiss set Lumar 31 (excitation BP565/30, emission BP620/60). The numerical aperture of the objective lens (40x) was 0.7 with a calculated maximal optical resolution of 0.37 μm.

4. RESULTS

4.1 Surface Characterization

The surfaces of EG_4-C18 SAMs were characterized by contact angle measurements, XPS, IRAS and ellipsometry.

4.1.1 Contact angle, surface energy and hysteresis

Table 2 shows the results of static water contact angle measurements, indicating that SAMs range from hydrophilic (θ_w=37.7) in EG_4 SAMs to hydrophobic (θ_w=94.8) in 10%C18 SAMs. Advancing and receding water contact angles are also presented in this table, and demonstrate that EG_4 SAMs have

the lowest hysteresis and that SAMs prepared from solutions with 5%C18 have the highest hysteresis, i.e. have the most heterogeneous or mobile surfaces.

Table 2. Water contact angles and surface hysteresis of EG_4-C18 SAMs.

	Water contact angle (°)			
	static	**advancing**	**receding**	**Hysteresis**
EG_4	37.7 ±0.5	41.8 ±0.6	33.3 ±0.2	8.5 ±0.2
0%C18	37.4 ±0.4	43.4 ±1.3	32.0 ±0.7	11.4 ±1.2
2.5%C18	55.9 ±1.3	67.2 ±2.2	40.6 ±1.4	26.6 ±1.0
5%C18	69.8 ±0.8	84.9 ±3.8	52.1 ±4.4	32.8 ±2.9
10%C18	94.8 ±2.6	104.9 ±1.1	81.9 ±1.7	23.0 ±1.1

Contact angle measurements using water and methylene iodide from Figure 1 show that the total surface free energy of the SAMs decrease with the increase of C18 percentage in solution, meaning that SAMs with –OH functionalities yield higher surface free energy than those terminated with methyl groups. Furthermore, it is possible to observe that the dispersive component of these surfaces increases with the increase of surface hydrophobicity, reaching almost 95% of the total surface free energy in 10%C18 SAMs.

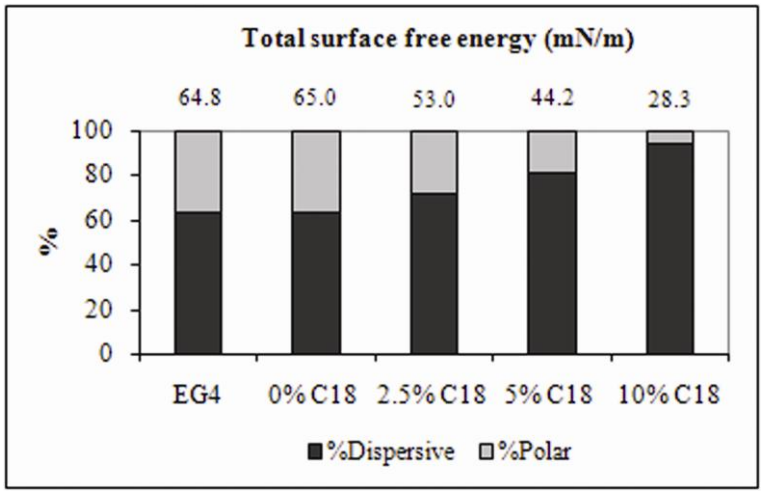

Figure 1. Dispersive and polar components of the total surface free energy of EG_4-C18 SAMs.

4.1.2 XPS

The surface atomic composition was determined by XPS. Survey spectra (data not shown) suggest the absence of contaminants since no elements other than the expected (Au, S, C, O, N) were found. Regarding high resolution spectra, the sulphur region demonstrates that S (2p) spectra were well fitted with a doublet structure centred at about 162.1 and 164.3 eV and a peak-to-area ratio of 2:1. As expected, all surfaces presented $\underline{C}H_2$ (285 eV) and $\underline{C}$-O (286.5 eV) peaks in the C (1s) spectra and C-$\underline{O}$H (533 eV) peak in the O (1s) spectra (data not shown).

The immobilization of C18 ligands can be detected through the reaction of the urethane group (-NHC=OO-) from the isocyanate and the hydroxyl terminated groups from the ethylene glycol moiety.

Figure 2 demonstrates the increase of nitrogen (from the N(1s) spectra), indicating the increase of C18 ligands on the surfaces. The presence of the (-NHC=OO-) (290 eV) and C-$\underline{O}$H (535 eV) peaks in the C (1s) and O (1s) spectra, respectively, have also confirmed the immobilization of ligands in 2.5%C18, 5%C18 and 10%C18 EG_4 SAMs (data not shown).

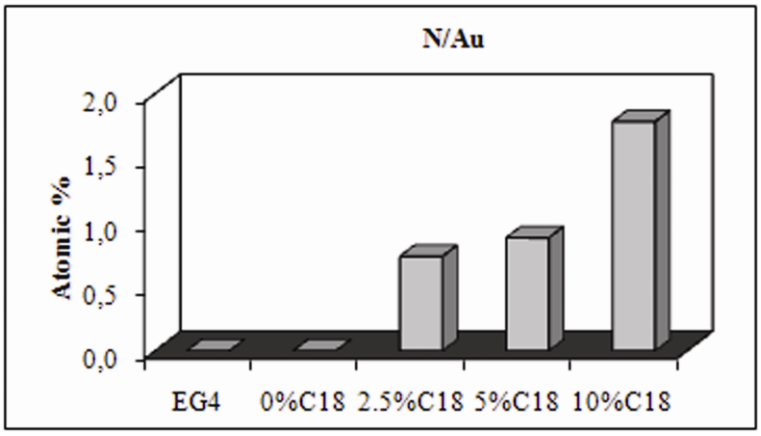

Figure 2. Relative atomic composition (%) of Nitrogen/Gold (N/Au) in EG_4-C18 SAMs determined by XPS.

4.1.3 IRAS

IR spectroscopy was performed in order to analyse the degree of crystallinity and molecular orientation of EG4 SAMs and to confirm the immobilization of C18 ligands on these SAMs.

The IR spectral region from 1000 to 1550 cm^{-1} for EG_4 SAMs on gold (Figure 3) shows a strong COC-stretching mode around 1130 cm^{-1} with a shoulder at ca. 1145 cm^{-1} and a CH_2-wagging mode at 1350 cm^{-1} with weak signal intensity, all characteristic of amorphous EG moieties.[21]

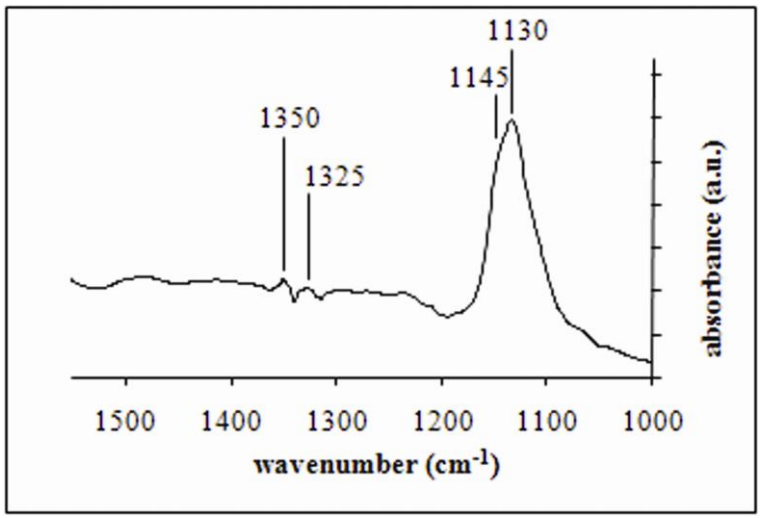

Figure 3. IR spectral region from 1000 to 1550 cm-1 for EG_4 SAMs on gold.

The orientation of the alkyl chains relative to the surface can be deduced from the relative intensities of their CH_2-stretching vibrations (Figure 4). A crystalline helical conformation with high packing density would have a characteristic very strong CH stretching peak at 2892 cm^{-1}. Instead, our EG_4 SAMs present a broad band from approximately 2840 to 2960 cm^{-1}, implying an amorphous and less oriented conformation of OEG groups on gold. These results are in accordance with other authors that have also reported that SAMs with these characteristics are protein resistant.[21,22]

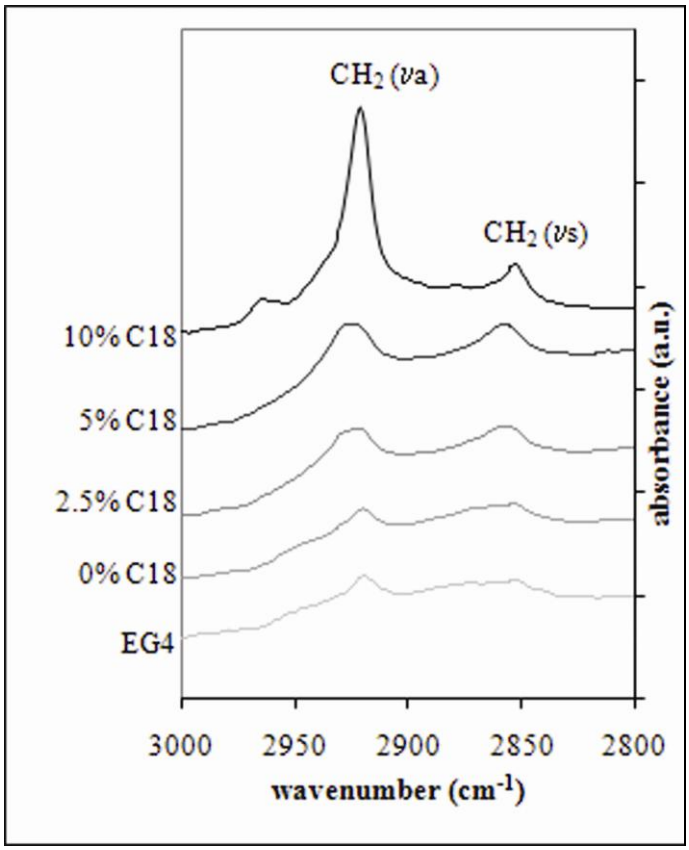

Figure 4. IRAS spectra for CH-stretching vibrations near 3000-2800 cm^{-1} of EG_4-C18 SAMs.

The asymmetric (ν_a) and symmetric (ν_s) CH_2 stretching modes for crystalline methylene units can be observed at c.a. 2920 and 2850 cm^{-1} in Figure 4. The increase of intensity of these peaks corresponds to the increase of C18 ligands immobilized on EG4 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.

4.1.4 Ellipsometry

Ellipsometry measurements were made using gold substrates with a refractive index of 0.9108 and an extinction coefficient of 2.6674. These values were used to calculate the thickness of each monolayer. Figure 5 shows the thickness of SAMs with and without octadecyl isocyanate ligands on

them. It is possible to observe that the background SAMs (EG_4 SAMs) have a thickness of ≈2.5nm, but as we increase the percentage of C18, SAMs get thicker to a maximum of ≈4.1nm in 10%C18 SAMs.

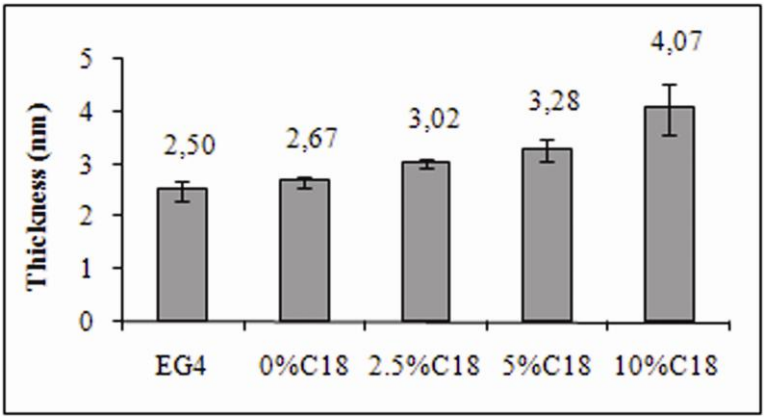

Figure 5. Thickness of EG_4-C18 SAMs determined by ellipsometry.

Surface characterization has demonstrated that, as expected, EG_4 SAMs and 0%C18 SAMs have similar results. For this reason and in order to simplify the following figures, only the results from EG_4 SAMs will be shown.

4.2 Protein adsorption

Figure 6 represents HSA adsorption on EG_4-C18 SAMs determined by radiolabeling (^{125}I). There is 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. The contact drop base diameter used in the calculations of the surface area ranged from 3.4 to 4.5 mm, depending on the type of SAM.

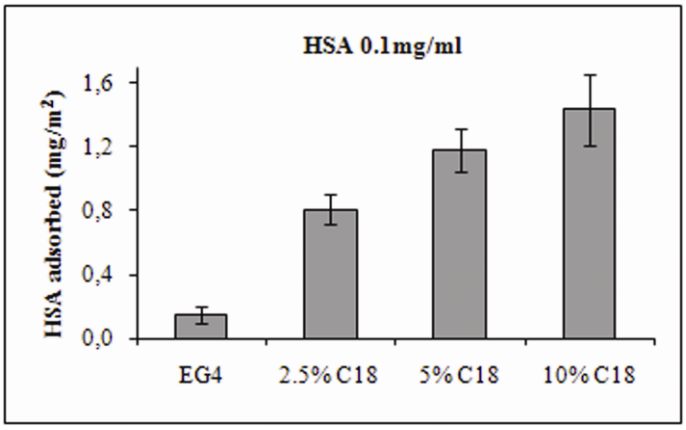

Figure 6. HSA adsorption on EG_4-C18 SAMs using 125I-HSA (0.1mg/ml; RT; 30 min).

SAMs with pre-adsorbed ^{125}I-labeled HSA (from Figure 6) were left for 24h in unlabelled 1mg/ml pure protein solutions (HSA and HFG), and PBS. The reversibility of albumin adsorption is presented in Figure 7.

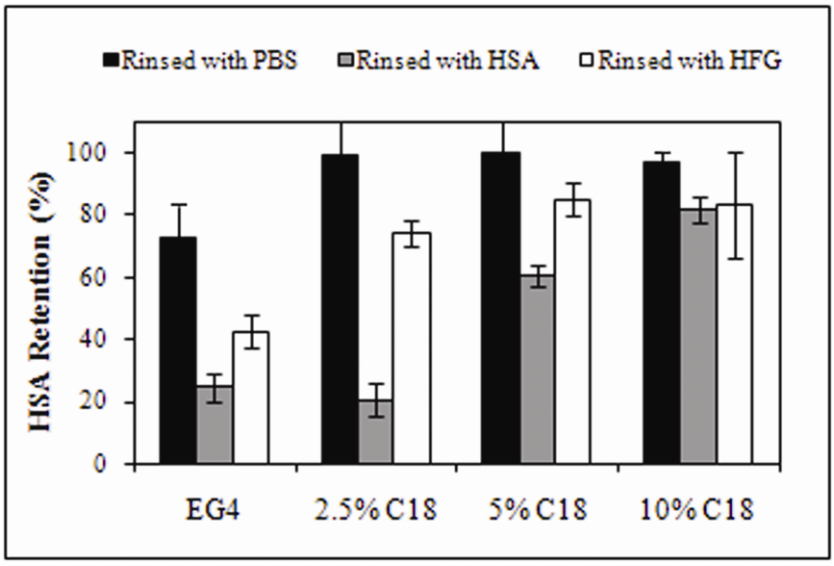

Figure 7. Retention of 125I-HSA previously adsorbed to EG_4-C18 SAMs (from Figure 6), after rinsing with unlabeled PBS, HSA (1mg/ml) or HFG (1mg/ml) solutions (RT; 24 h).

Background surfaces (EG_4 and 0%C18 SAMs) have released approximately 0.035 mg/m^2 albumin from the surface when in contact with PBS over 24h, meaning that 25% of the pre-adsorbed HSA was loosely bound. Regarding specific exchange, the two blood proteins are similar; background surfaces exchange ≈70% by other HSA and ≈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 20% by HFG. Regarding the other surfaces, 5%C18 SAMs retain higher amounts of ^{125}I-HSA, despite still having some selectivity on the exchange, but 10%C18 SAMs have 80% of the pre-adsorbed HSA irreversibly bound and do not have a preferred protein to exchange with.

Figure 8 shows the competitive adsorption between HSA and HFG in the same ratio as present in blood. Studies were made considering as 100% adsorption the concentration of HSA adsorbed using a pure HSA solution (presented in Figure 6).

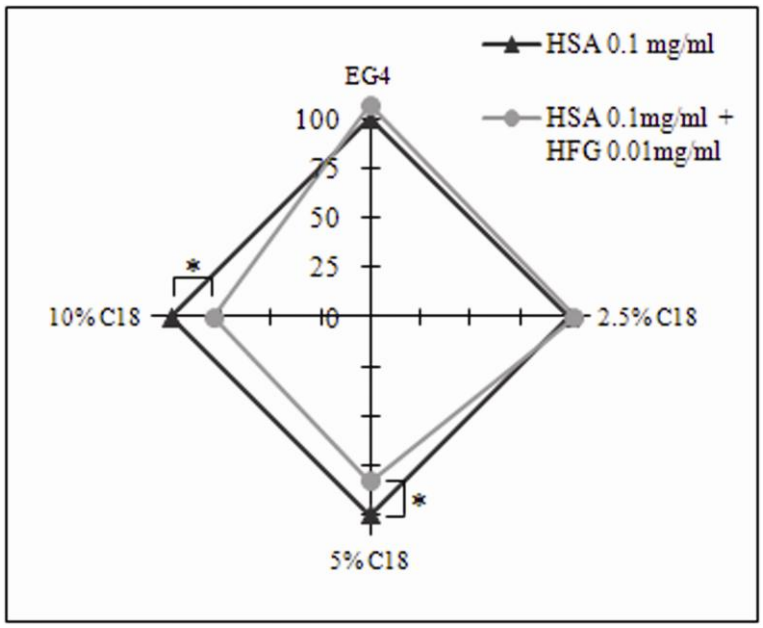

Figure 8. Competitive adsorption between HSA and HFG to EG_4 SAMs with different percentages of C18 (RT; 30 min). Calculations were performed considering as 100% adsorption the concentration of HSA adsorbed using a pure HSA solution from Figure 6. *Significant differences ($p<0.07$) between pure HSA solution and mixed HSA and HFG solution; Mann-Whitney Test.

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.

In order to evaluate competition of albumin with other blood proteins further studies were performed. Adsorption of HSA from 1% plasma was compared to adsorption from a pure HSA solution (in the concentration present in 1% plasma). Results presented in Figure 9 show that there is an increase of HSA adsorption (in both pure HSA and plasma) as the amount of C18 ligands increase. However, all surfaces adsorb more HSA from the pure solution than from plasma, suggesting that other proteins may adsorb to (compete for) the surface.

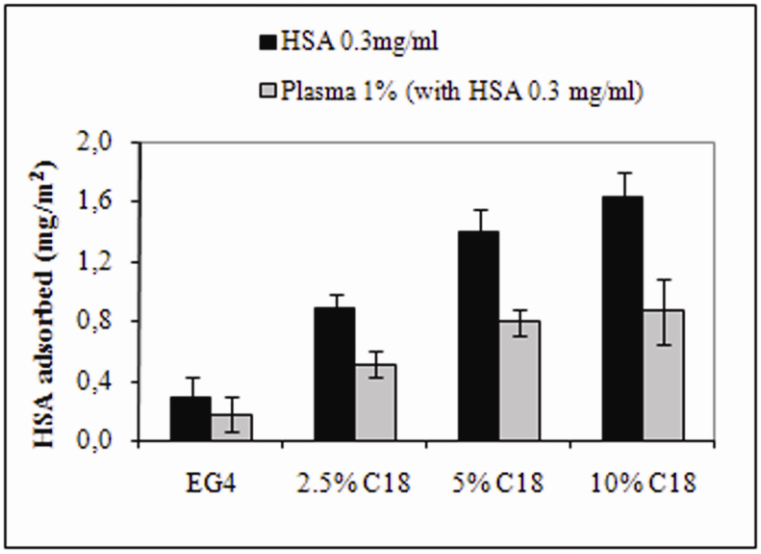

Figure 9. Competitive adsorption between HSA and other proteins from plasma on EG_4-C18 SAMs determined by radiolabeling (RT; 30min).

4.3 Platelet adhesion and activation

The effect of immobilized C18 ligands and pre-adsorbed proteins on platelet adhesion and activation was studied using SEM and GIFT. The quantitative analysis of platelet adhesion to EG_4-C18 SAMs pre-immersed in buffer (PBS), HSA and plasma is presented in Figure 10, and representative images are presented in Figure 11.

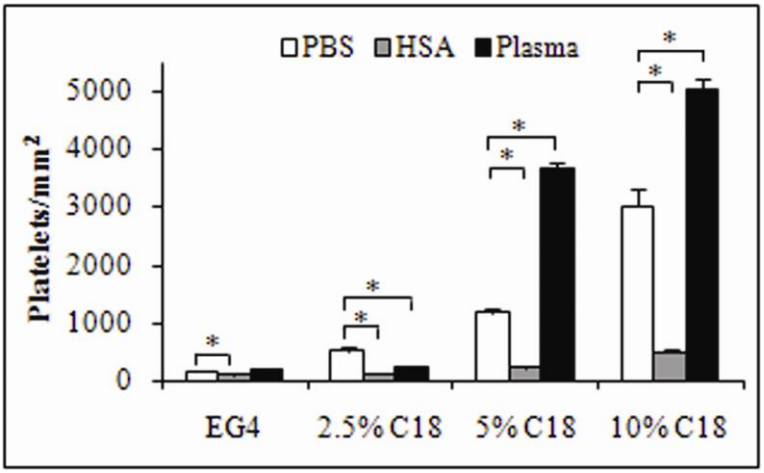

Figure 10. Number of adherent platelets on EG_4-C18 SAMs pre-immersed in PBS, HSA (0.1mg/ml) or plasma (1%) (RT; 30min). Platelet counts were statistically different ($p<0.05$) among the four surfaces within each pre-immersion; One-Way ANOVA. *Significant differences ($p<0.05$) comparing to PBS pre-immersion of the same surface; Independent-samples T Test.

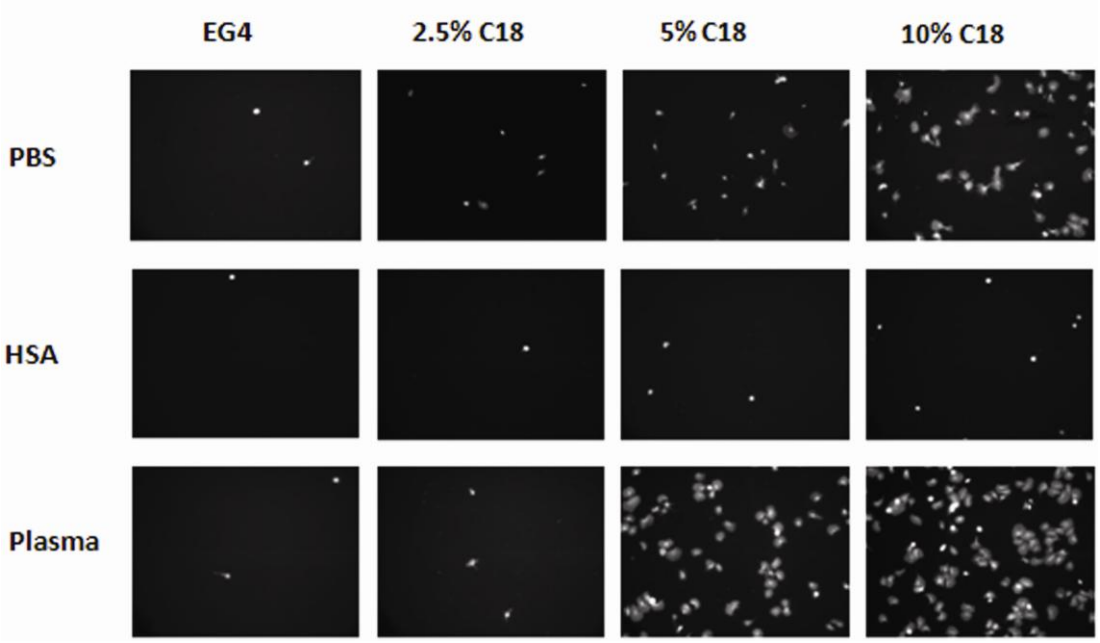

Figure 11. Representative GIFT images of adherent platelets on EG_4-C18 SAMs pre-immersed in PBS, HSA (0.1mg/ml) or plasma (1%) (original enlargement 630x).

As can be observed, for each pre-immersion condition, there is a statistically significant increase in the number of adherent platelets as the percentage of C18 increases. Regarding the effect of the pre-immersion, surfaces with pre-adsorbed HSA demonstrate a very significant decrease in the number of adherent platelets. EG_4 SAMs had a decrease of c.a. 35%, but the other surfaces decreased the number of adherent platelets by approximately 80%. The effect of plasma pre-immersion was nevertheless different. Compared with pre-immersion in buffer, EG4 SAMs maintained the number of adherent platelets whereas 5%C18 and 10%C18 increased substantially. However, 2.5%C18 SAMs were found to exhibit a decrease in the number of adherent platelets, despite not as much as in HSA pre-immersion, meaning it binds albumin from plasma, but is not 100% selective.

A qualitative analysis was performed by categorizing platelets according to their shape and degree of activation. Figure 12 shows the shape distribution of adherent platelets after pre-immersing the SAMs in PBS (control with no proteins), HSA (0.1mg/ml) and plasma (1%).

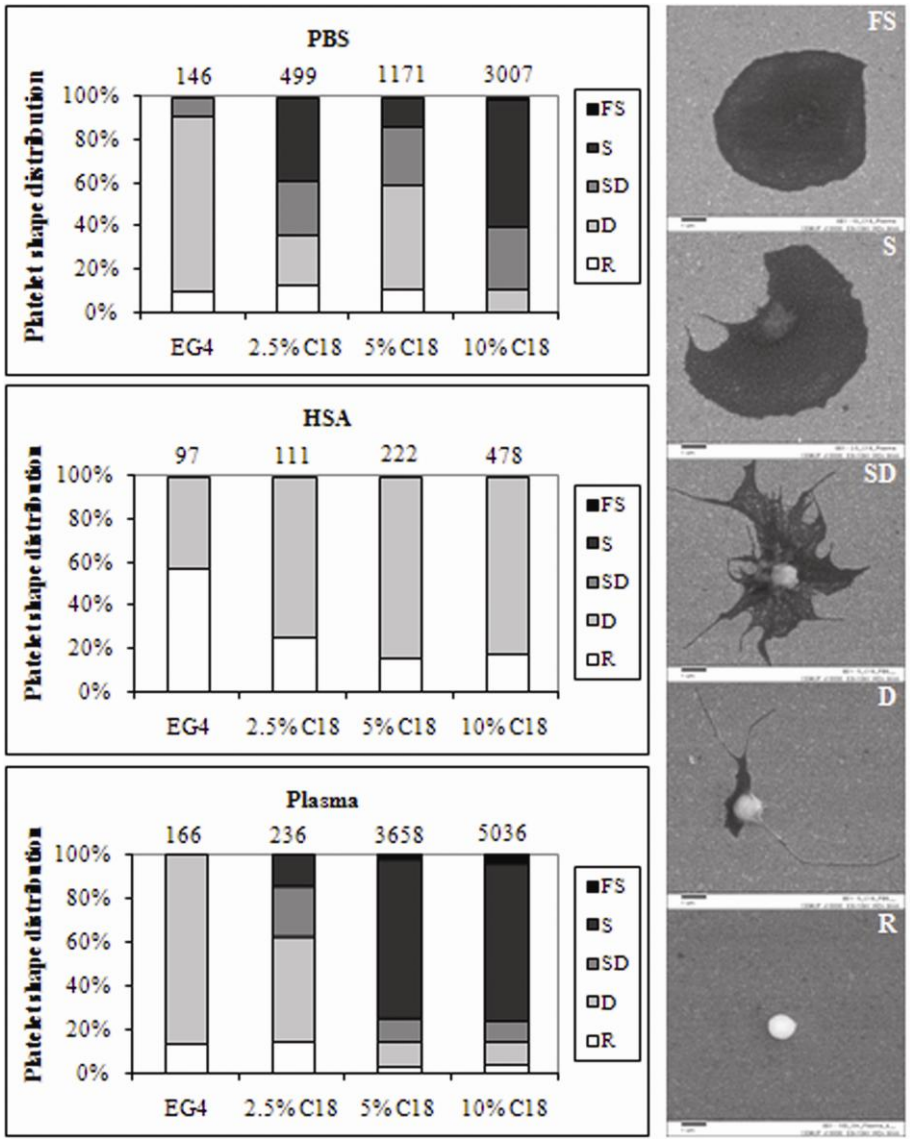

Figure 12. Shape distribution (% of the total number (in labels)) of adherent platelets on EG_4-C18 SAMs pre-immersed in PBS, HSA (0.1mg/ml) or plasma (1%). Legend: R=round; D=denditric; SD=spread dendritic; S=spread; FS=fully spread. On the right, SEM images illustrating the five types of platelet shapes.

Buffer pre-immersion demonstrates that the few platelets that adhere to the background surface (EG4 SAMs) are in a low activation state, and as surfaces get more hydrophobic (more C18 ligands), the platelets get more activated. When surfaces are exposed to albumin before contact with the platelet concentrate, all the adsorbed platelets are either round or dendritic, meaning that surfaces

covered by albumin are not activating the platelets. When SAMs are pre-immersed in plasma, the layers of adsorbed proteins on 5%C18 and 10%C18 SAMs have a strong effect on the platelets, since 80% of them have spread morphology, meaning they are activated. However, EG_4 SAMs and 2.5%C18 SAMs appear to perform better, since they are equal or less activating when comparing to buffer pre-immersion.

5. DISCUSSION

This work used oligo(ethylene glycol)-terminated SAM model surfaces as the background chemistry to study the effect of an 18 carbon (C18) ligand on albumin selective and reversible adsorption and subsequent platelet adhesion and activation.

An appropriate background surface should prevent protein non-specific adsorption and subsequent platelet adhesion and activation. EG_4 SAMs (Au-$S(CH_2)_{11}(OCH_2CH_2)_4OH$) were chosen as background surfaces since the $HS(CH_2)_{11}$–moiety provides a foundation for stable SAMs; while the oligo(ethylene glycol) headgroups should be substantially disordered and therefore show non-fouling properties.[22,23]

The advancing and receding contact angles of 42° and 33°, respectively, for EG_4 SAMs are higher than that obtained by other authors ($\theta_a \cong 38$ and $\theta_r \cong 24$).[13,24] However, these relatively high values of θ_a are consistent with an outer phase that exposes CH_2 groups to solution because is not closely packed. As shown by IRAS, the monolayers have an amorphous and non-oriented conformation of the OEG headgroups. These surface characterization techniques have therefore suggest that the EG-moieties should contribute to the non-fouling properties of the background surface. The packing density of 27 $Å^2$/ EG_4OH molecule (3.69 molecules/nm^2) was calculated by extrapolating the results from Herrwerth et al. [21], where there is a linear correlation between the packing density of oligoether-terminated SAMs and the number of EG groups on each OEG molecule (slope of -8.6148 nm^2 per EG group and intercept of 35.771). This packing density is therefore smaller than in an idealized, densely packed, and defect free alkanethiol SAM with 100% coverage, where the area occupied by a single chain is 21.4 $Å^2$ on Au(111),[22] corresponding to packing densities of 4.67 molecules/nm^2 on gold.[21]

On the top of the ethylene glycol terminated 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, 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 is expected since the C18 ligands are CH_3-terminated. Other authors [15,25] have obtained higher values of water contact angles (θ_w=110°) for pure, fully covered, CH_3 SAMs, suggesting that our 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 the carbamate N(1s) 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 ≈ 2920 cm^{-1} increased as the percentage of octadecyl isocyanate in solution increased. Regarding ellipsometry, assuming as did other authors [13,22] that d(CH_2)=1.1Å and d(OCH_2CH_2)=2.7Å, the theoretical thickness of a EG_4 SAMs is approximately 25.3 Å. Therefore, the thickness of 25Å 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.[13,24] 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.

Albumin adsorption results presented in this paper are in accordance with a previous work,[14] where there is an increase of protein adsorption with increase of surface hydrophobicity due to the increase of C18 ligands.

Concerning exchangeability of adsorbed ^{125}I-HSA, results showed that SAMs prepared from solutions with 2.5%C18 (2.5%C18 SAMs) replace 85% of the adsorbed HSA by HSA in solution, but not by HFG. In a previous work, studies were performed using the same C18 ligands but with a different background monolayer (OH SAMs).[14] The replacement percentage of adsorbed albumin by other albumin obtained using EG_4 SAMs increased 35% (for the same percentage of ligand in solution – 2.5%C18), meaning that this background surface has better non-fouling characteristics and therefore works better to inhibit non-specific interactions. This could also be concluded from the results in Figure 7, where EG_4 SAMs also have an exchangeability of ca. 80%. However, 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 adhesion and activation were evaluated on EG_4-C18 SAMs with and without prior immersion in HSA and 1% plasma to better understand the effect of the adsorbed layer of proteins in the behavior of platelets.

Following the trend observed in the protein adsorption studies, there is an increase of platelet adhesion as the percentage of C18 ligands increases (with buffer pre-immersion). Moreover, the morphology of the platelets changes from round to spread as they contact more hydrophobic surfaces. However, when a pre-immersion of HSA is performed, there is an 80% decrease in the number of platelets, and the few ones that adhere are not activated, 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 and activation of platelets as in buffer, but SAMs with 5% and 10%C18 demonstrated higher adhesion and activation than in buffer. These results can be justified by the competitive studies from Figure 8, where all SAMs maintain their preference for HSA when both HSA and HFG were present in solution, but 5% 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. These results were confirmed in competitive studies, where all surfaces adsorb less HSA in the presence of plasma than in pure HSA solutions, implying that other plasma proteins are competing with albumin, but not fibrinogen in EG_4 and 2.5%C18 SAMs (as demonstrated in Figure 8). Experiments from Jenney and Anderson have shown that PEO-coupled glass and octadecyl modified glass adsorb some vWF (~0.5ng/cm^2 and ~1.5ng/cm^2, respectively), despite in much smaller amounts than HSA (~40ng/cm^2 and ~100ng/cm^2, respectively).[26] Therefore, a potential competitive protein 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.

Surfaces prepared from a solution with 2.5% C18 have proved to have better performance than oligo(ethylene glycol)-terminated surfaces regarding selectivity in the exchange of proteins. However, they both behave similarly in competition studies between albumin and fibrinogen.

A long-term exposure of both materials to proteins would therefore be necessary to evaluate the benefits of the selective exchange of albumin on 2.5%C18 SAMs.

EG_4 SAMs and 2.5% C18 SAMs both adsorbed low numbers of platelets, although platelets that adsorb to the 2.5% C18 surface seem to be in a more activated configuration. The immobilization of C18 ligands does not seem to bring an advantage in platelet adhesion and activation but other aspects related to blood contact biomaterials, such as complement system activation and leukocyte adhesion and activation, should be studied. Sperling et al. have shown that complement activation is found to be positively related to the OH group content of the surface (surfaces with OH groups exhibit strong activation of complement factor C5a) and that it has strong chemotactic effects on leukocytes, which are known to shed a number of proinflamatory and procoagulant substances and therefore are prone to lead to incompatibility reactions.[27] The same authors showed that OH terminated surfaces revealed many deformed adherent leukocytes (628$\pm$181/mm^2) and almost no platelets (213$\pm$287/mm^2) and that CH_3 terminated surfaces revealed almost no leukocytes (2.3$\pm$7.4/mm^2) and many deformed platelets (10413$\pm$2305/mm^2). The same tendency in leukocyte adhesion to OH- and CH_3- terminated monolayers was also reported by Barbosa et al..[28]

Since HSA is not known to contain leukocyte adhesion ligands or promote the adhesion or fusion of macrophages, we believe that the 2.5% C18 SAMs would be a good compromise between these two

types of surfaces and would have the advantage of its the specificity in reversibility adsorption (exchange of HSA for other HSA).

6. CONCLUSIONS

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

The use of EG_4 SAMs as a background decreased non-specific adsorption and increased the preference for HSA, since all the surfaces presented more affinity to albumin than to fibrinogen. The importance of the concentration 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 using model surfaces has shown that a surface displaying a specific percentage of albumin-binding ligands (2.5% C18) on EG_4 SAMs can recruit albumin selectively from plasma and therefore minimize the adhesion and activation of platelets.

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CHAPTER V

LEUKOCYTE ADHESION TO OLIGO(ETHYLENE GLYCOL)-TERMINATED SELF-ASSEMBLED MONOLAYERS WITH C18 LIGANDS: EFFECT OF PROTEIN PRE-ADSORPTION

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1. ABSTRACT

Leukocyte adhesion to the surface is an important phenomenon in the evaluation of biomaterials since adherent leukocytes are often related to the inflammatory response after implantation. This study reports the adhesion of human leukocytes to tetra (ethylene glycol) self-assembled monolayers (EG_4 SAMs) after surface functionalization with different percentages of C18 ligands. The effect of plasma proteins on human leukocyte adhesion was also assessed by pre-immersing the different samples in phosphate buffered saline (PBS), human serum albumin (HSA), human fibrinogen (HFG) or human plasma before contact with the leukocytes. EG_4 SAMs induced minimum or no adhesion of leukocytes, regardless of the pre-immersion. Leukocyte adhesion to EG_4-C18 SAMs increased as the percentage C18 ligands immobilized on the surface increased until 5%C18. Adhesion of leukocytes to 10%C18 SAMs was similar so that on 5%C18 SAMs, except for surfaces pre-adsorbed with HSA, which increase even further leukocyte adhesion. Except for 10%C18 SAMs, pre-immersing the samples in buffer, single protein solutions (HSA or HFG) or complex protein solution (plasma) did not influence much leukocyte adhesion. Therefore, the surface chemistry, in this case the amount of C18 ligands on the surface, was found to be the main responsible for the differences observed in leukocyte adhesion to EG_4-C18 SAMs.

KEYWORDS: nanostructured surfaces, self-assembled monolayers, protein adsorption, human serum albumin, leukocytes.

2. 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 other foreign body, trigger an acute inflammatory response that is reflected by an *in situ* accumulation of leukocytes. The early inflammatory response involves the activation of humoral defenses such as the complement, coagulation, fibrinolytic and kinin systems.[2] Leukocyte adhesion to a biomaterial surface depends on many factors such as material surface chemistry and protein adsorption profiles.[3-5]

Regarding surface properties of the material, the intensity of acute inflammation depends mainly on the chemical composition, wettability, surface free energy, surface charge, porosity and roughness.[6,7]

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 *in vitro*, where serum or plasma is added to culture media to sustain cell growth.[8]

An investigation using non fouling self-assembled monolayers (SAMs) has been previously performed in order to determine the influence of different amounts of a C18 compound (0% to 10%C18) in the recruitment of albumin from plasma.[9] Recent work has proven that a surface displaying a specific percentage of albumin-binding ligands (2.5% C18) on EG_4 SAMs can recruit albumin selectively from plasma and minimize the adhesion and activation of platelets.[10] However, other aspects, such as complement activation and leukocyte adhesion, are also crucial in determining the fate of a blood contact biomaterial.[5]

In the present study we address the effect of surface properties, namely different percentages of immobilized C18 ligands, on leukocyte adhesion mediated by a protein layer pre-adsorbed from pure protein solutions such, as albumin or fibrinogen, and from a complex protein solution, namely plasma.

In the case of blood contact biomaterials, three host proteins predominate: albumin, IgG and fibrinogen. In this study, albumin was chosen because besides being abundantly present in blood and at sites of injury, it is known to "passivate" biomaterials surfaces, blunting proinflammatory and thrombogenic responses.[11,12] For that reason the design of a surface capable of adsorbing albumin selectively from plasma is the ultimate goal of this investigation. Fibrinogen was also selected since it is one of the most important competitors of albumin for surface adsorption and the main protein responsible for blood clotting, namely by inducing platelet adhesion and activation.[13] The presence of fibrinogen in the layer of adsorbed proteins is considered to attract more inflammatory cells than when fibrinogen is absent.[14] Fibrinogen is therefore of great importance in the initial acute inflammatory response to biomaterials.

3. MATERIALS AND METHODS

3.1 Preparation of EG_4-C18 SAMs

Preparation of EG_4-C18 SAMs comprises two steps: (i) preparation of 1-Mercapto-11-undecyl tetra(ethylene glycol) self-assembled monolayers (EG_4 SAMs) on gold; (ii) reaction of the OH groups from the EG chains with the isocyanate group of a compound with 18 carbons. This procedure has been described in a previous work.[10] Briefly, 1x1cm^2 gold substrates were cleaned and immersed in a 0.1 mM solution of 1-Mercapto-11-undecyl tetra(ethylene glycol) (EG_4; Assemblon) in ethanol (99,8%, Merck). After incubation at room temperature for 24 hours 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%, 1%, 2.5%, 5% and 10%) of octadecyl isocyanate (C18; 98%, Aldrich). After incubation for 2 hours at 40°C in a nitrogen environment, the samples were rinsed, dried and maintained in argon until used.

The surface of EG_4-C18 SAMs was examined by different surface characterization techniques. X-ray photoelectron spectroscopy (XPS) was used to obtain information on the chemical composition of the surface. Contact angle measurements were performed to study the hydrophilicity, hysteresis and

surface energetics. Fourier transform infrared reflection absorption spectroscopy (IRAS) was used to investigate the chain order and crystalline structure. Finally, the thickness of the surface was measured by ellipsometry. The methodology and results of these measurements have been previously described elsewhere and therefore will not be described in this work.[10]

3.2 Leukocyte adhesion assay

3.2.1 Preparation of leukocyte suspension

Buffy coat preparation has been performed at the Portuguese Blood Institute. Whole blood (450ml) anticoagulated with citrate phosphate dextrose (MacoPharma) was collected from non-medicated healthy donors. By centrifugation of a blood unit at 3900 rpm for 10 minutes at 22°C, an interphase between erythrocytes (at the bottom of the bag) and plasma (at the top of the bag) is formed. This interphase is termed the *buffy coat*, composed primarily of leukocytes and platelets. In order to remove some of the platelets, *buffy coat* was rinsed 3 times with a platelet additive solution (PAS; MacoPharma) by centrifuging at 1000 rpm for 10 minutes 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. For this, *buffy coat* was first diluted in two times its volume in phosphate buffered saline (PBS; Sigma). In 15 ml centrifuge tubes, 3 ml of Histopaque 1077 were smoothly added to 3 ml of Histopaque 1119 and then 6 ml of the diluted *buffy coat* were gently layered on the top. The tubes were centrifuged at 700g for 30 minutes at 25°C. After centrifugation, an opaque white ring could be observed, corresponding to leukocytes, whereas eventual contaminants like erythrocytes are deposited in the bottom of the tube. Leukocytes were transferred to a new tube and rinsed in ~10ml of PBS by centrifugation at 200g for 10 minutes at 25°C. After rejecting the supernatant, the cell pellet was re-suspended, and the rinsing step repeated. The last re-suspension was made in 5ml of PBS and cell counting was performed in a Neubauer chamber. Leukocyte concentration was afterwards adjusted to $1x10^6$ leukocytes/ml.

In order to determine the efficiency of the leukocyte separation process, a cellspin was prepared using a Cytocentrifuge (Cytospin 4, Shandon), by applying a leukocyte layer to a microscope slide

while preserving the cell structure. This preparation was fixed using the Hemacolor® Solution 1 (Sigma), rinsed with ddH_2O, stained with Hemacolor® dyes for blood cells microscopy, rinsed again and then observed by light microscopy.

3.2.2 Adhesion of leukocytes to SAMs

Leukocyte adhesion assays were performed in 24 well plates. In order to prevent leukocyte activation by the polystyrene from the plate, the wells were previously blocked with bovine serum albumin (BSA; Merck). For that, the wells were filled with 2 ml of 1% BSA, the plates were incubated for 1 hour at 37°C and then rinsed 5 times with PBS.

Since protein adsorption is known to influence cell adhesion, before leukocyte adhesion samples were pre-immersed in different protein solutions so as to investigate the effect of protein adsorption on subsequent adhesion of leukocytes. EG_4-C18 SAMs were pre-immersed for 30 minutes at room temperature in phosphate buffer solution (PBS; Sigma), 0.1mg/ml human serum albumin (HSA; Sigma), 0.1mg/ml human fibrinogen (HFG; Sigma) or 1% human plasma solutions. After protein adsorption, samples were rinsed 3 times with PBS to remove loosely bound proteins. Protein concentrations chosen in this study are similar to previous studies of protein adsorption and platelet adhesion and activation to these EG_4-C18 SAMs so that results could be related.[10]

After pre-immersions, EG_4-C18 SAMs were placed in the previously blocked 24 well plates containing 1 ml of RPMI 1640 culture medium (Gibco) supplemented with 10% heparinized plasma and 400 µl of leukocyte suspension ($1x10^6$ leukocytes/ml), being that the final leukocyte concentration in each well was $0.7x10^6$ leukocytes/ml. Samples were incubated for 30 minutes at 37°C and afterwards transferred to a clean 24-well plate with PBS and rinsed two more times with this buffer.

3.2.3 Preparation of samples for Light Reflection Microscopy

Adherent leukocytes were fixed by adding 300µl of Hemacolor® Solution 1 (Sigma) to the 24-well plate containing the rinsed samples. After 2 minutes, samples were rinsed twice with ddH_2O and allowed to dry at room temperature. Each sample was then glued to previously identified 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. Adherent leukocyte densities were determined by counting and photographing 10 different fields in each sample (4

replicates were used for each sample in each experiment). A 50x objective was used, and each field corresponded to $8x10^{-3}$ mm^2.

3.2.4 Data analysis

Five independent experiments were performed, each using different blood donors.

Data is presented by median and interquartile range. Normality of data was assessed by the non parametric test of Kolmogorov-Smirnov. Analysis of variance was used to compare different surfaces and pre-immersions. Due to lack of normality and homogeneity of variance, several power transformations were tried, but none was able to stabilize the variance. Thus, so that the 5 different experiments could be compared, another approach to the analysis of data was tried.

The results of each experiment were classified considering the amount of leukocyte adhesion distribution into three groups defined by the 25^{th} and the 75^{th} percentiles. Thus, for each experiment, the results were then ranked according to one of these three groups: below 25^{th} percentile (P25), between 25^{th} and 75^{th} percentile (P25-P75), and above the 75^{th} percentile (P75). Using this classification, the data of all experiments could now be considered all together as if a single experiment was performed. Thus, considering the data from all the experiments, different surfaces and pre-immersions were compared with respect to the number of observations in each interval defined by the quartiles. Chi-square tests were used to compare the distributions of the ranked observations by surface and pre-immersion. Differences were considered significant at the 95% confidence level ($p<0.05$).

4. RESULTS

The modified surfaces used in this study have previously been extensively characterized by XPS, FTIR-IRAS, contact angle measurements and ellipsometry. These results can be found elsewhere and therefore will not be presented here.[10] Very briefly, an increase of thickness and hydrophobicity was verified with the increase of C18 ligands immobilized on EG_4 SAMs.

Protein adsorption and platelet adhesion and activation studies have also been previously performed. It was shown that the concentration of adsorbed albumin increased as the percentage of C18 ligands

on the surface increased, regardless of albumin being in a pure solution or competing with fibrinogen in a mixed solution. The number of adherent platelets and their degree of activation also increased with the amount of immobilized C18. Pre-immersion of samples in HSA solution before contact with platelets revealed a 80% decrease in platelet adhesion and reduction of activation in all surfaces. Pre-immersion in plasma was only relevant in 2.5%C18 SAMs since this was the only surface to have less platelet adhesion compared to buffer pre-immersion.[10]

This study reports the effect of the immobilization of C18 ligands to EG_4 SAMs and the pre adsorption of different proteins on the adhesion of human leukocytes. The counts of the 5 experiments performed of leukocyte adhesion to EG_4-C18 SAMs pre immersed in PBS, HSA, HFG and plasma are presented in Figure 1. Data is represented by median and interquartile range.

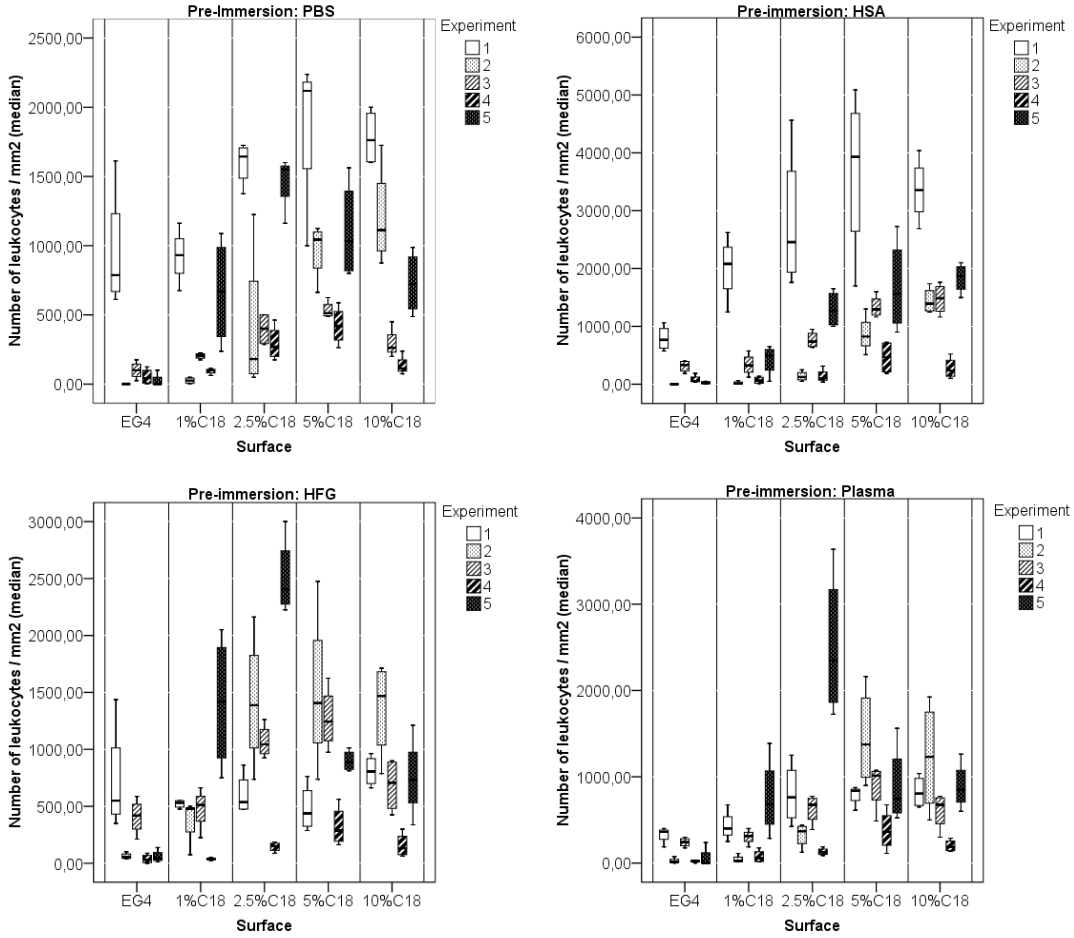

Figure 1 – Median of the number of adherent leukocytes to EG_4-C18 SAMs in the 5 experiments that were performed. Each graphic represents a different pre-immersion (PBS, HSA, HFG and plasma).

Each experiment was performed using different blood donors and therefore different leukocyte suspensions. Although the concentration of the leukocyte suspension was always adjusted to $1x10^6$ cells/ml and the concentration of leukocytes in each culture well was always the same ($0.7x10^6$ cells/ml), it was found that the absolute number of adherent leukocytes varied with the experiments, suggesting that the leukocyte adhesion may depend on the *buffy-coat* sample used. We discard the hypothesis of these differences resulting from the variability of the SAM samples since very small standard deviations and statistically significant differences were always found in surface characterization, protein adsorption and platelet adhesion and activation studies.[10]

Even though there is a divergence in the absolute number of adherent cells between the 5 different experiments (as can be observed in Figure 1), trendlines of the number of leukocytes adherent to EG_4-C18 SAMs after different pre-immersions could be determined using the median of the 5 experiments and are represented in Figure 2.

These results demonstrate that there is a tendency for leukocytes to adhere more as the percentage of immobilized C18 increases up to 5%C18. 10%C18 SAMs seem to have leukocyte adhesion similar to that observed for 5%C18 SAMs, except for surfaces pre-adsorbed with HSA, which increase even further leukocyte adhesion.

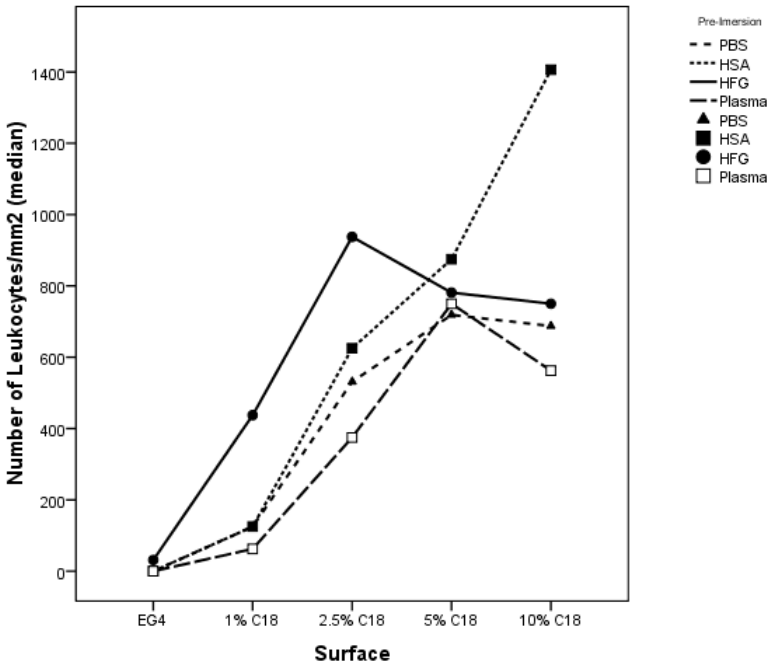

Figure 2 – Trendlines of the number of leukocytes adherent to EG_4-C18 SAMs after different pre-immersions determined using the median of the 5 experiments.

Representative images of the number of leukocytes adhered to EG_4-C18 SAMs after pre-adsorption without proteins (PBS), HSA, HFG or plasma solutions are given in Figure 3. As can be observed, the background surface (EG_4 SAMs) presents very low or no leukocyte adhesion, regardless of the pre-immersion solution. Eventhough samples with higher amounts of C18 induced more adhesion of

leukocytes comparing to the backgroound surface, the surface coverage is still relatively low (as can be seen in the images), indicating that the inflammatory reaction induced by EG_4-C18 SAMs is not likely to be high.

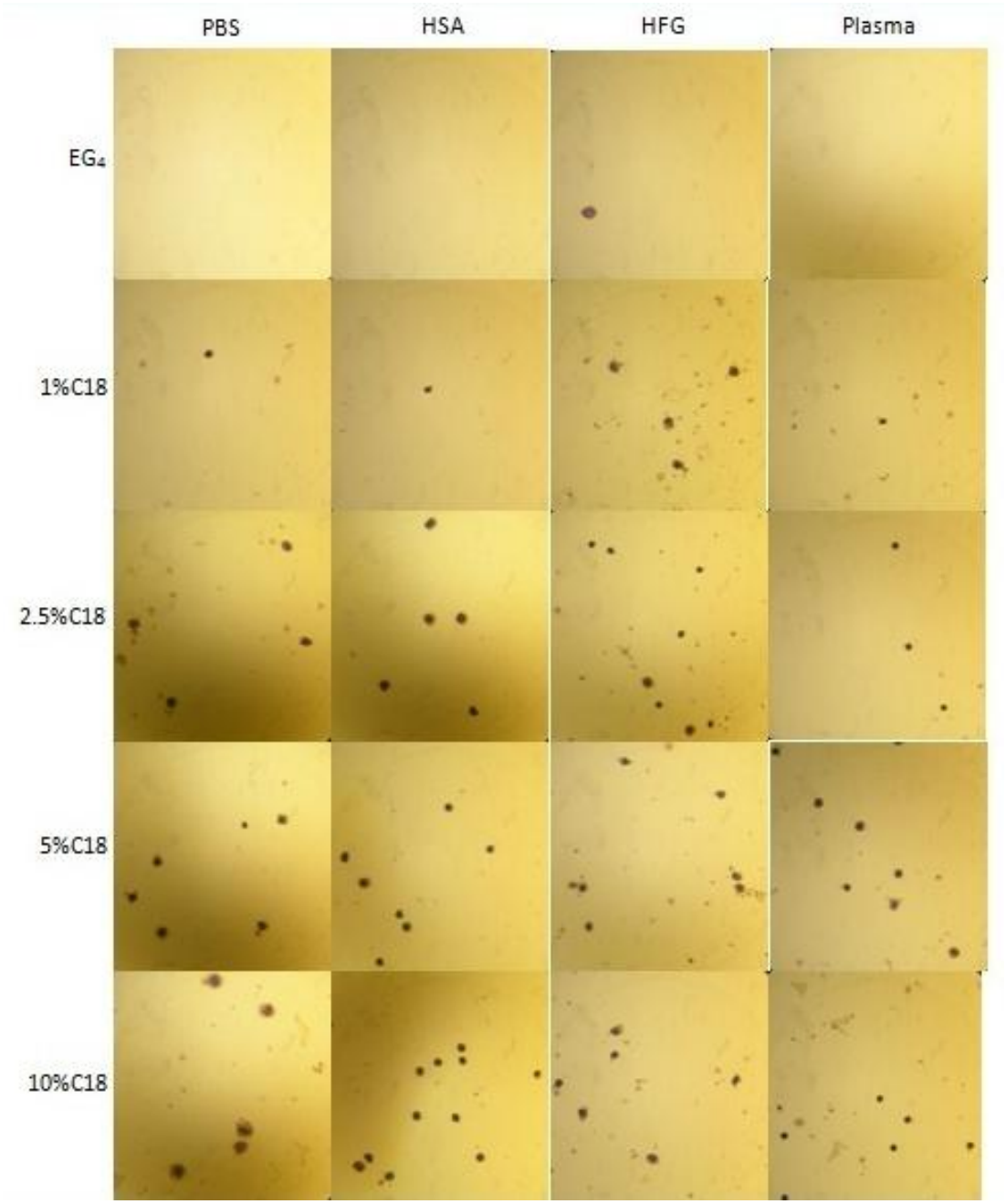

Figure 3 – Representative images of adherent leukocytes on EG_4-C18 SAMs pre-immersed in PBS, HSA (0.1mg/ml), HFG (0.1mg/ml) or human plasma (1%) solutions. Original enlargement: 125x.

In order to analyze more accurately the results from the 5 experiments and compare the surfaces and pre-immersions, data was homogenized considering as 100% the maximum adhesion in each experiment. The number of adherent leukocytes in each experiment was categorized in one of 3 categories: below percentile 25 (P25), for samples with low adhesion; between percentile 25 and percentile 75 (P25-P75), for samples with medium adhesion; and above percentile 75 (P75), for samples with high adhesion. Using this data approach it is possible to analyze the results of the 5 experiments all together as if a single experiment had been performed.

Figure 4 exhibits, for each surface, the influence of the pre-immersions in leukocyte adhesion. In EG_4 SAMs, 70% or more of leukocyte adhesion is observed in P25, meaning that this surface presents low adhesion of human leukocytes. In 1%C18 SAMs there is around 50% low adhesion and 50% medium adhesion, meaning that this surface induces more leukocyte adhesion than the background EG_4 surface. In 2.5%C18 and 5%C18 SAMs there is an increase of leukocyte adhesion to medium/high since the high adhesion bars (P75) represent 20 to 60% of the total adhesion. In 10%C18 SAMs, except for HSA pre-immersion, there seems to be a small reduction in leukocyte adhesion comparing to the latter surfaces since medium adhesion bars (P25-P75) increased and high adhesion bars (P75) decreased.

The surface that leads to higher adhesion of human leukocytes seems to be 10%C18 SAMs after pre-immersion in HSA. Within each surface no statistical differences were observed between pre-immersions, except for 10%C18 surface, where HSA pre-immersion induced higher leukocyte adhesion then PBS, HFG or plasma pre-immersions. However, 2.5%C18 SAMs and 5%C18 SAMs with pre-adsorbed plasma proteins seem to decrease leukocyte adhesion comparing to the same samples with the other pre-immersions (PBS, HSA and HFG).

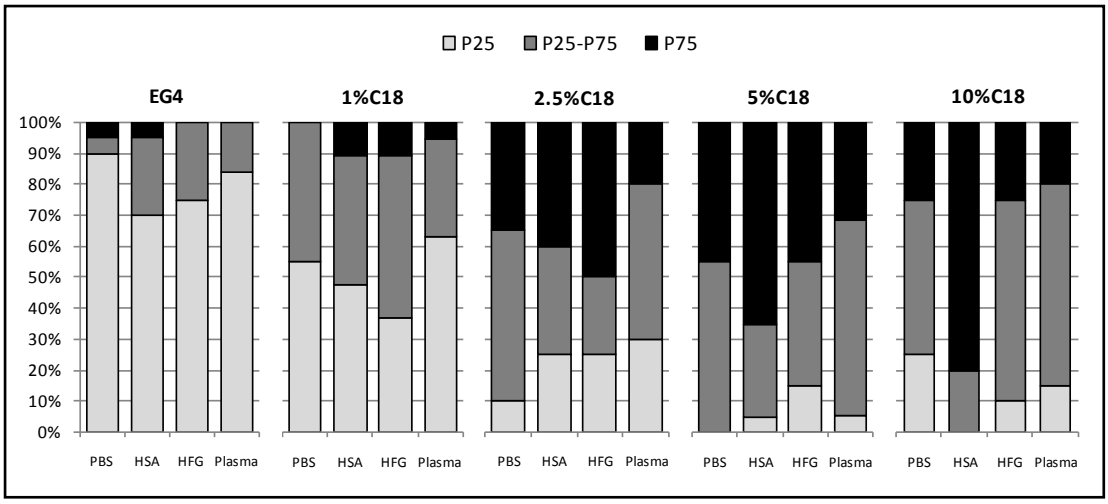

Figure 4 – Percentage of adherent leukocytes to each type of EG_4-C18 SAMs after different pre-immersions (PBS, HSA, HFG and plasma) represented as low adhesion (percentile 25 (P25)), medium adhesion (percentile 25 to percentile 75 (P25-P75)) and high adhesion (P75). Results show the effect of pre-immersions in each surface and were obtained using the medians of all 5 experiments.

Figure 5 exhibits, for each pre-immersion, the influence of the amount of C18 ligands in leukocyte adhesion. For the PBS pre-immersion, there is an increase in the adhesion of human leukocytes from EG_4 SAMs until 5%C18. 10%C18 SAMs induce less leukocyte adhesion than 5%C18, being comparable to 2.5%C18 SAMs. When SAMs are pre-immersed in HSA, there is a linear increase of leukocyte adhesion as the percentage of C18 increases until 10%C18. HFG and plasma pre-immersions presented similar results. In both cases no statistically significant differences were found between the background surface (EG_4 SAMs) and 1%C18, but surfaces with higher percentages of C18 (2.5%, 5% and 10%) presented higher adhesion of human leukocytes comparing to EG_4 SAMs.

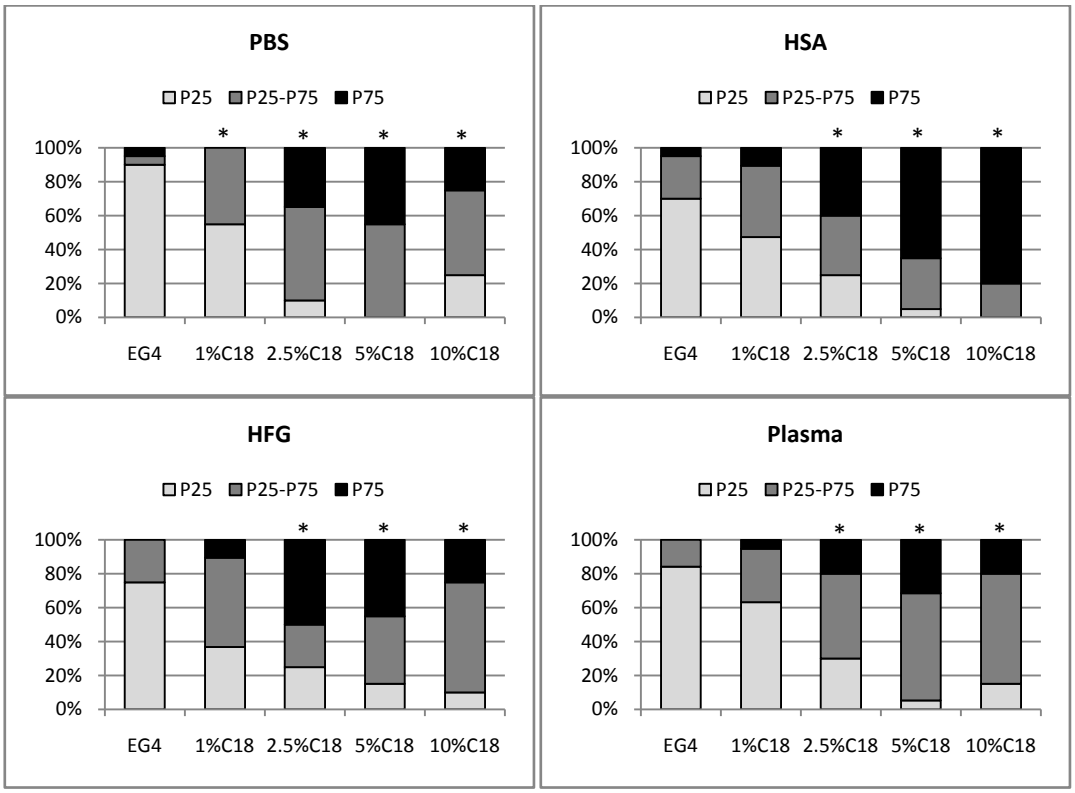

Figure 5 – Percentage of adherent leukocytes to each type of EG_4-C18 SAMs after different pre-immersions (PBS, HSA, HFG and plasma) represented as low adhesion (percentile 25 (P25)), medium adhesion (percentile 25 to percentile 75 (P25-P75)) and high adhesion (P75). Results show the influence of the amount of C18 ligands immobilized on EG_4 SAMs in each pre-immersion and were obtained using the medians of all 5 experiments. *significant differences ($p<0.05$) comparing to the background surface (EG_4 SAMs) of the same pre-immersion.

5. DISCUSSION

Leukocyte adhesion to artificial surfaces is an important phenomenon in the evaluation of biomaterials since adherent leukocytes are often related to inflammatory response after implantation.[15]

However, because implant surfaces spontaneously adsorb host proteins, this proteinaceous film is important in the subsequent attraction of leukocytes. Any pre-immersion treatment, and consequently pre-adsorbed proteins, are determinant.[3]

In the present study, adhesion of human leukocytes to EG_4 SAMs with different percentages of C18 ligands was investigated. The effect of plasma proteins on leukocyte adhesion was also assessed by pre-adsorbing no proteins (PBS), albumin, fibrinogen or plasma to the different surfaces.

Our results have demonstrated that EG_4 SAMs, the background surface, 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. in which SAMs containing three ethylene-oxide groups presented very low PMN leukocytes attachment.[15] It has been suggested that the ethylene oxide groups 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 cells.[16] 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.[17]

Results with EG_4-C18 SAMs showed that the adhesion of leukocytes increased as the percentage of immobilized C18 on the surface increased. It is important to bear in mind that C18 ligands are methyl terminated and wettability studies previously performed by contact angle measurements confirmed that EG_4-C18 SAMs become more hydrophobic as the amount of C18 ligands on the surface increase.[10] The contact angle of these SAMs ranges from 37.7° in hydrophilic EG_4 SAMs to 94.8° in hydrophobic 10%C18 SAMs.

Previous experimental observations by several authors are in accordance with our results since they revealed that hydrophobic surfaces adsorb higher amounts of PMN leukocytes.[15,18,19]

In contrast however, higher number of adherent leukocytes in hydrophilic OH-terminated SAMs and lower number in hydrophobic CH_3-terminated SAMs have been reported in *in vitro* studies by Sperling et al.[20] and both *in vitro* and *in vivo* by Barbosa et al.[3,21] and Lindblad et al.[2]. Sperling and co-workers believe that the complement activating potential of the OH-group bearing surfaces is the most important influence since complement activation has strong chemotactic effects on leukocytes.[20]

Another factor that may influence leukocyte adhesion to solid surfaces is surface free energy.[22] Neumann and co-workers showed enhanced leukocyte adhesion to surfaces with higher surface free energy or increased wettability.[23] 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), presented lower leukocyte adhesion than 10%C18 SAMs, that are hydrophobic and have lower surface free energy (28.3 mN/m).[10]

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 masking the surface properties of the underlying substrate.[15] Since protein adsorption from plasma-containing medium occurs rapidly, rendering direct recognition of surface functional groups by the leukocytes virtually impossible, functional groups are believed to affect cell adhesion indirectly via 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.[24]

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.[5,25] This process, called Vroman effect, is surface, time and plasma concentration dependent.[26] The first proteins to reach the surface are those present on plasma at higher concentration and with the lower molecular weight, which are subsequently replaced by other proteins with higher affinity to the surface.[27] 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, immunoglobumin G, fibrinogen, fibronectin and vitronectin adsorbed onto natural or synthetic surfaces.[5,28]

Elucidating the role of adsorbed and denatured protein in leukocyte adhesion and activation from the literature was found to be 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 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.[5] This effect of IgG in leukocyte adhesion has also been observed in studies by Collier et al., where the depletion of IgG from serum caused a significant decrease in initial adherent cell density.[4] 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.[29] Wattero et al. 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.[19]

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.[14,28] Adhesion of leukocytes to adsorbed fibrinogen is mediated by the β_2-integrin Mac-1[14,30] and intercellular adhesion molecule-1 (ICAM-1)[31], whereas fibrinogen-induced activation of these cells is mediated primarily by toll-like receptor-4.[32] 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.[33]

According to Jones et al., the adhesion of PMN leukocytes to immobilized fibrinogen is mediated by β2-integrins and their binding to the Arg-Gly-Asp (RGD)-containing peptide sequence present in the fibrinogen molecule.[34] However, Tang et al.[14], reported that the proinflammatory activity of fibrinogen resides within the D fragment, which contains neither the fibrin cross-linking sites nor RGD sequences, being that the major (and, perhaps, exclusive) proinflammatory sequence appears to be fibrinogen γ190-202. Altieri et al. have previously shown that this sequence mediates the binding of leukocytes via the Mac-1 (CD11b/CD18) β_2-integrin.[30]

Results herein presented do not reveal fibrinogen as a pro-inflammatory agent since no increase in leukocyte adhesion was verified in any of the surfaces when fibrinogen was pre-adsorbed comparing to when no proteins were pre-adsorbed. This was not expected based on the protein adsorption studies previously performed on these samples. EG_4-C18 SAMs have shown that protein adsorption increases as the % of C18 increases, and that EG_4 SAMs adsorb very low amounts of protein.

Competition studies using both albumin and fibrinogen in solution demonstrated that all the surfaces adsorb mainly albumin, and only 5%C18 and 10%C18 adsorb fibrinogen.[10] These results are in accordance with the generally accepted observation that hydrophobic surfaces adsorb more fibrinogen than hydrophilic surfaces.[13] The initial inflammatory activation by fibrinogen bound to the surface was therefore expected to be low on hydroxylated surfaces and high on hydrophobic ones. Given this, after pre-immersion in a fibrinogen solution no differences in leukocyte adhesion would be expected in EG_4 SAMs and samples with lower percentages of C18, but for hydrophobic 5%C18 and 10%C18 SAMs, which adsorb fibrinogen, higher leukocyte adhesion would be expected.

Lindblad et al. have postulated that it is possible that fibrinogen bound to the hydrophobic surface undergoes degradation before the arrival of the cells, thus offering a lower number of accessible cell binding sites. [2] In the same trend, Tegoulia et al. believe that the HFG conformation on the substrates is such that the RGD domain is not immediately available to the leukocyte receptors.[15]

The extent to which a protein unfolds during and after the adsorption process depends particularly 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 get internalized in the molecule, and polar residues get exposed at the surface. In general, for adsorption to substrates with hydrophilic surfaces, water-soluble proteins bind via their polar (surface) residues, and a layer of water molecules is embedded between the protein and the substrate. Adsorption of these proteins to substrates with hydrophobic surfaces, however, results in protein unfolding and binding via their non-polar (interior) residues directly to the substrate.[33] Previous results performed in EG_4-C18 SAMs have confirmed this, and revealed that around 80% of the albumin adsorbed to EG_4 SAMs can be exchanged by other albumin molecules, implying that it is adsorbed in a reversible way, whereas in hydrophobic 10%C18 SAMs only 20% of the adsorbed albumin is exchangeable, the remaining 80% being irreversibly bound and therefore probably denatured on the surface.[10]

Eventhough albumin is not known to contain any specific ligands for leukocyte adhesion[5,33], Brevig et al. 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.[33] Also, the latter group has performed studies showing that pre-denatured albumin was consistently more potent as cell-adhesion substrate when adsorbed to a hydrophobic than to a hydrophilic

surface. The high degree of albumin denaturation on 10%C18 SAMs with pre-adsorbed albumin is probably responsible for the high leukocyte adhesion to these surfaces.

The lack of significant differences in leukocyte adhesion density between the studied surfaces most likely results from protein masking. It should be noted that even nonpreadsorbed surfaces (PBS pre-immersion) will be rapidly adsorbed with plasma proteins when in contact with the leukocytes culture medium. When the material contacts a protein-rich environment like the 10% plasma supplemented medium that was used in our experiments, a protein layer immediately adsorbs to it. In this work we have studied only the influence of pre-adsorbed albumin and fibrinogen in leukocyte adhesion. However, it is important to consider that other proteins are also present in the plasma supplemented medium and may also adsorb and lead to leukocyte adhesion.

6. CONCLUSIONS

EG_4 SAMs with different amounts of immobilized C18 ligands were prepared and leukocyte adhesion to these surfaces was studied. EG_4 SAMs proved to be a good background surface to study the influence of C18 ligands on leukocyte adhesion, since negligible amounts of leukocytes adhered to these surfaces. It was showed that leukocyte adhesion increased with the increase of C18 ligands on the surface. The effect of pre-adsorbed proteins (albumin, fibrinogen or plasma) on leukocyte adhesion was also evaluated. Except for albumin pre-immersion, that induced higher leukocyte adhesion in samples completely covered by C18 ligands (10%C18 SAMs), no major differences were observed in the different pre-immersion conditions.

7. ACKNOWLEDGEMENTS

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CHAPTER VI

PROTEIN ADSORPTION AND CLOTTING TIME OF pHEMA HYDROGELS MODIFIED WITH C18 LIGANDS TO ADSORB ALBUMIN SELECTIVELY AND REVERSIBLY

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Submitted for publication

1. ABSTRACT

This work intended to create a nanostructured biomaterial that would bind albumin in a selective and reversible way in order to inhibit the adsorption of other blood proteins and therefore minimize activation of coagulation. Different levels of C18 ligand have been immobilized on poly(2-hydroxyethyl methacrylate) (pHEMA). We hypothesize that samples with intermediate amounts of C18 ligand would allow albumin to recognize them and bind through its hydrophobic pockets specific for long chain fatty acids. Surface characterization has confirmed increasing amounts of C18 ligand on pHEMA as the percentage of C18 in solution increases, with maximum coverage achieved in 10%C18 samples. Adsorption from pure albumin solution revealed a small decrease in albumin adsorption from pHEMA to 1%C18 and 2.5%C18 samples, but on surfaces with 5% or higher C18 the amount of adsorbed albumin increased as the percentage of C18 increased. Competitive adsorption studies in the presence of both albumin and fibrinogen, and in the presence of all plasma proteins showed that 1%C18 and 2.5%C18 were the only surfaces selective for albumin, and that the presence of all plasma proteins may even potentiate albumin adsorption. Reversibility studies demonstrated that both 2.5%C18 and 5%C18 samples exchange ^{125}I-albumin selectively in the presence of both unlabeled albumin and plasma, but 2.5%C18 samples presented higher exchangeability rates (58%). Clotting times using recalcified plasma revealed that samples with none or small amounts of C18 (pHEMA to 5%C18) did not shorten the clotting time compared to the negative control (polystyrene), indicating low activation of the intrinsic coagulation cascade.

KEYWORDS: poly(2-hydroxyethyl methacrylate), surface modification, protein adsorption, human serum albumin, clotting time

2. INTRODUCTION

When a foreign material comes into contact with blood, it rapidly adsorbs proteins onto its surface, and the adsorbed protein layer dictates subsequent cell and platelet responses. Albumin coatings have long been thought to passivate polymer surfaces since a pre-adsorbed layer of albumin inhibits subsequent adhesion of fibrinogen, producing a potentially thromboresistive effect.[1] Several groups have developed different methods to modify polymeric surfaces in order to improve albumin binding to surfaces.[2-9] However, problems associated with denaturation of albumin over time or exchange of the albumin with other proteins in solution make this strategy only satisfactory for short term use.

Noting that albumin in whole blood has a high affinity for circulating free fatty acids, Munro et al proposed the covalent binding of 16 to 18 carbon alkyl chains to polymers to create a surface selective for this protein.[7] Besides selectivity in albumin adsorption, exchangeability is also an important issue since a renewable albumin layer would prevent albumin denaturation on the surface. The use of a non-fouling background surface is thus important since it would create protein-resistant surfaces by avoiding non-specific protein adsorption; albumin affinity could then be achieved by immobilization of the alkyl chains on the surface. The use of non-fouling polymers on their own has also been suggested as a strategy to increase hemocompatibility of medical devices, but maintaining low fouling has proven difficult in long-term applications.

Another relevant aspect when immobilizing the alkyl chains is their concentration on the surface, so that albumin can actually recognize them and bind. If the surface is fully covered with C18 ligands, specificity is lost, probably due to steric considerations, and albumin will not bind in a selective and reversible way. It has been shown that ethylene glycol self-assembled monolayers (EG_4 SAMs) displaying a specific level of albumin-binding ligands (2.5% C18) have a higher affinity for albumin than to fibrinogen and are able to recruit albumin selectively from plasma and therefore minimize the adhesion and activation of platelets.[10]

SAMs are spontaneously organizing structures that have intrinsic stability and order, allowing precision molecular engineering. Their well defined stoichiometry facilitates their use as excellent model surfaces.[11] However, medical devices are made from bulk polymeric materials, and therefore successful transition of model SAM structures to real-world polymers is desirable. For that, and so

that we could use the same isocyanate chemistry employed in SAMs, we selected a polymer with available hydroxyl groups.

Kwok et al. [12] have previously reported a one step procedure method for preparing SAMs on polymeric biomaterials used in medical devices by constructing SAM structures on poly(2-hydroxyethyl methacrylate) (pHEMA). This polymer has a hydroxyl group in its side chain, thus allowing various modifications of the surface using primary alcohol chemistry. The pHEMA surface was derivatized using dibutyltin dilaurate-catalyzed isocyanate chemistry to form urethane linkages, the same chemistry we have used in former studies with SAMs.[10,13] For these reasons and because pHEMA is known as a low fouling polymer[14], an essential characteristic when designing a surface for selective and reversible protein adsorption, we have chosen this polymer for C18 ligand immobilization.

In this work we aimed to create polymers with different surface concentrations of immobilized C18 ligand, since not only the ligand but also its concentration, influences albumin selective and reversible adsorption. Albumin adsorption and exchangeability were studied in the presence of fibrinogen and other plasma proteins. The clotting time of recalcified plasma was also determined to investigate how the variation of the surface concentration of C18 on pHEMA impacts blood interactions.

3. MATERIALS AND METHODS

3.1 Preparation of pHEMA cross-linked gels

Cross-linked poly (2-hydroxyethyl methacrylate) (pHEMA) films were synthesized as previously described.[12,15] Briefly, 5 ml of 2-hydroxyethyl methacrylate monomer (HEMA; >99.5%, Polysciences, no. 04675) and 0.23 ml of tetraethylene glycol dimethacrylate (TEGDMA; Polysciences, no. 02654) cross-linking agent were added to a water/ethylene glycol (Sigma Aldrich, no. 9300) mixed solvent (1 ml/1.5 ml) with 1 ml of 40% ammonium persulfate (APS; 98+%, Aldrich, no. 24,861-4) and 15% sodium metabisulfite (SMB; 97+%, Aldrich, no. 25.555-6) as redox initiators to begin the radical

polymerization. The mixture was poured between two clean glass plates with a 1 mm (0.025 in) thick Teflon gasket and allowed to polymerize overnight (although the gel sets within an hour). The pHEMA film was released from the glass plates and soaked in distilled water for 4 hours (water renewed every hour) to leach out unreacted monomers, initiators and oligomer residues. The film was then cut into 8 mm diameter discs with a punch and pHEMA discs were vacuum-dried between two Teflon sheets and glass slides in order to flatten the surface. The samples were maintained in argon and, the day before derivatization, were rinsed twice in fresh THF for 2 min in an ultrasonic bath to remove potential contaminants. Finallly, they were vacuum-dried prior to surface derivatization with C18 ligands, because water molecules terminate the urethane linkage reaction between the hydroxyl group on the pHEMA surface and the C18 isocyanate.

3.2 Immobilization of C18 ligands in pHEMA

Solutions with different percentages (0%, 1%, 2.5%, 5%, 10% and 20%) of octadecyl isocyanate (C18; 98%, Aldrich) were prepared in tetrahydrofuran (THF; 99.9%, Aldrich) with 0.1% dibutyltin dilaurate (95%, Aldrich) as a catalyst, as described in previous work.[13] pHEMA discs were then immersed in the C18 solutions under a nitrogen atmosphere and incubated at 40°C for 2 hours with shaking (300 rpm). Afterwards, pHEMA-C18 samples were rinsed twice with fresh THF for 2 min in an ultrasonic bath to remove physically adsorbed C18 isocyanate. SAMs were blown dry with a stream of argon and maintained in a nitrogen environment until analyzed. The derivatized polymers will be identified by the percentage of C18 solution in which pHEMA was immersed (e.g. pHEMA immersed in 1%C18 solution will be designated 1%C18). The chemical structure of pHEMA-C18 is presented in Figure 1.

Control samples (0%C18) have been prepared, surface characterized and used in protein adsorption experiments, but since results were always identical to pHEMA, results will not be shown.

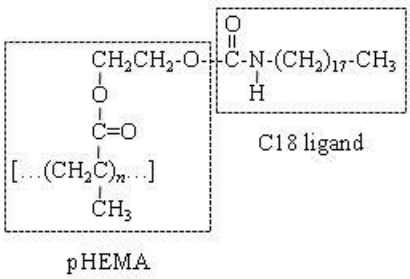


Figure 1 – Chemical structure of pHEMA derivatized with C18 isocyanate via urethane linkage.

3.3 Surface Characterization

The surface of pHEMA-C18 samples was analyzed by X-ray photoelectron spectroscopy (XPS), Fourier transform infrared spectroscopy with attenuated total reflectance (FTIR-ATR) and scanning electron microscopy (SEM). XPS was used to determine the chemical composition on the surface. FTIR-ATR was used to investigate functional groups and chain order/crystalline structure. SEM was used to acquire information on the topography of the surfaces.

3.3.1 XPS

Measurements were performed with a VG Scientific ESCALAB 200A (UK) spectrometer using a Al $K\alpha_{1,2}$ X-ray source to stimulate photoemission. All polymer samples were analyzed at a 90° takeoff angle (relative to surface sample), probing about 100 Å of the uppermost surface. Survey scans (0-1100 eV binding energy) were run at an analyzer pass energy of 50 eV with an X-ray spot size area of 10 mm^2 to determine the elemental composition of each surface. High resolution C(1s), O(1s) and N(1s) scans were obtained at a pass energy of 20 eV. The high resolution spectra were resolved into individual Gaussian peaks using an XPS peak fitting program (XPSPEAK version 4.1). All binding energies (BEs) were referenced by setting the maximum of the resolved C(1s) peak, corresponding to carbon in a hydrocarbon environment (CH_x), to 285.0 eV.

3.3.2 FTIR-ATR

Measurements were performed on a Perkin Elmer FTIR spectrophotometer, model 2000, coupled with a ATR accessory (SplitPea™ from Harrick Scientific) provided with a silicon internal reflection crystal and configured for external reflectance mode. All data were taken at 2 cm^{-1} resolution with a

total of 100 scans. Measurements were performed in the mid-IR frequency range (4000-600) and spectra were baseline corrected and smoothed.

3.3.3 SEM/EDS

The surface of all pHEMA-C18 samples was visualized by scanning electron microscopy (SEM) under different magnifications (200, 500 and 2000x) and photographed. pHEMA and 20%C18 samples were also fractured and their transversal sections were observed by SEM and analyzed by energy dispersive spectrometry (EDS) to determine the elemental composition of each zone of the sample.

Samples were coated with a thin layer of gold by sputtering. Observation was performed in a FEI QUANTA 400 FEG environmental scanning electron microscope coupled with a EDAX PEGASUS X4M using an accelerating voltage of 15 kV.

3.4 Protein adsorption to pHEMA-C18

3.4.1 Selective and competitive adsorption

Adsorption of proteins to biomaterials usually occurs from complex, multicomponent mixtures and involves competition of all the proteins in the mixture for the available surface sites. However, the relative effectiveness of the various proteins in the mixture in competing for occupancy of the surface sites is not readily measured from such complex mixtures.[16] In this work we intended to evaluate albumin adsorption to pHEMA-C18 samples and verify if albumin would preferentially adsorb to the surfaces in the presence of fibrinogen and all plasma proteins. Therefore, we performed adsorption from a pure albumin solution ([HSA] = 0.4 mg/ml) and two competitive adsorption studies, one from a binary mixture of albumin ([HSA] = 0.4 mg/ml) and fibrinogen ([HFG] = 0.03 mg/ml) in the same ratio as present in blood, and one from a complex mixture of blood plasma proteins (1% plasma having approximately 0.4mg/ml of albumin). The three studies were performed using the same radioactive labeled protein, albumin.

Human serum albumin (HSA; Sigma, ref. A1653) and human fibrinogen (HFG; Sigma, ref. F4129) were obtained as lyophilized powders. Human plasma was kindly provided by the Portuguese Blood Institute and was obtained from whole blood of nonmedicated healthy donors collected to a bag with CPDA1 anticoagulant (Macopharma). The buffer used in all protein adsorption experiments was a

freshly prepared citrate phosphate-buffered saline solution (cPBSzI) at pH 7.4, containing 0.01M citrate, 0.01M phosphate, 0.012M sodium chloride, 0.02% sodium azide and 0.01M NaI. The citrate acts as both buffer and calcium chelator, the latter function acting to inhibit the calcium-dependent proteases common to blood or blood products. The azide inhibits the growth of species requiring oxidative phosphorylation to grow. The sodium iodide prevents nonspecific uptake of free ^{125}I during protein adsorption.

HSA was labeled with ^{125}I using the Iodogen method, as described in a previous work.[13] The yield of the iodination reaction was 96% and was determined by precipitating the ^{125}I-HSA with 20% trichloroacetic acid (TCA; Merck).

The protein stock solutions were prepared by adding ^{125}I-labeled protein (HSA) to unlabeled protein solution (HSA, HSA+HFG or plasma) in order to obtain a final activity of 5 cpm/ng. These protein stock solutions had twice the desired final concentration, since the adsorption protocol requires mixing the protein with an equal volume of buffer that is in contact with the surface.

Samples were placed in 2 ml polystyrene cups containing 0.5 ml cPBSzI buffer and hydrated for 2 hours at 37°C. Adsorptions were initiated by adding 0.5 ml of 2x concentrated protein stock solution to the cups where the samples were being hydrated. After 1 hour at 37°C, adsorption was terminated by rinsing the samples via dilution-displacement of the protein solution with buffer. The dilution-displacement technique was performed by flowing approximately 20 volumes of buffer from an overhead reservoir through the cup containing the sample and the radioactive protein solution (the rinsing apparatus is similar to the one described by Horbet[16]). The protein solution was thus rapidly displaced, and exposure of the sample to an air-water interface was avoided. After rinsing, each sample was placed in a counting tube containing cPBSzI, and the retained radioactivity measured in a γ-counter to obtain the “initial rinse”. The tubes were then re-incubated at 37°C and 24h later the samples were rinsed again with cPBSzI and transferred to new counting tubes containing buffer and recounted to obtain the “24h soak” data. This soaking step was performed in order to minimize possible artifacts in protein adsorption, because even though NaI was added to the buffer solution, since pHEMA is a hydrogel, it might have the potential of retaining ^{125}I in its gel bulk. We performed an experiment (data not shown) with unlabeled HSA in the presence of free ^{125}I using PBS and PBSI (PBS with NaI) that revealed that the presence of NaI in the buffer did not decrease the

adsorption/absorption of free ^{125}I. However, the 24h soak released 97% of the free ^{125}I adsorbed/absorbed to the pHEMA.

HSA concentration was calculated by the following equation:

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

where the counts are the radioactivity measurements from the surfaces, the $C_{solution}$ and $A_{solution}$ are the concentration and the specific activity of the protein solution, respectively, and SA is the surface geometrical area of the sample.

3.4.2 Reversible adsorption

Albumin that is reversibly bound to a surface by association with a fatty acid (in this case, a C18 ligand) should be displaceable upon the addition of exogenous albumin, whereas irreversibly bound albumin should not be displaced. Samples with adsorbed ^{125}I-HSA from pure albumin solution (after the 24h soak) were immersed in different solutions with unlabeled proteins (cPBSzI, 1mg/ml HSA, 1mg/ml HFG or 1% plasma) for 24h at 37°C. The purpose of this study was to evaluate the reversibility of albumin adsorption in buffer and its selective exchangeability by free solution albumin, by fibrinogen or by other plasma proteins. After rinsing with buffer, each sample was placed in a counting tube containing cPBSzI and the retained radioactivity measured in a γ-counter.

3.5 Clotting time

The surfaces tested were pHEMA-C18 samples, glass (positive control), polystyrene (PS; well plate only - negative control) and some reference materials: medical grade poly(etherurethane) (PU; Pellethane 80 AE; Dow Chemical), poly(ethylene terephthalate) (PET; Goodfellow LS 179175NL, ES301400/9) and poly(dimethyl siloxane) (silicone rubber; NHLBI-DTB, Thoratec Laboratories Corporation). The reference polymers were cut into 8mm discs, rinsed in ethanol for 15 min and dried before use.

The recalcified plasma clotting time protocol was similar to that reported by Cao et al.[17] A 1M stock solution of $CaCl_2$ was prepared and added to human plasma (citrate anticoagulated; thawed at 37°C)

in order to obtain a final calcium concentration of 20mM. The recalcified plasma was then quickly mixed in a vortex, and 200µl plasma was immediately added to each well of the 48-well plate containing the samples. 48-well non-tissue culture treated PS plates were used. The 48 well plates were placed in an incubator at 37°C with shaking (150rpm) and the clotting time of the plasma was determined visually. The recalcified plasma clotting time was measured as the time it took for the plasma to undergo gelation, detected by loss of movement of the plasma in response to the rotation and shaking.

4. RESULTS

4.1 Surface characterization

A typical XPS spectrum of pHEMA is shown in Figure 2 (a), revealing that only carbon (C) and oxygen (O) are present on the surface, as expected. Figure 2 (b) is the spectrum of a pHEMA disc after immobilization of C18 ligands from a 20%C18 solution. We observe that, compared to the unreacted pHEMA sample, there is an increase of C relative to O. Furthermore, besides the C and O, nitrogen (N) is also present, indicating immobilization of the C18 ligands since the N is attributed to the post-reaction urethane linkage.

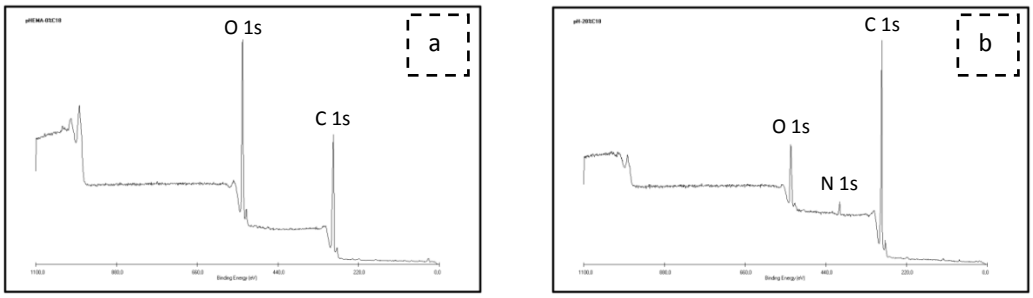
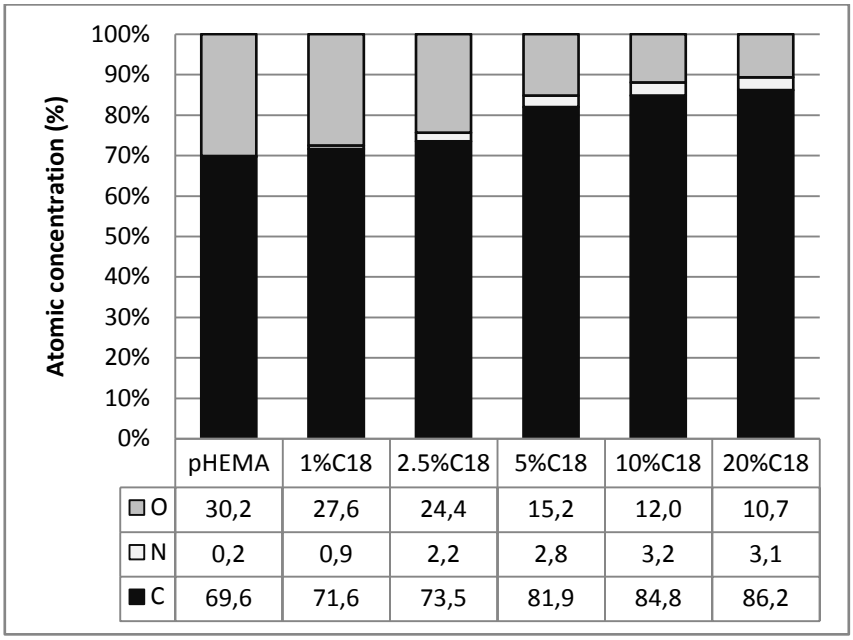


Figure 2 – Typical XPS spectra of pHEMA (a) and pHEMA derivatized with 20%C18 (b).

Figure 3 shows the atomic concentrations of pHEMA-C18 samples. The pHEMA films have about 70% of carbon and 30% of oxygen, a C/O ratio of 2.3, and present negligible levels of nitrogen within the experimental error of the technique. Results also demonstrate that as the percentage of immobilized C18 increases, there is a decrease of O and increase of C and N, resulting from the reaction between the hydroxyl groups from the pHEMA with the octadecyl isocyanate compound (C18).



	pHEMA	1%C18	2.5%C18	5%C18	10%C18	20%C18
O	30,2	27,6	24,4	15,2	12,0	10,7
N	0,2	0,9	2,2	2,8	3,2	3,1
C	69,6	71,6	73,5	81,9	84,8	86,2

Figure 3 – XPS atomic concentrations of the chemical components of pHEMA and pHEMA immobilized with increasing levels of C18 ligand.

Figure 4 shows representative high resolution C(1s) spectra from pHEMA and 20%C18, reflecting the types of carbon functional groups present. After spectral resolution into individual Gaussian peaks, it is possible to observe that both spectral envelopes are comprised of three primary C peaks: (1) a 285.0 eV peak corresponding to carbon with no bonds to oxygen (CH_x, hydrocarbons), (2) a 286.6 eV peak corresponding to carbon with one oxygen bond (C-O, ethers and alcohols), and (3) a 288.9 eV peak corresponding to carbons with three bonds to oxygen (O=C-O, esters). As expected, the 20%C18 spectrum shows increased CH_x peak amplitude, resulting from methylene chain addition (from the C18 ligands) via urethane bonding.

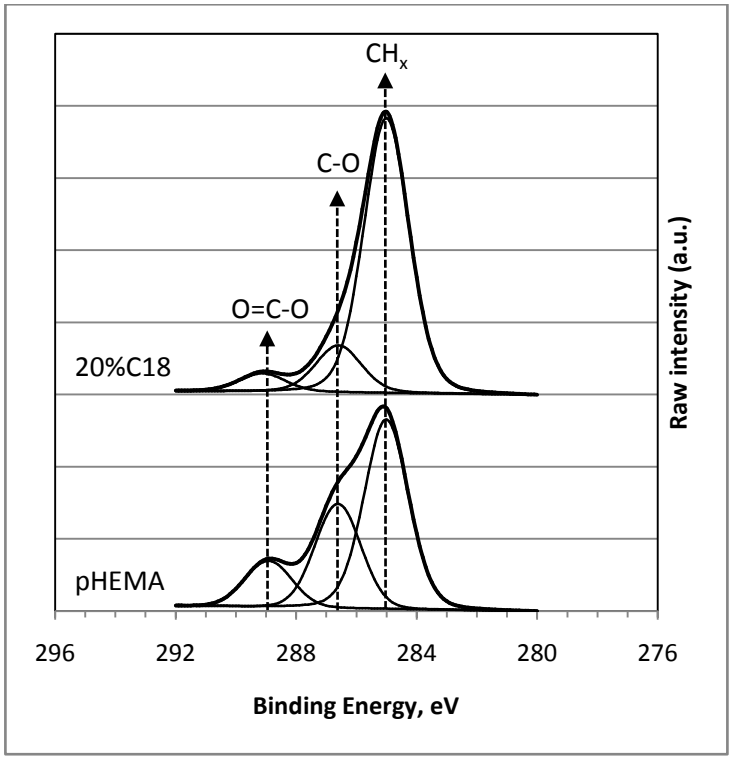


Figure 4 – Typical XPS high resolution C(1s) spectra of pHEMA (a)and pHEMA derivatized with 20%C18 (b). From left to right we can see the peaks corresponding to O=C-O, C-O and CH_x.

Figure 5 shows the low frequency regions of the ATR-FTIR spectra for the pHEMA-C18 samples. The characteristic pHEMA absorption bands at 1720 cm^{-1}, from the ester stretching band of the carboxyl group (C=O), at 1075 cm^{-1}, from the stretching band of the alcohol group (C-O), and at 1150 cm^{-1}, from the carboxylic acid esters, clearly show for the pHEMA gel. Furthermore, the later two bands decreased as the percentage of C18 increased, revealing immobilization of C18 ligands.

As the fraction of of C18 ligand on the surface increase, there is also an increase of the absorbance of the CNH stretching peak at 1530 cm^{-1} and the methylene scissoring vibration frequency at 1453 cm^{-1}.

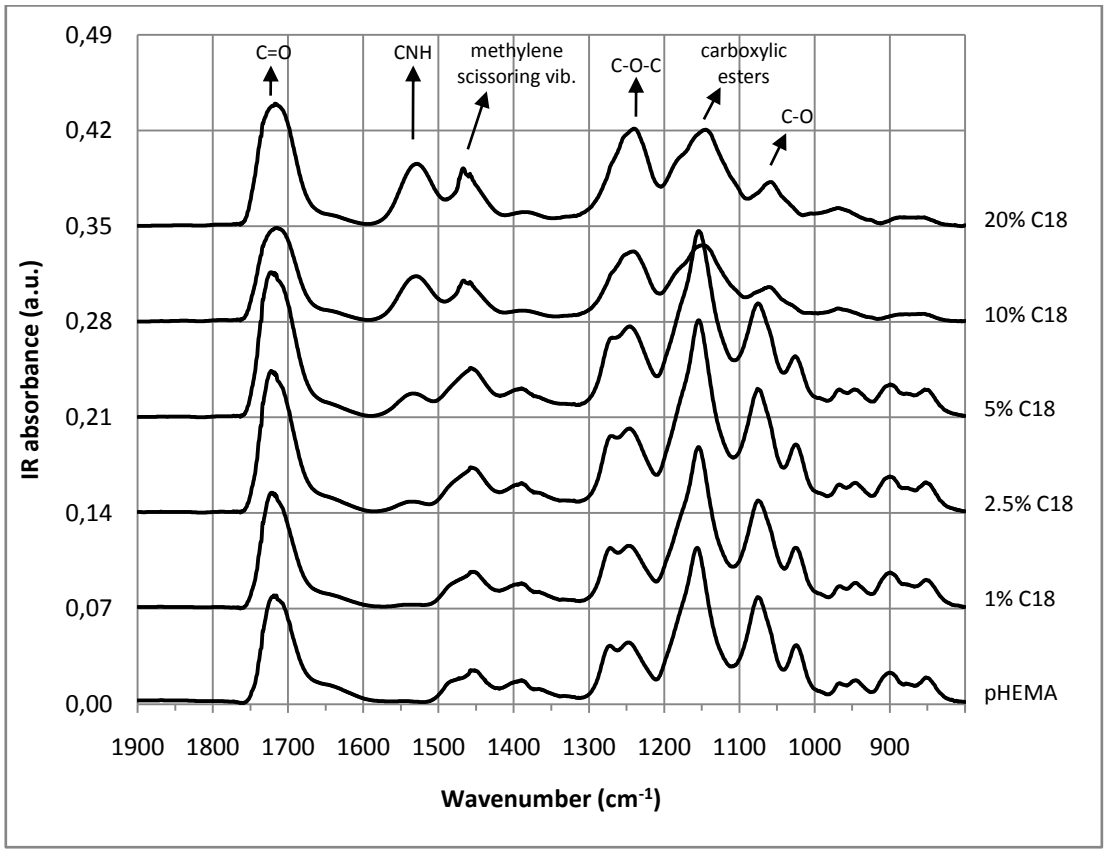


Figure 5 – Low frequency region from IR absorbance spectra of the derivatized pHEMA-C18 samples.

The high frequency region of the IR spectra of pHEMA with different levels of immobilized C18 is shown in Figure 6. As the amount of C18 ligand on the surface is increased, the strong, broad band around 3400 cm^{-1}, typical of the hydroxyl region of pHEMA, narrowed and shifted to the right, showing characteristics of an NH stretching peak. All the IR results obtained suggest a successful attachment of the C18 methylene chains to pHEMA via covalent urethane bonding.

Another important observation is the shift of the CH_2 stretching frequencies at 2850-2920 cm^{-1}. Figure 6 demonstrates that the asymmetric (νa) and symmetric (νs) CH_2 stretching bands increased in amplitude as the percentage of C18 increased. Moreover, in samples 10%C18 and 20%C18, both the CH_2 (νa) and CH_2 (νs) stretching frequencies were found to be stabilized at 2919 and 2851 cm^{-1},

typical peak frequencies of CH_2 units in the trans state, indicating the immobilized methylene chains ordered themselves in a crystalline structure.

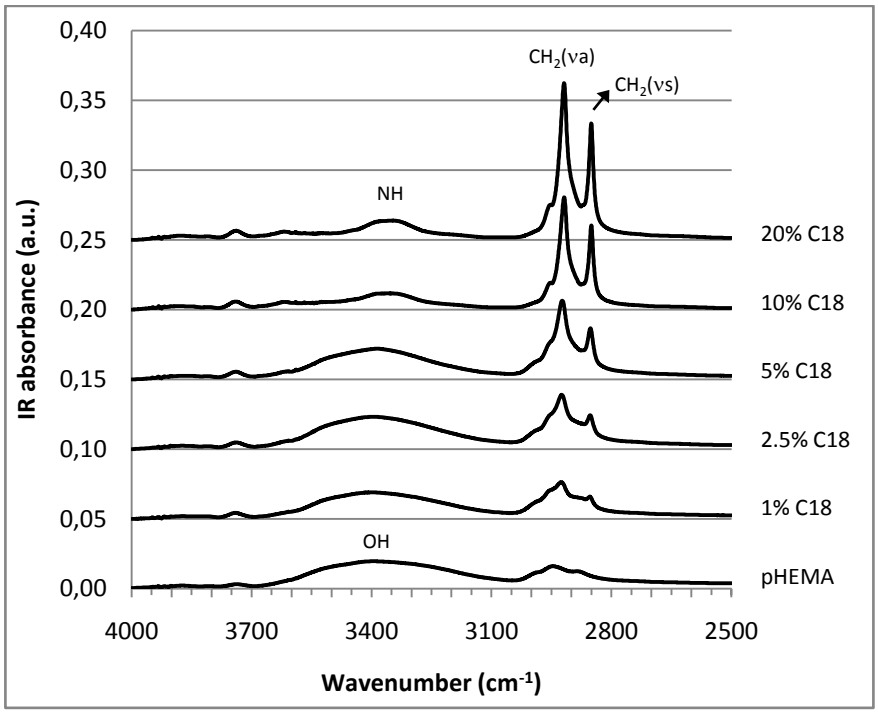


Figure 6 – High frequency region of the IR absorbance spectra of the derivatized pHEMA-C18 samples.

The IR spectra were also checked for characteristic bands for allophanate formation during the surface reaction. Allophanate is formed by an attack on the active hydrogen in the urethane bond with a free isocyanate molecule, and can lead to disorganized, multilayer films. Allophanates are normally characterized by a triplet of intense bands at 1220, 1280 and 1310 cm^{-1} associated with the skeletal vibrations of the allophanate group as well as unique NH bands at 3298, 3267 and 3233 cm^{-1}.[18] The absence of these bands in the IR spectra demonstrated that undesirable allophanate formation did not occur. Furthermore, the absence of the NCO band at 2270 cm^{-1} confirmed that samples were well rinsed and no free isocyanates were physisorbed to the surface (data not shown).

pHEMA-C18 discs were observed by SEM and representative images were taken. Figure 7 reveals that pHEMA discs have a smooth surface, and that immobilization of 1%C18 and 2.5%C18 does not lead to

changes that can be visualized by SEM, i.e., the surface is similar to pHEMA. In 5%C18 samples, however, it is possible to observe that microscopic features appear on the surface. Total surface coverage (as suggested by XPS and IR) is achieved in 10%C18 and 20%C18 samples, even though these samples have a significantly reticulated surface.

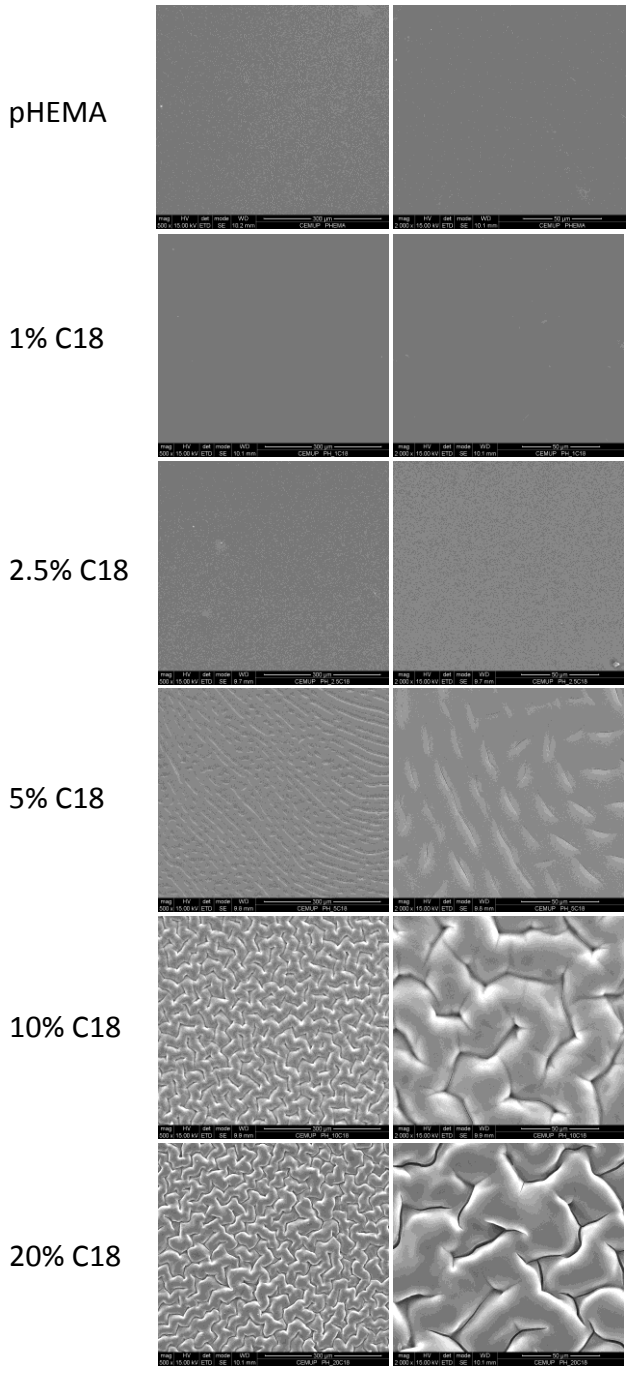


Figure 7 – Scanning electron micrographs at two different magnifications (left image: 500x; right image: 2000x) of a pHEMA crosslinked gel and pHEMA derivatized with different levels of C18 ligands.

In order to evaluate how deep the C18 ligands were penetrating into the pHEMA, pHEMA and 20%C18 samples were fractured and observed by SEM. Figure 8 shows their transversal section and EDS analysis. The image of the pHEMA cross-section confirms that the surface is microscopically smooth. The surface of 20%C18 samples is rough and the changes in morphology resulting from C18 ligand immobilization are clearly visible. EDS enabled us to confirm that zone 1 (Z1) from 20%C18 has less oxygen (second peak) than zone 2 (Z2) and zone 1(Z1) from pHEMA, indicating penetration of C18 ligands into the upper ~20 µm of pHEMA. The gold peak (third peak) present in the EDS spectra results from sample treatment for SEM visualization (sputtered layer of gold).

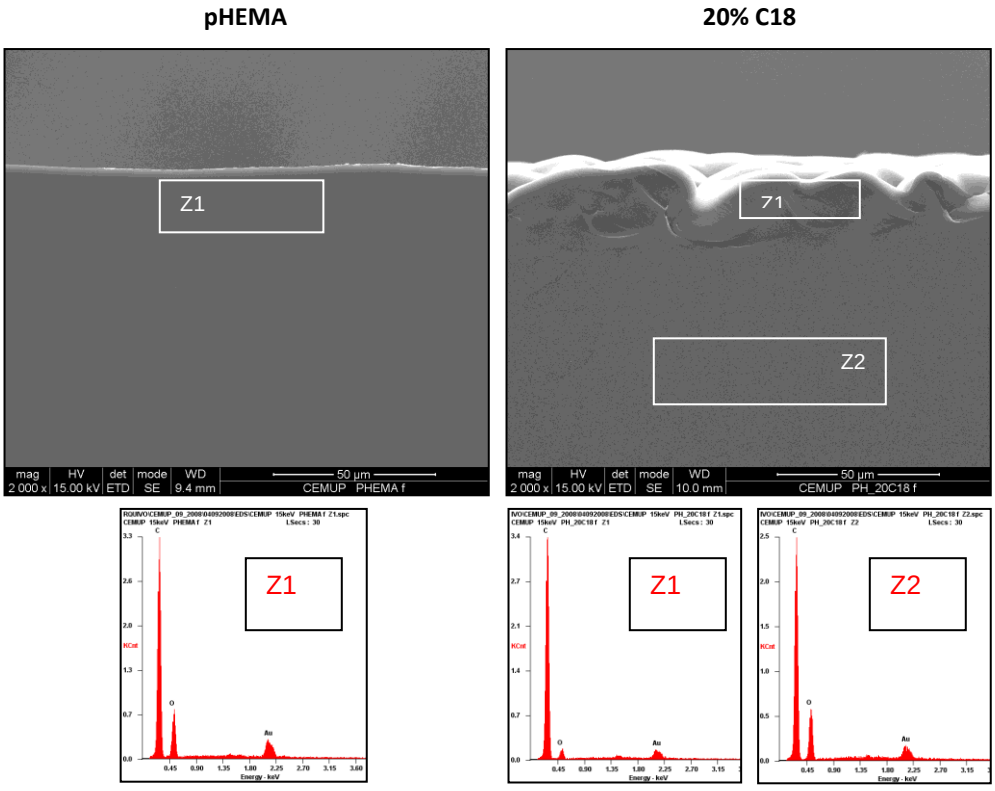


Figure 8 – Scanning electron micrographs of transversal sections of pHEMA and 20%C18. The EDS x-ray spectra from the zones (Z) marked in the pictures are represented below each picture.

4.2 Protein adsorption

The adsorption of human serum albumin (HSA) onto pHEMA-C18 samples from a pure albumin solution (0.4 mg/ml) is presented in Figure 9. When, after 1h protein adsorption, only the initial rinse is performed, about 750 ng of HSA/cm^2 are found on pHEMA. No statistically significant differences were found between this control polymer and 1%C18, 2.5%C18 and 5%C18 samples. However, after the 24h soaking step, only approximately 100 ng/cm^2 remain adsorbed to pHEMA. A similar decrease of around 90% was verified on 1%C18 and 2.5%C18, but samples with 5%C18 presented a smaller reduction (about 83%) after the 24h soak. The pHEMA discs with 10%C18 and 20%C18 had roughly half the initial adsorbed amount compared to the other surfaces after the initial rinse, and behaved very differently after the 24h soak since most of the albumin remained adsorbed (78% and 82%, respectively).

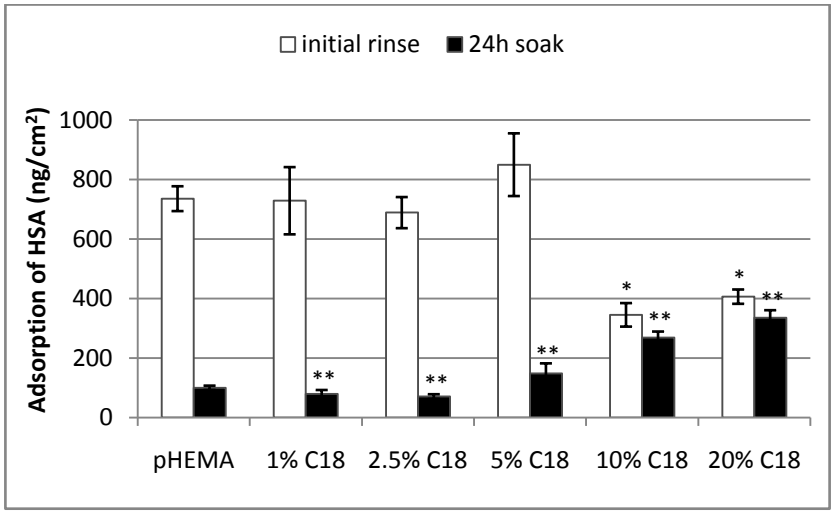


Figure 9 - Albumin adsorption to pHEMA-C18 discs. The "Initial rinse" refers to to the albumin adsorbed to the samples after 1h adsorption from albumin pure solution (0.4mg/ml) at 37°C and following rinsing with cPBSzI buffer. The "24h soak" refers to the albumin adsorbed to the samples after submitting the samples that were rinsed after adsorption (initial rinse) to a 24 hour soak in buffer and then rinsing them again. Within each surface, adsorption values are always statistically different ($p<0.05$) between the initial rinse and 24h soak; Independent-samples T Test. *,**Significant differences ($p\leq0.05$) compared to initial rinse pHEMA surfaces (*) and 24h soak (**); One-Way ANOVA.

Figure 10 shows albumin adsorption to pHEMA-C18 samples from different protein stock solutions. It illustrates the competition between albumin and fibrinogen (HSA+HFG) and between albumin and other plasma proteins (plasma).

Looking first at the influence of C18 ligands on albumin adsorption from each protein solution, it is possible to observe that, when a pure HSA solution (black bars) is used, there is a statistically significant small decrease from pHEMA to 1%C18 and 2.5%C18. On surfaces with 5%C18 or higher levels of C18 there is an increase in albumin adsorption as the percentage of C18 ligands increase. When both albumin and fibrinogen are in solution (dark grey bars), 1%C18 and 2.5%C18 adsorb less albumin than pHEMA. Surfaces with 5%C18 adsorb the same amount as the former. Only 10%C18 and 20%C18 increased the adsorption of albumin compared to pHEMA. Finally, when albumin adsorption occurs from a plasma solution (light grey bars), values are the same for pHEMA, 1%C18 and 2.5%C18 and increase as the concentration of C18 ligand increase from 5%C18 onwards.

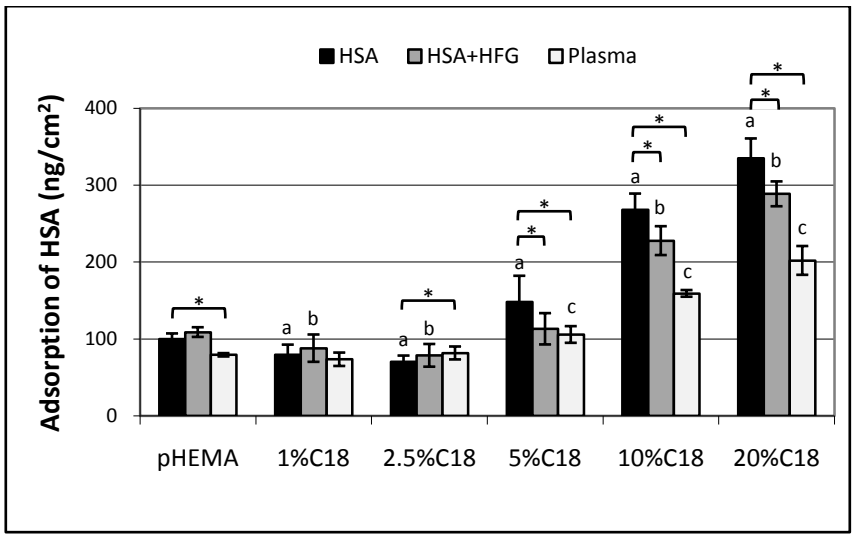


Figure 10 – Competitive adsorption of proteins to pHEMA-C18 discs. The bars represent the albumin adsorbed (ng/cm^2) from pure 0.4 mg/ml HSA solution (HSA), from a solution with both 0.4 mg/ml HSA and 0.03 mg/ml HFG (HSA+HFG) and from a 1% plasma solution (that has 0.4mg/ml HSA). Adsorption was performed for 1 hour at 37°C and samples were rinsed, soaked in buffer for 24h, rinsed again and then counted for radioactivity. *Statistically significant different samples ($p \leq 0.06$); Mann-Whitney test. $^{a, b, c}$ Samples significantly different from pHEMA in HSA, HSA+HFG and plasma solutions, respectively ($p \leq 0.05$); Mann-Whitney test.

When comparing adsorption from mixed protein solution (HSA+HFG) and plasma solution to adsorption from pure albumin solution on the same surface, it is possible to observe (Figure 10) that, on pHEMA discs, albumin adsorption remains at the same level when fibrinogen is present, but decreases in the presence of the plasma protein complex mixture. In 1%C18 samples, adsorption of albumin was not influenced by the presence of competitive protein since adsorption values are statistically the same, indicating that these surfaces are selective for albumin. Samples with 2.5%C18 showed no difference when both albumin and fibrinogen were present, but revealed a statistically significant increase in albumin adsorption from plasma solution, suggesting that other proteins enhance albumin adsorption to this surface. Discs with 5%C18 presented a similar 25% decrease in adsorption from both mixed protein and plasma solutions, suggesting that in addition to albumin, fibrinogen, but little of other proteins, are adsorbing to the surface. Both 10%C18 and 20%C18 exhibited a decrease in albumin adsorption when fibrinogen was present, meaning that this protein is competing with albumin for surface adsorption. An even larger decrease in albumin was seen when adsorption occurred from a plasma solution, indicating that other proteins besides fibrinogen are also adsorbing to these surfaces.

The reversibility of albumin adsorption to different surfaces was evaluated by the exchange of preadsorbed ^{125}I-labeled HSA by unlabeled HSA, HFG and plasma in solution. Figure 11 shows that about 36% of the albumin preadsorbed to pHEMA is reversibly bound, but does not demonstrate a selective exchangeability since retention values are the same regardless of the exchange solution used. Samples with 1%C18 present higher exchangeability of albumin when immersed in an albumin solution (65% exchange) then when immersed in any of the other solutions (50% exchange), indicating that albumin exchanges for albumin when only this protein is present in solution, but when all other plasma proteins are present it does not exchange selectively. 2.5%C18 and 5%C18 samples seem to be selective for albumin in their exchange since retention is lower in both HSA and plasma solutions ($p<0.09$) than in buffer, but no statistically significant differences were found after immersion in buffer and HFG solutions. However, exchangeability values of 2.5%C18 (58% in HSA and plasma) are higher than on 5%C18 (46% in HSA and plasma), meaning that more ^{125}I-HSA remains irreversibly adsorbed on the last surface. The samples that showed the lowest exchangeability are 10%C18 and 20%C18, with retention values in buffer around 95% and 100%, respectively. Thus, despite their selectivity in albumin exchange by solution albumin (either from pure HSA solution or

from plasma), about 80% of the preadsorbed albumin is still irreversibly bound. Furthermore, these surfaces, unlike the others, exchange 5%-10% of albumin with fibrinogen, since there is a decrease of retention compared to buffer.

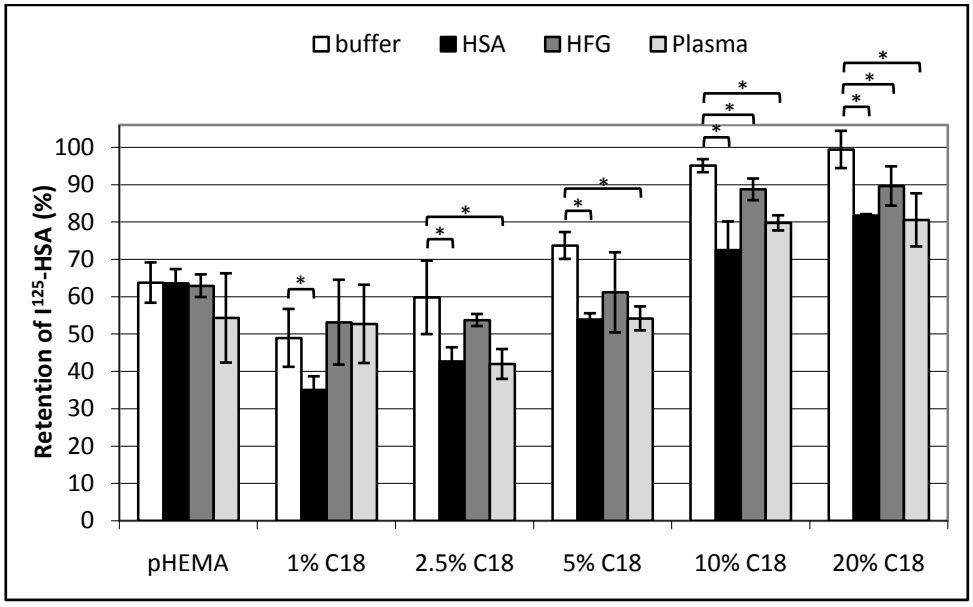


Figure 11 – Retention of previously adsorbed ^{125}I-HSA (see "24h soak" from Figure 9) in pHEMA-C18 discs after immersion in unlabeled buffer, 1 mg/ml HSA, 1 mg/ml HFG or 1% plasma solutions for 24h at 37°C. *Statistically significant different samples (p≤0.1); Mann-Whitney test.

4.3 Clotting time

Clotting times of recalcified plasma in the presence of different surfaces are presented in Figure 12. The recalcified plasma solutions in blank wells gave a clotting time of ca. 20 min, which should be the upper limit of the clotting time for the protocol used (negative control). When the glass disks were put in contact with the recalcified plasma solution, the clotting time decreased to 6 min. Glass had the shortest clotting time as expected, suggesting that it was potent in activating the intrinsic coagulation cascade.

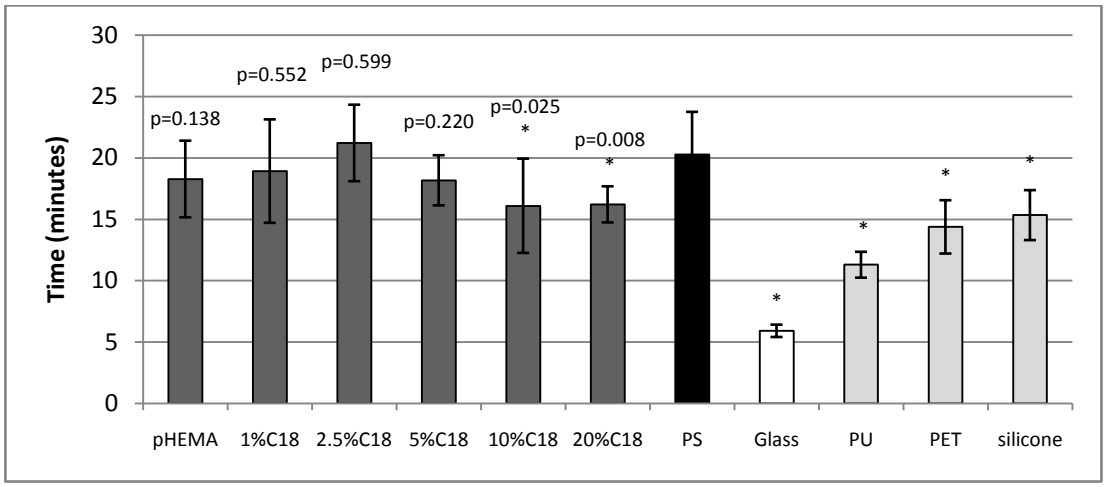


Figure 12 – Clotting time of recalcified plasma in the presence of pHEMA-C18, polystyrene (PS), polyurethane (PU), poly(ethylene terephthalate) (PET) and silicone. All samples ($n \geq 6$) were put in 48 well non-tissue culture treated PS plate (PS sample means the well plate only). *Statistically significant different samples from PS ($p<0.05$); p values of pHEMA-C18 samples comparing to PS are indicated above the corresponding column; Mann-Whitney test.

Statistically significant differences were not found between PS and pHEMA, 1%C18, 2.5%C18 or 5%C18. However, samples with 2.5%C18 appear to have unique performance. This can be verified by analyzing the evolution of *p* values, that show there is a greater probability of clotting time values from 2.5%C18 samples being the same as PS than the other samples'. On the other hand, 10%C18 and 20%C18 samples are statistically different from PS and decrease the clotting time of recalcified plasma, being more prone to induce coagulation.

Since clotting times from pHEMA-C18 samples were very similar to our negative control (PS), we have tested and included in Figure 12 the clotting times of other polymers so as to demonstrate that the protocol we have used to analyze the clotting time is sensitive to polymer composition.

5. DISCUSSION

The aim of this study was the preparation of a hydrophilic polymeric surface to be used as a background non-fouling support for the immobilization of C18 ligands in order to investigate which C18 ligand concentration better induces albumin adsorption in a selective and reversible way.

A pHEMA cross-linked gel cast onto glass was chosen as background polymer since it possesses hydroxyl groups available to react with the octadecyl isocyanates (C18) and is known to adsorb low amounts of plasma proteins.[19] This polymer was successfully prepared, as demonstrated by XPS and FTIR-ATR. The elemental composition of pHEMA determined by XPS of 69.6% C and 30.2% O, is close to the composition expected based on the structure of pHEMA (66.7% C and 33.3% O) and also to results obtained by Castner et. al[20] (68.3% C and 31.7% O) and other authors[12,21] (69.7% C and 30.3% O). All the characteristic pHEMA bands were detected by FTIR-ATR and are consistent with published literature values.[12,22,23]

The porosity of hydrogel systems can be controlled during synthesis. If the monomer is polymerized and/or cross-linked in the presence of a good solvent for both itself and the polymer formed, an optically clear gel results. This type of hydrogel has been referred as “homogeneous” or microporous.[24] The pHEMA discs that were prepared in this work are optically clear, suggesting that the hydrogel is homogeneous. This aspect was confirmed in the SEM micrographs taken, showing that the crosslinked pHEMA used as the background has a homogeneous smooth surface.

XPS, FTIR-ATR and SEM-EDS have been used to confirm the immobilization of different levels of C18 ligand on pHEMA. The presence of nitrogen is a good indicator of successful incorporation of C18 ligands in pHEMA since pHEMA does not contain this element. Another parameter important to assess the effectiveness of the derivatization is the increase of total % carbon on the surface, resulting from the long alkyl chains of the C18 ligands. All surface characterization techniques used revealed that increasing the concentration of C18 isocyanate in solution resulted in an increased surface coverage of C18 ligands.

As stated above, the C/O ratio for a pHEMA film ($C_6H_9O_3$) is expected to be around 2. Pure octadecyl isocyanate ($C_{19}H_{37}NO$) would have a C/O ratio of 19. Therefore, a derivatized monomer unit consisting of one pHEMA unit + one octadecyl isocyanate would have a C/O ratio of 6.25. XPS, which has a sampling depth of approximately 10 nm, has detected values of the C/O ratio slightly higher

than this for 10%C18 and 20%C18 samples. These results indicate that the reaction of C18 ligands with pHEMA hydroxyl groups went to completion and that C18 ligands penetrate deeper than 10 nm (the XPS sampling depth). If the reaction depth were less than 10nm we would have a C/O value between 2 and 6.25. Further analysis of the surface using SEM and EDS has confirmed that the C18 ligands derivatize pHEMA as deep as 20 µm. Moreover, the increase of surface coverage is macroscopically visible since the transparent pHEMA samples become more opaque and stiff as the percentage of C18 increases. Previous work performed by Kwok et al. had already demonstrated the extensive surface zone coverage of pHEMA with a dodecyl isocyanate (C12 isocyanate) but saturation of the pHEMA surfaces was obtained using just a 5% C12 solution since higher temperatures were used (60°C, instead of 40°C).[12]

As mentioned before, in this work we intended to create a surface with intermediate amounts of C18 ligand so that albumin could recognize them and bind through its hydrophobic pockets specific for long chain fatty acids. If C18 ligand concentration on the surface is too low or too high, albumin will either not bind because the background surface is non fouling or bind non specifically and eventually denature, losing its capacity to unbind, due to the hydrophobic nature of the outermost surface of the densely-packed n-alkyl chain layer (C18 ligands are methyl terminated).

Samples were tested for albumin adsorption from a pure albumin solution and from solutions with competing proteins and also for exchangeability after albumin preadsorption. Results from albumin adsorption after the initial rinsing and 24h soak (Figure 9) suggest that, as we suspected, some ^{125}I was initially being retained in the gel bulk of the pHEMA with none or low levels of ligands (0% to 5%C18), but was released after a 24h soak. In the pHEMA discs with 10%C18 and 20%C18, however, the surface is fully covered by C18 ligands that act as a barrier to the free iodine uptake and therefore adsorption values are roughly the same after the 24h soak. For this reason, all further studies were performed using a 24h soak as the first rinsing step and results will be discussed taking this into account.

The maximum theoretical protein monolayer coverage can be calculated for a given uniform conformation/orientation by assuming a rectangular close-packed monolayer of proteins.[25] Based on human serum albumin dimensions (heart shaped/8.7 x 8 x 6 nm)[25] and molecular mass (Mw = 66438 Dalton = 1.096×10^{-10} ng)[26], it is possible to estimate the surface area occupied by the molecules lying perpendicular or parallel to the surface and to convert this to the mass of albumin per unit surface.

The theoretical maximum monolayer coverage for HSA is 158 ng/cm^2 if it adsorbs with the long axis parallel to the surface (side-on) and 228 ng/cm^2 if it adsorbs with the long axis perpendicular to the surface (end-on).

The pHEMA surface adsorbed around 100 ng of albumin/cm^2 from pure 0.4 mg/ml HSA solution, meaning that approximately 50% of the surface is covered by this protein. These adsorption values are similar to 90 ng/cm^2 obtained by Garret et al.[27] and Mequanint et al.[28] but lower than 200 ng/cm^2 obtained by Horbett et al.[29] and 300 ng/cm^2 obtained by Martins et al.[5]. However, the pHEMA described in these two papers was graft polymerized onto other polymers, generating rough surfaces with micron-sized features, which may lead to the adsorption of higher amounts of protein because of increased surface area.

In an ideal non-fouling surface one would expect lower protein adsorption than that obtained on pHEMA in this work. However, exchangeability after 24h in different solutions (buffer, HSA, HFG and plasma) was around 36% and about 60 ng/cm^2 of albumin remained adsorbed.

Intriguingly, in 1%C18 and 2.5%C18 surfaces, albumin adsorption from pure albumin solution decreased compared to the pHEMA background surface, with values of 80 and 70 ng/cm^2, respectively. These results were not expected since C18 ligands were supposed to increase albumin adsorption. Yet, all these three surfaces presented selectivity in albumin adsorption when both albumin and fibrinogen are present. Neither the 1%C18 nor the 2.5%C18 surfaces would offer an advantage compared to pHEMA. However, considering competitive adsorption results from plasma and exchangeability studies, the 2.5%C18 surfaces present behaviors not seen in other surfaces. The presence of all plasma proteins seem to potentiate albumin adsorption to the 2.5%C18 surfaces. Furthermore, the exchangeability of preadsorbed albumin after 24h in buffer is high (58%), and these surfaces also exhibited some selectivity in the exchange since pre-adsorbed radiolabeled albumin exchanged more readily when immersed in unlabeled albumin and plasma (leaving only 31 ng of albumin/cm^2 adsorbed to the surface) then when immersed in unlabeled buffer or fibrinogen.

Surfaces with 5%C18 also demonstrated selectivity in exchange. In addition, albumin adsorption values from pure albumin solution increased with ligand immobilization compared to pHEMA, which is consistent with the aim of this work. However, competition studies with fibrinogen and plasma

revealed that this surface adsorbs other plasma proteins, including fibrinogen, therefore reducing interest of 5%C18 since it can potentially activate platelets.

The albumin adsorption values from pure albumin solution of 270 and 335 ng/cm^2 for 10%C18 and 20%C18, respectively, suggest that more than a monolayer of albumin is adsorbing to these samples. Although proteins usually adsorb as monolayers, multilayer adsorption is not uncommon, especially when the solution concentration is high. A monolayer is usually achieved with solution concentrations of 0.05–0.15 mg/ml proteins.[30] The solution concentration of 0.4mg/ml of albumin used here may therefore be responsible for the formation of a multilayer on these surfaces. Another aspect that can explain this high adsorption to 10%C18 and 20%C18 samples is the increased surface roughness. The micron-sized lumps that were visualized by SEM lead to surface area increases that allow more surface sites for protein adsorption.

The binding of tethered alkyl chains to albumin binding sites as a mechanism for increasing albumin's affinity for a surface seems promising, but some issues have been identified. Under some conditions, it is observed that the adsorption of relatively hydrophobic proteins (such as albumin) increases as the polymer substrate becomes more hydrophobic. This increase in adsorption is attributed to a general increase in the hydrophobic interactions between the protein and the polymer. The entropy increase as surface-structured water is displaced by these hydrophobic interactions is one of the dominant driving forces in protein adsorption,[31] and addition of alkyl chains to a surface makes the surface more hydrophobic.[10,13] We considered the possibility that the increase of albumin adsorption results from non-specific binding to a surface which is more hydrophobic than the underivatized polymer, and not necessarily from the specific binding of albumin to the alkyl chains of the C18 ligand.[31] In this work, however, we did not find a linear increase of albumin adsorption as the percentage of C18 increased as would be expected if only hydrophobic forces were involved. 5%C18, 10%C18 and 20%C18 samples indeed presented higher albumin adsorption than pHEMA, increasing with the increased amount of ligand. However, 1%C18 and 2.5%C18 samples showed a small decrease compared to the background surface. Also, the existence of selectivity in albumin adsorption in the presence of competitive proteins and in exchangeability studies suggests that adsorption through binding pocket interactions with the C18 ligands is taking place.

Coagulation, triggered through the intrinsic pathway, begins with contact activation of factor XII, high-molecular-weight kininogen (HMWK) and prekallikrein (PK). The addition of of Ca^{2+} (Factor IV)

into anti-coagulated human plasma will induce the intrinsic blood coagulation system to activate prothrombin (Factor II) converting it into thrombin, and the thrombin will cleave fibrinogen leading to insoluble fibrin, the framework of the thrombus.[32]

In the recalcified plasma clotting time study, glass (positive control) had the shortest clotting time (6 minutes) compared with all other surfaces. Similar fast glass clotting times of 4 minutes[17,33] and 9 minutes[34] were obtained by other authors. This was expected, as it is well known that surfaces such as glass initiate the intrinsic pathway via contact activation by adsorbing factor XII, HMWK and PK.[35] In contrast, PS (from the 48 well plates), used as a negative control, demonstrated a prolongation of the clotting time to 20 minutes. These results are consistent with the average of the clotting times of PS obtained by other authors (14 minutes[17], 21 minutes[34] and 26 minutes[33]).

It should be pointed out that samples were placed in the bottom of the wells, leaving some lateral PS from the well exposed to the plasma solution. As a result, clotting tests were influenced by the clotting time of the PS wells in this experimental protocol, making it more difficult to compare the anticoagulant properties of the different samples. However, differences were observed between samples completely covered by C18 ligand (10% and 20%C18) and the other pHEMA-C18 samples. Taking into account the results from competitive adsorption studies (Figure 10), that revealed that 10%C18 and 20%C18 samples adsorb around 60% albumin and 40% of other plasma proteins, it is predictable that some of the plasma proteins that might have adsorbed to these surfaces are factor XII, HMWK and PK, leading to contact activation of the coagulation and therefore faster clotting times than PS. Both pHEMA and 5%C18 samples adsorbed around 20% of plasma proteins other than albumin, but we propose that not many of the adsorbed proteins were factor XII, HMWK and PK since clotting times were similar to the negative control (PS). Samples with 1%C18 and 2.5%C18 adsorbed 100% of albumin in the presence of all plasma proteins, which justifies the clotting times similar to PS.

Previous work carried out in self-assembled monolayers (SAMs) had already demonstrated that the concentration of C18 ligand on the surface would greatly influence albumin adsorption and that a specific percentage of ligand in solution (2.5%C18) would have the best performance in binding albumin in a selective and reversible way. In the present study we have succeeded in transposing the knowledge we had obtained using model surfaces to a polymer. Indeed, in both cases samples with 2.5%C18 were found to have the best behavior. The fact that 2.5%C18 samples did not accelerate

coagulation of recalcified plasma compared to pHEMA, reinforced the idea obtained in protein adsorption studies that the presence of this specific level of ligand on a pHEMA background may be beneficial as a blood contacting biomaterial. The mechanical properties of PHEMA gels are not adequate for many blood contact applications. However, this polymer can be easily grafted or polymerized on the surface of other biomaterials that have the appropriate mechanical properties but lack other desirable attributes such as blood compatibility, low protein adsorptivity and the presence of hydroxyl groups useful for further bio-functionalization.

6. CONCLUSIONS

Different levels of C18 ligand were successfully immobilized onto a pHEMA polymer from solutions with different fractions of C18 isocyanante, achieving maximum coverage in 10% C18 and 20%C18 samples. An increase of albumin adsorption was verified when pHEMA was derivatized with 5%C18 or more. Even though 2.5%C18 samples showed lower albumin adsorption than the pHEMA background surface, samples with this specific level of ligand appear to induce albumin adsorption in a selective and reversible way, since they adsorb albumin preferentially in the presence of fibrinogen and other plasma proteins and they exchange 58% of the albumin that binds when in contact with pure albumin solution or plasma. Furthermore, samples with this specific percentage of C18 ligand are not prone to activate intrinsic coagulation since recalcified plasma clotting times are similar to the negative control.

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CHAPTER VII

GENERAL DISCUSSION, CONCLUDING REMARKS AND FUTURE PERSPECTIVES

1. GENERAL DISCUSSION AND CONCLUDING REMARKS

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. Albumin acts as a bystander molecule in the case of many surface contact-activated biological reactions because it contains none of the peptide sequences known to interact with either adhesion receptors on cell membranes or enzymes in the coagulation and complement cascades.[1] Furthermore, if albumin is bound on the surface other thrombogenic proteins, like fibrinogen, cannot adsorb. An albumin coating may thereby increase the biocompatibility of the surface.

The design of such surface had two competing demands: the immobilization of a ligand on the surface with specificity to albumin, and simultaneous minimization of the adsorption of all other proteins in a complex protein mixture like plasma (which adsorb by the so-called "non-specific adsorption").

The strategy herein presented of immobilizing ligands on a surface, so that endogenous albumin can be attracted from the bloodstream, has two main advantages. In contrast to the use of exogenous albumin, either chemically or thermally crosslinked to a surface, specifically adsorbed albumin retains its native conformation. Additionally, adsorbed host albumin is inherently non-immunogenic. The rationale behind this approach is that albumin forms a platelet compatible surface, but is probably degraded with time *in vivo*. If adsorbed albumin could be renewed by fresh albumin molecules, the surface might maintain its platelet-compatible properties.

C18 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 had to be appropriate, so that albumin could recognize the alkyl chains, bind to them by the hydrophobic pockets specific for fatty acids, and cover the surface. If ligand concentration were too high, the proteins would just sense a homogeneous layer of methyl groups on the surface and specificity would be lost.

The understanding of the mechanism of protein adsorption at the molecular scale needed the utilization of stable models with a well-defined surface structure, which are not achieved when using conventional polymer surfaces. Self-assembled monolayers (SAMs) of alkanethiols on gold were chosen since they are well-ordered organic surfaces that allow control over the properties of the interface at a molecular scale. Using SAMs, instead of polymers, to study the effect of the concentration of C18 alkylation on albumin selective and reversible adsorption has the advantage of C18 ligands not being able to rearrange towards the bulk polymer, away from the adsorbing protein.

In this investigation, C11OH SAMs were first used as background surface for the immobilization of C18 ligands. Samples with different amounts of immobilized C18 ligands were obtained, the maximum coverage being achieved in a 10%C18 solution. The increase of the percentage of C18 ligands in solution resulted in SAMs with increasing atomic percentage of N (from 0%N in C11OH to 3.1%N in 10%C18) and increased thickness (from 1.4nm in C11OH to 3.0nm in 10%C18). An increased intensity of IRAS peaks corresponding to CH_2 groups with increased chain order was also observed with C18 ligand immobilization, indicating that C18 ligands in fully covered 10%C18 SAMs are densely packed. With the increase of immobilized C18 in SAMs, we also observed an increase of hydrophobicity (from 25° in C11OH to 91.3° in 10%C18) and a decrease of the surface energy.

Protein adsorption studies showed that albumin adsorption increases with the amount of C18 ligands immobilized in C11OH SAMs (from 1.3mg/m^2 in C11OH to 3.5mg/m^2 in 10%C18). Nevertheless, the amount of bound protein is not the only important parameter; selectivity and reversibility of albumin adsorption are also key parameters for the adequate implementation of the strategy herein presented. SAMs obtained from solutions with 2.5%C18 presented considerable albumin adsorption in a selective and reversible way. However, 50% of the adsorbed albumin remained irreversibly bound to these surfaces. This study demonstrated that not only the ligand, but also its surface concentration, are important to adsorb albumin in a selective and reversible manner.

In the subsequent part of this study, instead of C11OH SAMs, oligo (ethylene glycol)-terminated SAMs (EG_4 SAMs) were used as background, in order to improve non-specific adsorption and increase the reversibility (displaceability) of HSA adsorption previously obtained. EG_4 SAMs (Au-$S(CH_2)_{11}(OCH_2CH_2)_4OH$) were chosen as background surfaces since the $HS(CH_2)_{11}$–moiety provides a foundation for stable SAMs, while the oligo(ethylene glycol) headgroups should be substantially disordered to show non-fouling properties.[2-4] The ethylene oxide groups present on the surface of

the EG_4 SAMs provide a template for water adsorption thus creating a stable interfacial water layer that prevents direct contact between the surface, proteins and the cells.

Following the same strategy to bind albumin, different percentages of 18-carbon (C18) ligands were immobilized on the top of the EG_4 SAMs. Surface characterization of EG_4-C18 SAMs was performed similarly to C11OH-C18 SAMs. The contact angle of EG_4-C18 SAMs ranges from 37.7° in hydrophilic EG_4 SAMs to 94.8° in hydrophobic 10%C18 SAMs. EG_4 SAMs are less hydrophilic than OH SAMs, possibly because of the exposure of the methylene groups and oxygen atom of the terminal ethylene glycol units at the outer surface.[3] This has been confirmed by IRAS that revealed that EG_4 SAMs are amorphous and not well oriented. Regarding samples completely covered by C18 ligands, despite having similar water contact angles and thicknesses (1.6nm of C18 ligands thickness when the background is subtracted), XPS results revealed that EG_4-10%C18 SAMs only have about 1.7% of N, comparing to the 3.1%N in C11OH-10%C18 SAMs, suggesting that fewer C18 ligands can be immobilized in SAMs with EG_4 as background.

The use of EG_4 SAMs as background decreased non-specific adsorption since albumin adsorption to the background surface dropped from 1.3mg/m^2 in C11OH SAMs to 0.14mg/m^2 in EG_4 SAMs. It also increased the preference for albumin, once all the surfaces presented more affinity to albumin than to fibrinogen (when both proteins are in solution in the same proportion as present in blood). The importance of the concentration of C18 ligands was stressed in the reversibility studies since EG_4-2.5%C18 SAMs are the only surfaces to exchange almost all the pre-adsorbed albumin by albumin in solution, but not by fibrinogen. The replacement percentage of adsorbed albumin by other albumin molecules, that was obtained when using EG_4 SAMs, increased 35% (for the same percentage of ligand in solution – 2.5%C18), implying that this background surface, comparing to C11OH SAMs, has better non-fouling characteristics and therefore performs better in inhibiting non-specific interactions.

Following the trend observed in the protein adsorption studies, there is an increase of platelet adhesion as the percentage of C18 ligands increases (with buffer pre-immersion). Moreover, the morphology of the platelets changes from round to spread as they contact more hydrophobic surfaces. However, when a pre-immersion in albumin is performed, there is an 80% decrease in the number of platelets, and the few ones that adhere are not activated, stressing the passivant effect of albumin for blood contact surfaces. 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.

The adhesion of human leukocytes was found to increase as the percentage of C18 ligands on EG_4 SAMs increases, until 5%C18. However, even in samples where higher leukocyte adhesion occurred, surface coverage by cells is low. This work revealed that the amount of C18 ligands is the main responsible for the differences in leukocyte adhesion to EG_4-C18 SAMs. Unlike results with platelets, an albumin passivant effect was not observed in leukocyte studies after pre-immersion in protein solutions. Actually, no major differences in leukocyte adhesion were observed in samples pre-immersed in buffer, albumin (except for 10%C18 SAMs), fibrinogen or plasma. However, it is important to bear in mind that this study has a limitation that might also have influenced the results. The fact that the leukocyte culture medium was supplemented with plasma proteins, which ultimately may mask the effect of pre-adsorbed proteins, may be the explanation. Despite this, medium with plasma had to be used in cell-adhesion experiments in order to obtain a physiologically relevant response, since the adhesion of leukocytes is mediated by a number of cell surface molecules, including β2 integrin (Mac-1) which, according to Koyama et al.[5], does not function without serum and divalent cations.

The work performed using model surfaces confirmed that, by manipulating the amount of C18 ligands immobilized on the surface of SAMs it is possible to obtain a surface that adsorbs albumin in a selective and reversible way. It also reduces platelet adhesion and activation, but a reduction of leukocyte adhesion has not been verified.

Since a large proportion of medical devices are made from polymers, the next step of the investigation was to transpose the knowledge obtained using SAM structures to a polymeric material.

The same isocyanate chemistry used to immobilize C18 ligands in the hydroxyl terminating groups of SAMs was used to immobilize the C18 ligands in the hydroxyl groups available in poly(2-hydroxyethyl methacrylate) (pHEMA). Optically clear pHEMA discs with a homogeneous smooth surface were prepared and derivatized with different amounts of C18 ligands, the maximum coverage being achieved with 10%C18 solutions.

Unlike what happened with SAMs, an increase of albumin adsorption from pure solution was only verified when pHEMA was derivatized with 5%C18 or more. Competitive adsorption studies in the

presence of both albumin and fibrinogen, and in the presence of all plasma proteins, showed that 1%C18 and 2.5%C18 were the only surfaces selective for albumin, and that the presence of all plasma proteins may even potentiate albumin adsorption. Reversibility studies demonstrated that both 2.5%C18 and 5%C18 samples exchange ^{125}I-albumin selectively in the presence of both unlabeled albumin and plasma, but 2.5%C18 samples presented higher exchangeability rates (58%).

Recalcified plasma clotting times revealed that samples with no or small amounts of C18 (pHEMA to 5%C18) did not shorten the clotting time comparing to the negative control, indicating low activation of the intrinsic coagulation cascade.

The work presented in this thesis demonstrates that the concentration of C18 ligands on the surface greatly influences albumin adsorption. A specific percentage of C18 ligands (2.5%C18 in solution) induces best performance in binding albumin in a selective and reversible way and may be beneficial for blood contact applications. Furthermore, this investigation has succeeded in transposing the knowledge obtained using model surfaces to a biomedical polymer.

2. FUTURE PERSPECTIVES

The results reported in this thesis have put in evidence that the immobilization of intermediate amounts of C18 ligands on the surfaces of both model surfaces, using self-assembled monolayers – SAMs, and a polymeric material, poly(2-hydroxyethyl methacrylate) – pHEMA, induce albumin adsorption in a selective and reversible way.

Eventhough samples prepared from solutions with 2.5%C18 have been identified has having the best performance, the distribution of the C18 ligands on the surface was not investigated. Such study could be done using TOF-SIMS (time-of-flight secondary ion mass spectroscopy).

Protein conformation is very important for the hemocompatibility of a surface. Protein exchangeability studies using radiolabeling and protein pre-immersion studies followed by platelet or leukocyte adhesion have indirectly provided information on protein conformation. However, further investigation could be done in order to directly determine the conformation of adsorbed proteins. Ellipsometry or SPR associated with monoclonal antibodies can be used to study alterations on the protein conformation during adsorption. In order to determine the conformation of proteins already adsorbed to a surface, ATR-FTIR and IRAS can be adapted using a liquid cell and ELISA (enzyme linked immunosorbent assay) can be performed using monoclonal antibodies.

Regarding leukocytes adhesion studies, further experiments may prove important. It would be interesting to study which subpopulation of leukocytes are adhering to the samples, by isolating each type of leukocytes and performing adhesion assays separately, as well as studying their degree of activation.

The complement system destroys and removes substances, either by direct lysis or by mediating leukocyte function in inflammation. The alternative pathway of the complement system is triggered directly by foreign surfaces, such as man-made biomaterials. It would therefore be very interesting to study the effect of the immobilized C18 ligands in the complement system. Complement activation could be studied by quantification of the adsorption of complement proteins.

PHEMA has the disadvantage of having weak mechanical properties, restricting its use in most applications as blood contact biomaterial. However, when pHEMA is derivatized with C18 ligands, it

becomes stiffer with the increasing amount of ligands. This may be an interesting property for some applications. A proper evaluation of the mechanical properties of pHEMA-C18 should therefore be done, so that future applications can be envisaged. Also, this polymer can also be easily grafted or polymerized on the surface of other biomaterials that have the appropriate mechanical properties but lack other desirable attributes that pHEMA possesses, such as blood compatibility, low adsorptivity and the presence of hydroxyl groups useful for further bio-functionalization.

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