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Tese de Doutoramento em Engenharia Biomédica

Trabalho realizado sob a orientação do
Professor Doutor Rui L. Reis
Professor Doutor John A. Hunt

Setembro de 2007

To my parents

(Aos meus pais)

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To my brothers and especially to my mother and my father, this thesis is dedicated to you. You have truly supported me showing all your respect for my aims and decisions. You and your love were always present and continue to give me the essential strength even when we are away.

ABSTRACT

The Role of Protein Interactions with Novel Starch Based Polymeric Implant Materials on Determining the Correspondent Host Responses

The importance of cell-protein-surface interactions is commonly accepted as a key step for the successful application of any biomaterial. Immediately upon contact with physiological fluids many proteins adsorb to the implant surface, subsequently promoting nearby cells to indirectly interact with the material. Either in *in vivo* or *in vitro* conditions, cells are known not to interact with biospecies-free surfaces. Along with a number of other interfacial processes, the amount, type and conformation of adsorbed proteins regulate the bio-integration of an implant, thus defining its final outcome: integration or rejection.

The paradigm of cell material interactions, which considers that protein adsorption as the first event following contact and determines the later interactions of the cell, could be central to the design of new strategies for biocompatibility and tissue engineering. Many authors consider protein adsorption as a material/surface dependent phenomenon and many attempts have been made in order to achieve perfect compromise between materials and biocompatibility by means of manipulating protein behaviour at interfaces. From a functional point of view, it is accepted that qualitative and quantitative assessment of the affinity of proteins to surfaces is essential to evaluate cell mechanisms upon attachment to the surfaces and thus develop improvements in the properties of implanted materials.

The materials investigated in this research have already been utilised for a range of applications in the biomedical field. Blends of corn starch with cellulose acetate (SCA), ethylene vinyl alcohol (SEVA-C) and polycaprolactone (SPCL); hydroxyapatite reinforced SCA (SCA+10%HA); and poly[D,L-lactic acid] (PDLLA) were studied. Starch based biomaterials (SBB) and PDLLA have shown to exhibit an *in vitro* and *in vivo* biocompatible behaviour. Although several cell studies were previously conducted, the adsorption behaviour of biomolecules relevant for the cell-biomaterial interactions had yet to be understood.

For that, the PhD work plan that culminated in this thesis was conducted to determine different aspects, directly related with *surface-protein* and *surface-protein-cell* issues: (*i*) to investigate protein distribution onto the surface of starch based polymers; (*ii*) to analyse the effect of surface chemistry in the adsorption of proteins; (*iii*) to assess the kinetics and adsorption isotherms of clinically relevant proteins; (*iv*) to explore protein competition, exchange and desorption from the surfaces; (*v*) to evaluate the influence of proteins-surface systems in cell adhesion, proliferation and morphology; and finally, (*vi*) to investigate protein behaviour onto the surface of implanted materials.

In the initial evaluation of protein adsorption onto SBB surfaces, single, binary and complex protein solutions were used. The distribution of the molecules was found to depend on the surface studied regardless of the protein specie. In single protein systems a preference for fibronectin and vitronectin adsorption in comparison to albumin was demonstrated, the laters adsorption potential was further decreased in competitive conditions. SPCL presented the highest protein adsorption levels. For the studied serum concentrations all the surfaces generally indicated good vitronectin adsorption. In the second part of

this study, the effect of proteins on modulating leukocyte adhesion showed short and long-term effects in cell adhesion developed by vitronectin and albumin, respectively.

The effect of oxygen-based plasma treatment on the different surfaces and the influence of proteins adsorption on modulating bone-cells behaviour were studied. Both SBB and PDLLA surface properties were affected by the selected surface modification technique, which increased albumin and fibronectin adsorption onto treated PDLLA. The crucial role of adsorbed proteins in mediating the response of osteogenic cells to the treated PDLLA surface was demonstrated. Regarding SBB surfaces, the absence of pre-incubated proteins showed to improve osteoblast-like cells proliferation onto plasma treated SPCL. Moreover, the pre-adsorbed proteins primarily defined MG63 cells morphology on SEVA-C surfaces, while on SPCL it was mainly affected by the plasma treatment.

The correlation between the surface characteristics and protein adsorption isotherms from unitary and complex protein systems was investigated onto starch based materials. Albumin adsorption on SBB was affected by the material composition as well as by the concentration of the protein solution, preferentially adsorbing onto SCA and SPCL. Fibronectin adsorption reached higher values on SEVA-C and SPCL. There was no effect on the adsorption of albumin and fibronectin onto SPCL in competitive conditions. In the presence of albumin, fibronectin adsorption was reduced on SCA, while the opposite situation was observed for SEVA-C. Fibronectin demonstrated a different adsorption activity for the different materials as assessed by single and competitive adsorption with albumin.

The adsorption/desorption study of plasma proteins on SBB surfaces was performed by multiloop Dynamic Contact Angle (DCA). In this study, the analysis of the hysteresis, advancing and receding wetting tensions indicated that adsorbed proteins could desorb more readily on SCA and SPCL than on SEVA-C. In the later case, stronger interactions such as hydrophobic forces were established, which could indicate the rearrangement of protein conformation.

The physicochemical relationship between different biological molecules and SEVA-C was investigated *in vivo*. Results indicated that albumin and vitronectin were absent from the immediate tissue-implant interface and diffused into the bulk of the material. In contrast, fibronectin adsorption presented multilayer patterns that displaced fibrinogen from SEVA-C surfaces.

In general, the adsorptive potential of albumin, fibronectin and vitronectin was characterized and SPCL surfaces showed increased adsorption levels for both unitary and complex protein systems. Protein distribution and desorption profiles were obtained and SEVA-C showed the lowest desorption ability. *In vitro* cell studies showed that PDLLA and SPCL were highly sensitive to the surface modification, and that cell response to SEVA-C was highly modulated by pre-adsorbed molecules. Finally, *in vivo* studies provided further insights into the dynamics established between different proteins and SEVA-C material.

RESUMO

Estudo do Efeito da Interacção de Proteínas com Novos Biomateriais à Base de Amido de Milho e da Respectiva Influência na Determinação da Resposta Biológica

A importância atribuída à interacção entre células, proteínas e superfícies é geralmente considerada como um passo fundamental para a aplicação bem sucedida de qualquer biomaterial. Após o contacto com fluidos fisiológicos, muitas proteínas adsorvem às superfícies implantadas, induzindo a interacção indirecta das células com o material. Nas condições *in vivo* e *in vitro* é sabido que as células, geralmente, não interagem com as superfícies implantadas na ausência de biomoléculas. À semelhança de outros processos de interface, a quantidade, tipo e conformação das proteínas adsorvidas regulam a biointegração de um implante, definindo, assim, o seu resultado final: integração ou rejeição.

O paradigma das interacções entre células e superfícies, que considera a adsorção de proteínas o primeiro acontecimento depois da existência de contacto e que determina os processos celulares consequentes, pode ser fulcral para o desenvolvimento de novas estratégias de biocompatibilidade e engenharia de tecidos. A adsorção de proteínas é considerada por muitos autores como um fenómeno dependente do material/superfície. Diversos estudos foram desenvolvidos com o intuito de atingir um compromisso perfeito entre material e biocompatibilidade através da manipulação do comportamento das proteínas nas superfícies. Do ponto de vista funcional, o estudo qualitativo e quantitativo da afinidade de proteínas a superfícies é essencial na avaliação dos mecanismos posteriores às interacções célula-superfície permitindo, deste modo, melhorar as propriedades de biomateriais implantados. Os materiais utilizados neste trabalho foram estudados para diversas aplicações na área da biomedicina, designadamente, misturas de amido de milho com acetato de celulose (SCA) etileno álcool-vinílico (SEVA-C) e policaprolactona (SPCL); SCA com hidroxiapatite (SCA+10%HA); e poli-D,L-ácido láctico (PDLLA). Os materiais à base de amido de milho e o PDLLA demonstraram, tanto *in vivo* com *in vitro*, um comportamento biocompatível. Embora vários estudos de biologia celular tenham sido efectuados, o efeito da adsorção de biomoléculas com relevância clínica nas interacções entre células e superfícies ainda não é totalmente conhecido.

Neste contexto, foi delineado um plano de trabalhos, que deu origem a esta tese, com o objectivo de avaliar diferentes aspectos da interacção entre proteínas, células e superfícies dos materiais, nomeadamente: (*i*) a distribuição de proteínas nas superfícies em estudo, (*ii*) o efeito da química de superfícies na adsorção proteíca, (*iii*) a cinética de adsorção de proteínas, (*iv*) aspectos de competição, troca e desadsorção de biomoléculas, (*v*) a influência das proteínas na adesão, proliferação e morfologia celulares, e finalmente, (*vi*) o comportamento de moléculas na interface implante-tecido.

Numa fase inicial da avaliação da adsorção de proteínas foram utilizadas soluções proteicas simples, binárias e complexas. Concluiu-se que a distribuição das moléculas depende da superfície em estudo, e não do tipo de proteínas utilizadas. Em sistemas proteicos simples verificou-se a preferência pela adsorção da fibronectina e vitronectina em comparação com a albumina, cujo potencial de adsorção diminui em condições competitivas. Os níveis mais altos de adsorção de proteínas foram obtidos pelo SPCL. Todas as

superfícies demonstraram, em termos gerais, uma boa adsorção de vitronectina nas concentrações de soro estudadas. Numa segunda fase deste estudo, a influência das proteínas na adesão de leucócitos apresentou efeitos a curto e a longo prazo na adesão celular desenvolvidas, respectivamente, pela vitronectina e albumina.

Foi ainda estudado o efeito do tratamento por plasma de oxigénio nas diferentes superfícies e a influência da adsorção de proteínas no comportamento das células do osso. As propriedades do PDLLA e dos materiais à base de amido de milho foram modificadas pela técnica seleccionada, resultando no aumento de adsorção de albumina e de fibronectina nas superfícies de PDLLA. Demonstrou-se também a função essencial das proteínas adsorvidas na resposta celular ao PDLLA modificado por plasma. Relativamente aos materiais à base de amido de milho, a ausência de proteínas pré-adsorvidas melhorou a proliferação de osteoblastos nas superfícies de SPCL modificadas. Além disso, as proteínas pré-adsorvidas influenciaram a morfologia das células MG63 nas superfícies de SEVA-C, enquanto que no SPCL as maiores alterações resultaram do tratamento por plasma. A relação entre as características das superfícies dos materiais à base de amido de milho e a adsorção de proteína foi estudada usando soluções proteícas simples e complexas. A adsorção de albumina foi influenciada pela composição dos materiais e pela concentração da solução proteíca, adsorvendo preferencialmente às superfícies de SCA e de SPCL. Por outro lado, a adsorção de fibronectina atingiu valores mais elevados na superfície de SEVA-C e de SPCL. Em condições competitivas nenhum efeito foi observado na adsorção da albumina e fibronectina ao SPCL. Na presença da albumina, a afinidade da fibronectina ao SCA diminuiu, enquanto que o contrário foi observado para o SEVA-C. Verificou-se que o comportamento da fibronectina na presença de albumina em condições competitivas difere do demonstrado em sistemas unitários.

A adsorção/desadsorção de proteínas do plasma sanguíneo aos materiais à base de amido de milho foi estudada utilizando a técnica de Ângulo de Contacto Dinâmico. Neste estudo, a análise da histerese e das tensões de molhabilidade indicou que a desadsorção das proteínas é mais rápida nas superfícies de SCA e de SPCL do que nas de SEVA-C. Neste último material, estabeleceram-se interacções mais fortes (ligações hidrofóbicas), o que pode indicar o rearranjo da conformação proteíca. A relação físico-química entre as diferentes biomoléculas e as misturas poliméricas de SEVA-C foi estudada *in vivo*. Os resultados obtidos demonstraram a ausência de albumina e vitronectina na interface tecido-implante e a sua difusão no interior do material. Por outro lado, a fibronectina adsorveu em padrões de multicamadas, deslocando o fibrinogénio da superfície dos materiais implantados.

Em termos gerais, o potencial de adsorção da albumina, fibronectina e vitronectina foi caracterizado. Nas superfícies de SPCL observaram-se os níveis mais elevados de adsorção utilizando sistemas proteicos unitários ou complexos. A distribuição das proteínas e os perfis de adsorção foram estudados e verificou-se que a mistura de SEVA-C apresenta menor capacidade de desadsorção. No que respeita aos estudos celulares, concluiu-se que o PDLLA e o SPCL foram os materiais mais afectados pelo tratamento por plasma e que o SEVA-C foi o material mais influenciado pela pré-adsorção de proteínas. Por último, os estudos *in vivo* permitiram aprofundar os conhecimentos sobre a dinâmica estabelecida entre diferentes proteínas e o SEVA-C.

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LIST OF ABBREVIATIONS

A

Alb - Albumin

ALP - Alkaline Phosphatase

ATR - Attenuated Total Reflection

B

BSA - Bovine Serum Albumin

D

DCA - Dynamic Contact Angle

DMEM - Dulbecco's modification of Minimum Essential Medium Eagle

E

ECM - Extracellular matrix

ELISA - Enzyme-linked immunosorbent assay

EtO - Ethylene Oxide

F

FBR - Foreign body reaction

FBS - Fetal Bovine Serum

Fbg – Fibrinogen

Fn – Fibronectin

FRC - Fetal rat calvaria

FTIR-ATR - Fourier-transformed infrared spectroscopy with attenuated total reflectance

H

H – Hysteresis

HA – Hydroxyapatite

HBSS - Hank's Balanced Salt Solution

HBP - Human blood plasma

HFbg - Human fibrinogen

HFn - Human fibronectin

HSA - Human serum albumin

HVn - Human vitronectin

I

IgG - Immunoglobulin

L

LSCM - Laser Scanning Confocal Microscopy

M

MALDI - Matrix-assisted laser desorption/ionization mass spectroscopy

MEM - Minimum Essential Medium Eagle

MW - Molecular Weight

P

PBS - Phosphate Buffered Saline

PDLLA - Poly(D,L-lactic acid)

PEG - Poly(ethylene glycol)

PEO - Poly(ethylene oxide)

PHSRN - Proline-histidine-serine-arginine-asparagine

R

RDGS - Arginine-aspartic acid-glycine-serine

REDV - Arginine-glutamine-aspartic acid-valine

RfGD - Radio Frequency Glow Discharge

RGD - Arginine-glycine-aspartic acid

RGDS - Arginine-glycine-aspartic acid-serine

S

SBB - Starch-Based Material

SCA - Blend of corn starch/cellulose acetate blends

SEM - Scanning electron microscopy

SEVA-C - Blend of corn starch with ethylene-vinyl alcohol

SPCL - Blend of corn starch/polycaprolactone blends

T

TCPS - Tissue culture polystyrene

TGFb-1 - Transforming growth factor beta-1

V

Vn - Vitronectin

X

XPS - X-ray photoelectron spectroscopy

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SHORT *CURRICULUM VITAE*

Catarina M. Alves was born in 1978 in Lisboa, Portugal. At the present she lives in Braga and works, as a researcher, in the 3B's Research Group (Biomaterials, Biodegradables and Biomimetics), one of the Units of the Institute for Biotechnology and Bioengineering (PT Government Associated Laboratory), under the supervision of Prof. Rui L. Reis.

Her background includes a four-year graduation in Applied Biology, by the School of Sciences, University of Minho (with 15 over 20). She has just submitted her PhD thesis on Materials Science and Technology - Biomedical Engineering to the University of Minho, Portugal, which was prepared in cooperation with the University of Liverpool, United Kingdom.

During the last year of her graduation, C. M. Alves worked in the Biomaterials filed, in the 3B's Research Group in cooperation with the Department of Biology of the University of Minho. Her work focused on the *in vitro* protein adsorption and cellular response to natural based materials.
In 2000 she formally joined the 3B's Research Group, where she has been working ever since on the field of surface-protein interactions and surface-mediated cell response. During this phase she worked for different time periods at the University of Liverpool, under supervision of Professor John A. Hunt and at the University of Texas Health Science Center at San Antonio, under supervision of Professor C. Mauli Agrawal, under the same scope of study.

As a researcher of the 3B's Research Group she has been involved in the supervision of undergraduate students. C. M. Alves has also been involved in the preparation of several grant proposals, both at the National and European levels, including the EU-funded project HIPPOCRATES. Moreover, she has been involved in the EXPERTISSUES – the only EU funded Network of Excellence in Tissue Engineering. C. M. Alves has participated in the Conference Organization Team of the 3B´s Research Group and was actively involved in the preparation of the following proposals: two NATO – ASI (Advanced Study Institute) Courses, in InVENTS (Marie Curie Series of Events), ESF-EMBO Symposium on "Stem Cells in Tissue Engineering: Isolation, Culture, Characterisation and Applications", and is currently part of the organization of the 2008 Annual Meeting of TERMIS-EU (Tissue Engineering and Regenerative Medicine International Society - EU Chapter). Presently she has been the elected representative the Students and Young Investigators of TERMIS-EU.

As a result of her research work she has attended several important international meetings in the present field of research. Presently she is the author of 10 papers in international refereed journals (6 published and 4 submitted), 6 book chapters and 41 abstracts published in international conference proceedings. C. M. Alves was awarded 3 conference travel grants and 2 poster prizes.

LIST OF PUBLICATIONS

The work performed during this PhD resulted in the following publications:

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- PAPERS IN REFEREED JOURNALS

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INTRODUCTION TO THE THESIS FORMAT

This thesis is divided in four sections containing nine chapters: Section 1 (*Chapter I*), Section 2 (*Chapter II*), Section 3 (*Chapter III to VIII*) and Section 4 (*Chapter IX*). According to the tradition of the 3B's Research Group, the thesis format is based on papers. Section 3 includes 6 Chapters of experimental data, each consisting of a paper published or submitted to an International Journals with *referee*. The contents of each chapter are summarized bellow.

SECTION 1 (*Chapter I*)

Chapter I is based on a book chapter written in review style. It gives a comprehensive overview on the process of protein adsorption: from one protein systems to the complex multi-protein environment, tracing protein dynamics, instability, practical details, techniques, limitations and the success of adsorption manipulation on the control of cell response.

SECTION 2 (*Chapter II*)

Chapter II presents the options selected to accomplish the proposed work plan. This Section includes a concise and explicit description of the techniques and methodologies used for the characterization of the studied biodegradable materials; for the *in vitro* analysis of protein and cell interactions; and for the *in vivo* assessment of protein adsorption.

SECTION 3 (*Chapters III to VIII*) consists of six chapters of experimental work.

Chapter III presents immunostaining studies performed to assess potential effects of the surface properties on the adsorption and competition of human proteins. The effect of proteins preadsorption in cell response was assessed by the co-culture of lymphocytes and monocytes/macrophages onto starch based biomaterials (SBB).

Chapter IV and V, focuses on the effect of oxygen-based radio frequency glow discharge (rfGD) on protein adsorption onto the surface of starch based biomaterials and poly(D,L-lactic acid), respectively. Moreover, it described the response of bone related cells to pre-adsorbed proteins.

Chapter VI explores the kinetics of protein adsorption onto starch based biomaterials. Langmuir and Freundlich empirical models were applied and the competition of proteins in binary solutions was investigated using fluorimetry and laser scanning confocal microscopy (LSCM).

The further investigation of the fundamentals of protein adsorption, adsorption/desorption experiments and the study of protein orientation are presented in ***Chapter VII***. The interactions between molecules and SBB surfaces were studied by dynamic contact angle (DCA) and LSCM using different proteins and competitive systems.

In ***Chapter VIII***, the *in vivo* protein adsorption onto SEVA-C (polymeric blend of corn starch with ethylene vinyl alcohol) was assessed. The screening of different proteins and their diffusion potential was analysed.

SECTION 4 (*Chapter IX*) contains the general conclusions regarding the overall work carried out under the scope of this thesis, as well as some final remarks.

SECTION 1

CHAPTER I

INTRODUCTION: PROTEIN AND CELL INTERACTIONS WITH BIODEGRADABLE SYSTEMS

CHAPTER I

INTRODUCTION: PROTEIN AND CELL INTERACTIONS WITH BIODEGRADABLE SYSTEMS*

1. SURFACES, SOLUTIONS, PROTEINS AND CELLS: THE FOUR KEY ELEMENTS

Whenever a protein solution contacts with a solid surface, molecules spontaneously accumulate at the solid-liquid interface. In the last decades, protein adsorption was reported by several authors[1-5] as the initial step following the contact of an artificial surface with blood. This phenomena was early related to the initiation of thrombosis by foreign surfaces;[6] and its dynamic and complexity initially indicated from Watson and Sodersquist observations[7] as from Vroman effect concept.[8] Protein adsorption was earlier found to alter the sorbent surface and in many cases also the properties of the adsorbed molecules.[9] More specifically, the adsorption of a certain protein to a surface is often accompanied by a change in its structure or three-dimensional rearrangements. The interaction protein-surface has been of major concerning on a number of fields such as medicine,[10-12] pharmacology[13,14] and biotechnology[15-17] once several biological processes depend upon protein adsorption onto biosurfaces.

During the last years of research in the biomaterials field, predicting, controlling and manipulating protein adsorption onto biomaterial surfaces has been one of the main aspirations. New experimental techniques were developed and the design of theoretical and descriptive models cold in some cases be achieved.

The definition of what is desirable or undesirable regarding protein adsorption is evidently related to the application one is dealing with. In turn, this same application success is intimately defined by the biological reactivity starting from the surrounding environment: the fluid composition and the cellular profile.

In the biomaterials field, the performance of several devices depends on different aspects of the protein adsorption phenomena that affect cell response and determine the implant performance (see Figure 1). On the other hand, controlling the adsorption of proteins from aqueous solutions or from the blood serum is only viable by means of a complete understanding of its specific properties, organization and dynamic protein-interface mechanisms. In turn, this demands for a detailed characterization of the biomaterials surface composition and molecular structure, since the primary interactions between a biomaterial surface and the biological environment occur in the atomic level and in a very thin interface of less than 1 nm in thickness.[18]

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*This chapter is based on the following publication:

C. M. Alves, R. L. Reis, "Protein and cell interactions with biodegradable systems"; in Biodegradable Systems in Tissue Engineering and Regenerative Medicine, Eds. R. L. Reis, J. S. Roman, CRC Press, Boca Raton, (2004), 399-427.

Early on, under this perspective, the opinion of the authors was already the need for plenty of information to complete a protein adsorption study. Empirical answers were to be achieved:[19] what is the classical protein adsorption isotherm? What is the adsorbed amount as function of time? What is the protein orientation/three dimensional (3D) conformation? Protein mixtures: how proteins compete with one another? Do proteins desorb or exchange? How the surface does changes all this? How surface-protein systems modulate cell response?

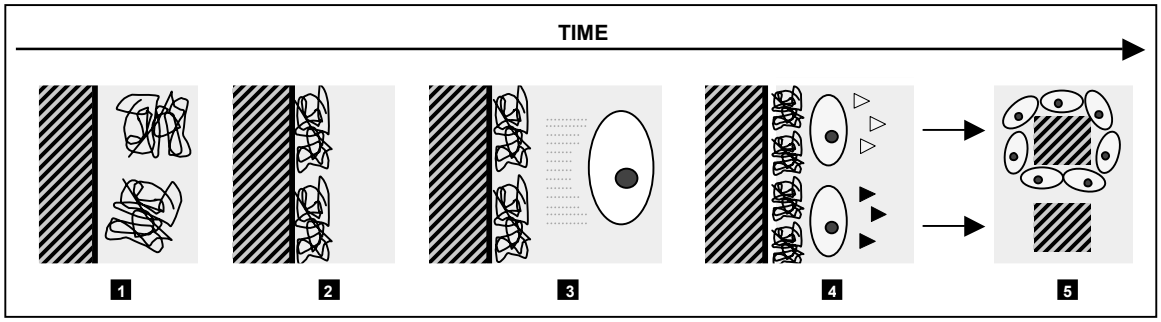

Figure 1. Schematic representation of the interaction between surfaces, proteins and cells. Legend: proteins and surface before (1) and after (2) interacting; the proximal cells (3) by means of interacting with the surface/protein layer, initiates signalling mechanisms (4) which can lead in the end to a cell covering or to a cell resistant surface (5).

In this review chapter a comprehensive overview on the process of protein adsorption is presented: from one protein systems to the complex multi-protein environment, tracing protein dynamics, instability, practical details, limitations and the success of adsorption manipulation on the control of cell response. Finally, the interface surface-protein is considered and presented in terms of its relevance to understand the biological performance of biodegradable materials. Furthermore, complexity, concerns and potential problematic issues are also described.

2. THE IMPORTANCE OF SURFACE PROPERTIES

Polymers are macromolecules composed of many small monomers added to each forming linear, branched or crosslinked structures.[20] Many of the most popular natural origin polymers are polysaccharide-, protein- or polynucleotide-based structures. These are manly distinguished by their degradation rates. In the biomedical field, the control and rate of degradation is critical for the assigned function.

The human body is a hostile environment for implanted polymers, due to the reactivity that fluids and biological surfaces develop.[19,21] The surface and to a later extent, the bulk of the material will undergo significant changes starting from time zero of implantation.[19,22-24] Biodegradable materials are susceptible of incorporating ions and compounds from the surrounding environment but also to send to

solution products of the degradation.[23] Material surface can easily alter the phenotypic expression of bone related cells. Namely molecular weight, polydispersity, wettability and cristallinity can perturb normal cell bioprocesses.[25,26] On the other hand, local tissue response initiate surface erosion mechanisms originating degradation by-products, which can affect the pH of the neighbouring surrounding.[27] The entire environment that is settled around the implanted device will affect the overall cell response conditioning tissue activity and, finally, the recover of the patients health condition.[28,29]

On of the most well-know examples of applications of biodegradable systems in clinical situations, is on the field of bone-related substitution.[30-32] Bone is known to phase between formation and resorption, a turnover process of osteoblast-osteoclast interactive cycles.[33,34] This balanced synchronism involving these two mechanisms is controlled and extremely important for bone normal development. Several pathologic condition bioprocesses arise simply from disturb of normal bone homeostasis.[35-37] When biodegradable biomaterials are implanted its rate of resorption or degradation must go together with bone formation and moreover, the decrease in the mechanical properties of the device are required to protect the bone tissue by a simultaneously reducing of its strength.[38] In other words, a balance between rigidity, strength and elasticity of bone and material properties is to be scaled.[39-41]

Protein adsorption will obviously have a profound effect on the biostability and interfacial properties of the implanted surface, including surface tension, water affinity or even surface charge and structure.[42-44] More important is the study of the opposite effect. Protein adsorption to surfaces is currently known to directly depend on general physicochemical surface properties such as:[9,45-49] wettability, chemical composition, roughness and surface charge energy and tension. Their effect and modification onto protein adsorption and later cell behaviour have been extensively analyzed,[50-54] with many researchers persisting on the study of different surface stimuli to optimize the short-term and long-term performance of biomaterials.

The strategy of surface modification of different biomaterials has been adopted over the years in order to alter the area of the biomaterial that first comes in contact with the biological environment. Surface modifications methodologies have been used in a variety of applications for preventing or improving adsorption of proteins and adhesion of cells to biomaterial surfaces.[46,55-64]

Hydrophobicity and hydrophilicity of the surfaces have been extensively exploited. For instances, studies with chitosan[56] show that an increase in hydrophobicity for values of around 100º of water contact angle lead to increased protein adsorption regarding the more hydrophilic non-modified surfaces. In this case, hydrophobic interactions govern the protein adsorption and the majority of blood proteins form proteinaceous layers over the surfaces.[10,46,56] On the other hand, very hydrophilic surfaces also favour high biocompatibility due to the preferential adsorption of albumin which firmly binds in high concentration.[10,46] Albumin being highly concentrated and diffusive over the solution

medium reaches the surface and binds, leading to thrombogenicity lowering effect.[65] In opposition,[54,66] other authors state that strongly hydrophobic or hydrophilic surfaces present a very low ability for protein adsorption.

Other group of authors state[10,67-69] that the higher water content or higher water uptake ability and minimal interface energy could minimize protein-material interactions and thus decrease the thrombogenic effect. In disagreement, Andrade work[70] showed that hydroxymethacrylate and methylmethaclylate formulations of high water content presented increased thrombogenicity. Recent studies[71,72] with biodegradable poly(D,L-Lactic Acid) showed a relation between contact angle and surface energy with protein adsorption: preferential albumin and fibronectin was adsorbed onto surfaces of improved hydrophilicity and surface energy.

Polymer surface dynamics and relaxation on a solution environment is exhibited for interfacial free energy minimization: polar components tend to dominate.[73,74] The contact protein-surface is function of the chemistry of the polymer in equilibrium with the water and its ions, and with the protein chemical properties. By contacting a surface, protein dynamics takes place in response to the characteristics found in the interface.[75] Surface physic-chemical characteristics such as the surface free energy and electric charge do also affect protein adsorption.[76-78]

3. PROTEINS, ADSORPTION AND KINETICS

The process of protein adsorption onto a solid surface is considered for years as a complex type of interactions of physical and chemical origin that is established between surface, solvent and proteins.[73,79-89] Characterizing the complexity of protein covered surfaces is required,[90] demanding advances in surface science instrumentation together with new material science and molecular biology technologies. Finally, a full understanding of the properties of the proteins in study is fundamental for reaching the complexity of the adsorption phenomena.

3.1. Assessing protein adsorption

The success of protein adsorption studies depends directly on the selected techniques of analysis. Surface science models for application in biological systems are not fully developed. If that was the case, ideally one would be provided with an understanding of how the surface chemistry and structure of a material can be used to control the biological reactivity of a cell interacting with such a surface. To accomplish this goal, understanding cell reactivity and characterizing the complexity of protein covered surfaces is clearly required.[90]

In the last quart of century, advances in surface science instrumentation together with new material science and molecular biology technologies greatly improved the ability for characterizing interfaces of biological importance. Nevertheless, the majority of the popular techniques are only an approximation of the ideal non-artefact generating characterization tool. Single protein solutions are generally emphasized over the complex protein mixtures when it comes to the simplicity of the study that must be performed.

The analysis of the amount of proteins that adsorb onto a specific interface must be performed by means of applying highly accurate methods once the amount of adsorbed proteins in function of the surface area is typically very low.[15]

Techniques that give information about the adsorption process and nature of the protein layer are summarized on Table 1, together with respective references for allowing the reader to obtain further detailed information. Several techniques have been used to quantify adsorbed proteins but the preferred strategy is always to combine different techniques in one study allowing for complementing and adding new information. In the literature several overviews[19,90-92] relate the analysis methods with the variable in study, simultaneously presenting limits and advantages, highlighting new approaches, techniques and models.

The most traditional technique used over the years for analysis of protein adsorption is the solute depletion technique.[19] In this case, adsorption is determined as the difference between final and initial protein amount following the contact and incubation protein-surface. If the obtained result approximates zero, an adsorption rate near to 100% was achieved.[19] This methodology is coupled to other protein detection methods such as: immunoassays,[123] colorimetric[140] and fluorescence[141] techniques that can also be used for total protein quantification. Simultaneously, radiolabelling has been frequently used[142-144] for determining protein adsorption concentration. This methodology as practical limitations including the easy alteration of the protein affinity for the surface following radiolabelling.[145] For the understanding of the complexity of the molecular aspects of protein adsorption and denaturing, computational chemistry is becoming a very attractive technique.[146,147] Real biomaterials surfaces are still not fully represented but the contribution given so far by this methodologies, may influence the way we look for these phenomena.[147] The authors believe there will be a great future for such type of methodologies. Techniques and their applicability will be further explored along this chapter while related to topics such as competitive protein adsorption onto a particular surface.

Table 1. Methodologies and techniques used to study different aspects of protein adsorption onto surfaces compiled from references [93-139].

Method	Obtained Information	Refs.
Solute Depletion	Adsorbed amount of proteins	93, 94
Direct Weighing	Adsorbed amount of proteins	95, 96
Surface Plasmon Ressonance (SPR)	Rate of adsorption and thickness of adsorbed layer	97-99
Ellipsometry	Thickness of adsorbed layer	100-103
X-Ray Photoelectron Spectroscopy (XPS)	Adsorption amount	104, 105
Radiolabelling	Adsorption quantification	106, 107
Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOFMS) and MALDI-MS	Protein adsorption distribution, quantification, composition and conformation	108-110
Circular Dichroism (CD) Spectroscopy	Conformation	111, 112
Atomic Force Microscopy (AFM)	Visualization of Conformation	113-115
Confocal Microscopy	Detection and conformation of protein adsorption	116, 117
Fourier Transform Infrared Attenuated Total Internal Reflection (FTIR-ATR)	Conformation of adsorption	118-122
Immunoassays	Detection and conformation of adsorbed proteins. Adsorption patterns	123-126
Microcalorimetry	Enthalpic changes	127
Raman Spectroscopy	Protein Conformation	126
Elution method coupled to SDS Electrophoresis	Qualitative analysis of protein composition	93, 128, 129
Time-of-Flight Secondary Ion Mass Spectrometry (ToF SIMS)	Molecular structure	130
Scanning Probe Microscopy (SPM)	Spatial resolution	131
Near Edge X-Ray Absorption Fine Structure (NEXAFS)	Chemical Specificity	132, 133
Total Internal Reflection Fluorescence (TIRF)	Protein adsorption kinetics, competition, conformation and lateral mobility	134-136
Axisymmetric Drop Shape Analysis-Profile (ADSA-P)	Chronological determination of protein adsorption	137-139

3.2. Proteins in definition

Chemically, proteins are unbranched co-polymers of twenty-two different aminoacids of varying hydrophobicity. Some of the *R* groups of aminoacids are acidic or basic conferring to the protein molecules an ambivalent character (see Figure 2), and due to their differences in polarity, proteins are rendered surface-active macromolecules of amphiphilic properties.[19,80,148-151] Aminoacids are linked by polycondensation, head to tail, from carboxyl group to amino group through the formation of an amine linkage designated peptide bond.[151]

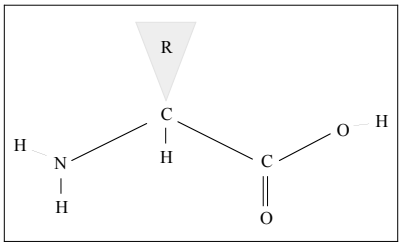

Figure 2. The amino acid monomer structure. R, represents the side chain different in each amino acid.

The primary structure of proteins is the polypeptide chain or amino acid sequence. It is important to state that all the information needed for the protein molecule to achieve its architecture is contained within its amino acid sequence.[19,151] The formation of hydrogen bonds between peptide units, results in the well-known α-helix or β-sheet, two different non-compact types of secondary structure.[19,150,152] By means of ionic interactions, salt-bridges, hydrophobic interactions, hydrogen bonding and covalent bonds create a more compact structure, assigned tertiary structure.[19,150,152] The final possible association is between two polypeptides of organized primary, secondary and tertiary structures, giving rise to the quaternary structure of proteins.[19,148-152] Whereas the primary structure of a protein is determined by covalently linked amino acids residues, other organizational levels are mainly determined by non-covalent forces.[152]

Any protein adsorption study can not be useful without first fully understanding the properties of the surfaces or specially while ignoring the properties of the proteins playing the game. Some of the well-studied proteins in the biomaterials field are:[153-155] serum albumin, fibronectin and vitronectin.

Albumin is the most abundant protein in the human blood serum and due to its concentration and ability to bind other molecules is seriously considered as a model protein also in terms of protein competition.[156-158] More specifically, albumin primary role is the transport of fatty acids, but also, the maintenance of colloidal osmotic blood pressure and detoxification.[156-160] Human serum albumin is a heart-shaped and monomeric protein composed by 585 amino acids that compose a total of 66 400 Da of molecular weight.[157,160]

Most of the proteins contain short carbohydrate sequences and are therefore, called glycoproteins.[19,151] Fibronectin (FN) is a large glycoprotein formed by two disulfide bounded polypeptides that can be

found in blood plasma and other fluids on its soluble form but also as structural protein on solid tissues.[161-163] FN is an extended molecule folded in globular domains of particular functions and linear arrangement of repeating units of amino acids, know as type I, II and III.[164,165] Several research studies[166-170] have proved the influence of this molecule in interacting with integrin and non-integrin cell surface receptors, through which, cell adhesion, migration, proliferation and differentiation are affected. In central location of the chain, type II repeats were identified to include both arginine-glycine-aspartic acid (RGD) motif and the proline-histidine-serine-arginine-asparagine (PHSRN) synergistic sequence.[164,171]

Vitronectin (VN), also found in plasma and ECM is a multifunctional glycoprotein of approximately 75 kDa, which comprises the important RGD peptide that is known for mediating attachment and spreading of cells.[172] On the other hand, by binding to plasminogen activation inhibitor-1,[173] this protein can potentially regulate the proteolytic degradation of the extracellular matrix, and is also involved in the immune response and clot formation.[108,174]

3.3. Adsorption and desorption kinetics

In an adsorption study several parameters are always to be considered. In Figure 3 a simplistic schematic representation of several factors affecting the phenomena of protein adsorption are plotted regarding the tri-element situation: surface-solution-protein. On what concerns to surface characteristics some of the fundamental aspects are:[19,148,175-178] surface hydrophobic nature, topography, heterogeneity, surface composition, chemistry, interfacial dynamics and surface stability in water. On the other hand, protein characteristics as the isoelectric point, charge distribution, three-dimensional conformation and stability, the distribution and nature of hydrophobic domains and the ability to bind low molecular weight species need to be considered.[19,178] The pH, ionic strength, ionic compounds, the buffer nature, the presence of low molecular weight species and also the solutions temperature, pressure or hydrodynamic flow are decisive in terms of the final protein-surface interactions.[19,178-181]

The adsorption of proteins at interfaces has been shown to be a complex phenomenon that includes the diffusion of the protein species through an aqueous medium and its collision and interaction at the interface,[182] where the major driving factor is the ensuing entropy gain.[152] Several papers[147,148,152,183-188] are available in the bibliography were models of the thermodynamics of adsorption isotherms are described.

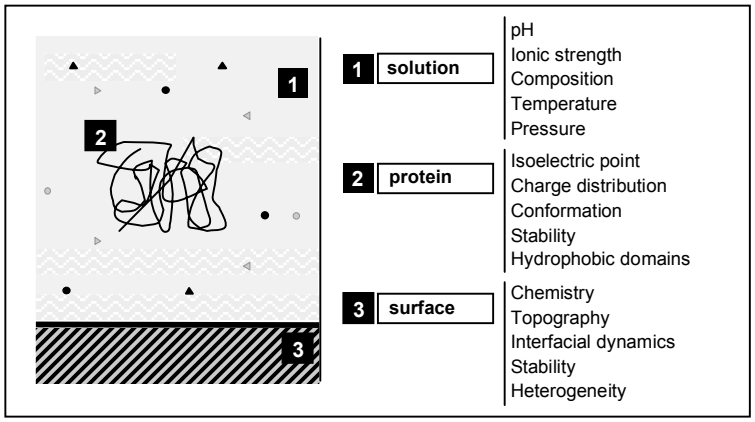

Figure 3. Simplistic cartoon representing some of the solution, protein and surface factors that affect the protein adsorption event.

Over the last two decades, several authors proposed models to explain the adsorption phenomena. Examples are Beissinger and Leonard,[189] Soderquist and Walton,[190] and Lundström and Elwing.[191] The complexity of this last model already includes the concepts of:[191] adsorption constant (k_a), desorption constant (k_d,) conformational changes and exchange constants, (k_e) and (k_c), respectively. According to Norde,[152] the process of adsorption kinetics is typically divided in five steps: transport to the surface; adsorption; time-dependent re-arrangement; desorption or exchange; and diffusion away from the surface. Measuring the interaction protein/surface is one of the major goals in this field translating the affinity of a certain protein to a surface. In 1986, Horbett and Brash[182] proposed that this affinity phenomenon could be deduced from kinetic observations under diffusion limit, which would directly lead to the sticking coefficient. According to the authors, the sticking coefficient reflects the number of collisions that lead to adsorption being a function of the molecular interactions between the protein and the surface.[182,192] The study of the sticking coefficient has been intimately discussed with another concept, the *elastic barrier*. [182,192] Within this concept, a certain protein in solution and approaching the surface has probability Φ to adsorb and 1-Φ to be reflected. Also considered, is the *random walk* of this particle, or in other words the *journey* performed by the molecule in the proximity of the surface.[182,192]

Different studies[145,193] have been reporting the difficulties and practical limitations of the estimation of protein adsorption onto polymeric surfaces. One of the prerequisite for the analysis of the biological reactivity of a material is the kinetic association constant of high information value over the early adsorption times.[193] The difficulty for achieving this quantitative element regards the complexity of the determination of the adsorbed proteins over the first seconds of adsorption. In this case, several techniques can be used, but the most successful one as been reported to be ellipsometry.[54,102,194-197] Also, the steric hindrance and mass transport considerations hinder the accurate determination of

k_a.[19,47,193,198] The interaction of the water components with the surface generates potential adsorption sites and the balance between protein concentration, surface area and volume for diffusion make experiments a difficult issue to control. Other of the difficulties is when surfaces became in contact with the air, the protein layer will denature by conformational changes, which explains the air avoiding need of the techniques and models developed on the scope of the kinetics studies.[43] A spontaneous structural arrangement is expected when a protein molecule in solution touches a solid surface. Binding and orientation of proteins is one of the most studied topics of the protein adsorption issue.[199]

Desorption of proteins from surfaces has been reported to be nonexistent or very slow, which in other words means: irreversible, partially reversible,[152,200] and also reversible process.[201] pH changes and ionic strength can completely remove molecules from surfaces, which are often used for protein adsorption analysis methods, as two dimensional (2D) electrophoresis.[19,91,202,203] New ionic strength conditions allow for bound proteins to be eluted from the surface into the solution in a more pronounced way for hydrophilic surfaces than for hydrophobic ones.[19,152,178,200,204-206] The discussion of desorption mechanisms cannot be dissociated from two other concepts: exchange and protein competition, both characterized as a high speed interfacial phenomena.[196,207-210]

4. EXCHANGE AND COMPETITION OF PROTEINS: BLOOD PLASMA AND COMPLEX SOLUTIONS

The complexity of the issue protein adsorption was very well reviewed by Andrade et *al*.[211] using a axis concept which ranges from relatively simple proteins to the very complex ones following the multi-component protein solutions such as blood plasma and tears.

By means of simply performing single protein adsorption studies neither the complexity of blood bioenvironment nor the biocompatible potential of biomaterial surfaces can be assessed.[97,212] When it comes to the adsorption from blood, plasma surfaces are enriched on a number of protein species. The limited number of adsorption sites per unit area drives to a selection process regulated by the intrinsic ability of some plasma proteins for preferential adsorption in opposition to others.[91,135,213,214] In this sense, competition phenomena can only be measured by means of studying multi-protein solutions, such as blood plasma[97,123,215,216] or mixtures of plasma proteins.[97,109,123,217]

Essentially, all the biological fluids are multi-protein systems. Blood plasma was firstly studied by Vroman and Adams in 1969.[218] Proteins were suggested to adsorb sequentially starting from the abundant low molecular weight ones, like serum albumin, and ending, for longer time periods and after species exchange, with kininogen as preferentially adsorbed onto the formed layer.[218] These experimental findings gave rise to the so called Vroman effect.[8,219-225] More specifically after the contact of blood or plasma with crystal and glass modified surfaces, the absorbate composition

changes with time as a result of the consecutive replacement of the adsorbed proteins. In the early stages smaller and higher concentrated protein species will reach easily the surface for adsorption being later exchanged by higher surface activity ones and less concentrated proteins. According to their observations, Vroman and Adams[8,222,223,226-228] proposed the following sequence of adsorption onto blood contacting surfaces: albumin, immunoglobulins, fibrinogen, fibronectin, high molecular weight kininogen, and factor XII.

The effect of concentration coupled to the residence time was found to modulate the amount and composition of the protein layer.[97,217] Although these observations became generalized for several proteins and surfaces, systems were observed to be excluded from this concept.[229,230] Both, the degree of dilution[230] and the type of surface[231] affect the kinetics and sequence of exchange, thus influencing the absorbate composition. Besides residence time and surface properties, the protein nature, unfolding rate, diffusion constants, surface affinity or ability for irreversible binding will determine the conformational change and interaction with the surface to achieve the most favourable energetic state.[135,230,232]

Considering this, competition and exchange of proteins cannot be understood as separate mechanisms. It is frequently observed that proteins desorb into solution at a very low rate, in opposition to the situation were new or other protein species are present.[135] In the early 80s, Jennisen proposed a molecular explanation for these observations.[233] Proteins adsorb forming multiple contact points with the surface, which are unlikely to disappear at the same time in accordance to the observed low spontaneous desorption rate. If other more active proteins start adsorbing in this newly created free spaces, eventually the old protein will be replaced by the establishment of contact points between the new protein and the surface (Figure 4). Thus the increasing desorption rate could be promoted by protein exchange.[233]

Protein competition is expected to happen simultaneously to the adsorption of the molecules to a presented surface. Competition between proteins is conditioned by several parameters[207,234] such as diffusion coefficient, molecular mass, polarity or electrical charge of the proteins in a determined evolving fluid. Surface properties are known to greatly affect adsorption of single protein systems.[156,205]

Although inappropriate for simulating the bioenvironment complexity, attempts[1,19] have been made to find general rules for relating adsorption from single protein solutions, to the adsorption in competitive environment. Still in this context and on the biotechnological level, the development of new techniques to evaluate complex protein solutions in contact with the material surfaces is becoming urgent.

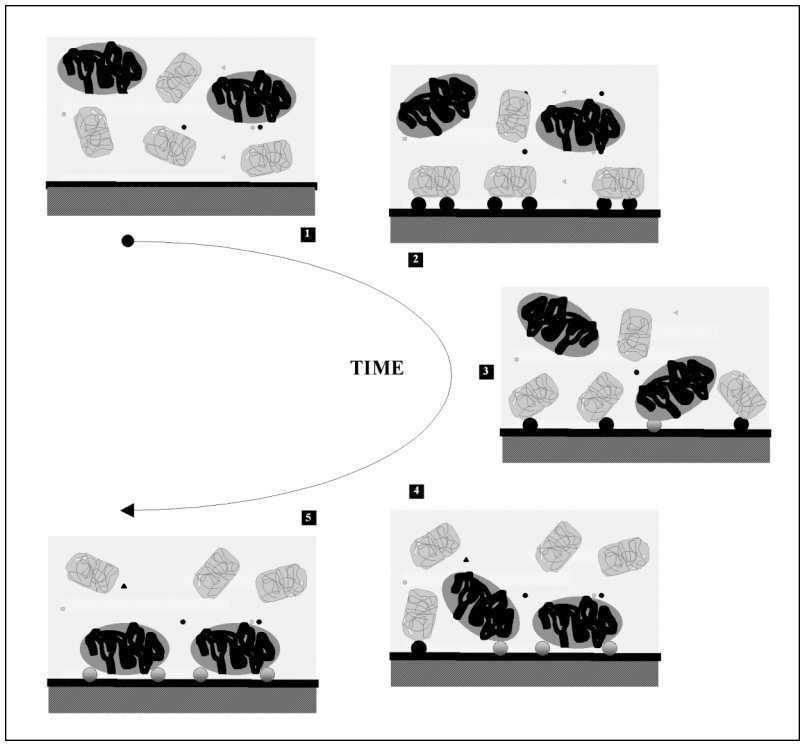

Figure 4. Simplistic cartoon representing the exchange and competition of proteins from the earliest to the later stages. Concentrated small proteins with higher diffusion constants and lower concentrated proteins with higher dimension and binding affinity are symbolized as previously indicated.

Exchange reaction models have been proposed for the analysis of complex protein solutions.[231] and several techniques have been adapted for this application. The most common ones are radiolabeling,[235-237] fluorescence[238] and ellipsometry.[239,240] More recently, methodologies such as surface plasmon resonance[97] and X-ray photoelectron spectroscopy (XPS) coupled to surface matrix-assisted laser desorption/ionization (MALDI) mass spectroscopy[109,241] have been applied. Methodologies based in antibody-specific binding properties, as the enzyme linked immunoassay (ELISA) system have been also selected and applied by several authors.[123,242-244]

Norde and Lyklema showed that the stability/rearrangement ability of the proteins strongly affects preferential adsorption of less stable proteins in favour of the most stable ones.[204]

Lassen and Malmsten[217] have shown in 1997 that human serum albumin (HSA), immunoglobulin (IgG) and fibrinogen (Fbg) extensively adsorbed onto both hydrophilic surfaces of different charges, but negatively charged ones evidenced simultaneously, slower adsorption kinetics, and HSA exchange under the presence of other proteins. Regarding protein competition, Fbg was predominantly adsorbed on both these surfaces while for a third hydrophobic surface IgG and albumin dominated the protein layer.[217] Earlier on, the same authors demonstrated the ability of albumin for blocking other proteins adsorption on the presence of hydrophobic polymer surfaces due to irreversible adsorption associated

to conformational changes of albumin.[245] On another study,[236] adsorption of collagen was reduced in the presence of albumin and to dramatically decrease with the increase of surface hydrophobicity.[236]

The use of antibodies to label human proteins allows for obtaining reproducible and useful for understanding protein adsorption on biodegradable surfaces (Figure 5).[123] Immunoassays are aimed to detect a specific *target protein* (Figure 5A *2*), using *blocking proteins* (Figure 5A *3*) for minimizing the non/specific binding of the *primary antibody* (Figure 5A *4*) to the *surface* (Figure 5A *1*). To this anti-target protein antibody, specifically binds the *secondary*, which in turn binds *Alkaline Phosphatase* (ALP). In the presence of the *substrate*, enzyme *catalysis* takes place and *a different colour* is produced on the surface (Figure 5A –step *5, 6, 7, 8 and 9, respectively*). On Figure 5B, labels 1 and 2 show colour production and absence, respectively, onto the surface of a starch and cellulose acetate (SCA) polymeric blend reinforced with hydroxyapatite. Antibody labelling technique has been used with different polymeric blends of starch with cellulose acetate (SCA), ethylene vinyl alcohol (SEVA-C) and polycaprolactone (SPCL).[123] Besides the synthetic phase being different for each material, the percentage of starch is also variable: 50% for SCA and SEVA-C and 30% for SPCL starch based blends. Single, binary and serum diluted solutions were prepared using human sources and the proportion of the proteins in the human blood serum was considered.[156] Results allowed to observe that after 24 hours, fibronectin (FN) and vitronectin (VN) adsorbed in higher amounts than the more concentrated HSA.[123] When studying binary systems, FN in the presence of HSA was found to adsorb less than albumin on the SEVA-C surface, in opposition to the observations obtained for single protein solutions. Both, the chemistry of the material and the presence of other proteins was found to be determinant for the final adsorbed layer. When diluted human blood serum was used as the protein source, differences in protein adsorbate after 24 hours of incubation were observed.[123] As a general trend, the competitive potential of albumin to adsorb onto starch-based surfaces was decreased.[123] This can be related to the lower activity, size, higher concentration of this protein and also with the residence time.[97,156-158,217] In agreement, vitronectin, and to some extent, fibronectin were the most highly adsorbed proteins independently of the surface used. In terms of polymeric blend types of it was also found that corn starch with polycaprolactone presented the highest protein adsorption levels independently of the protein specie.

These results show the influence of the polymer nature and the protein specie onto the final adsorbate layer.[123] Furthermore, these results agree with earlier performed experiments of Fabrizius-Homan and Cooper,[236] which showed that following the contact of polymeric surfaces with diluted serum, plasmatic proteins do adsorb but a significant enrichment of vitronectin is observed in the surface.

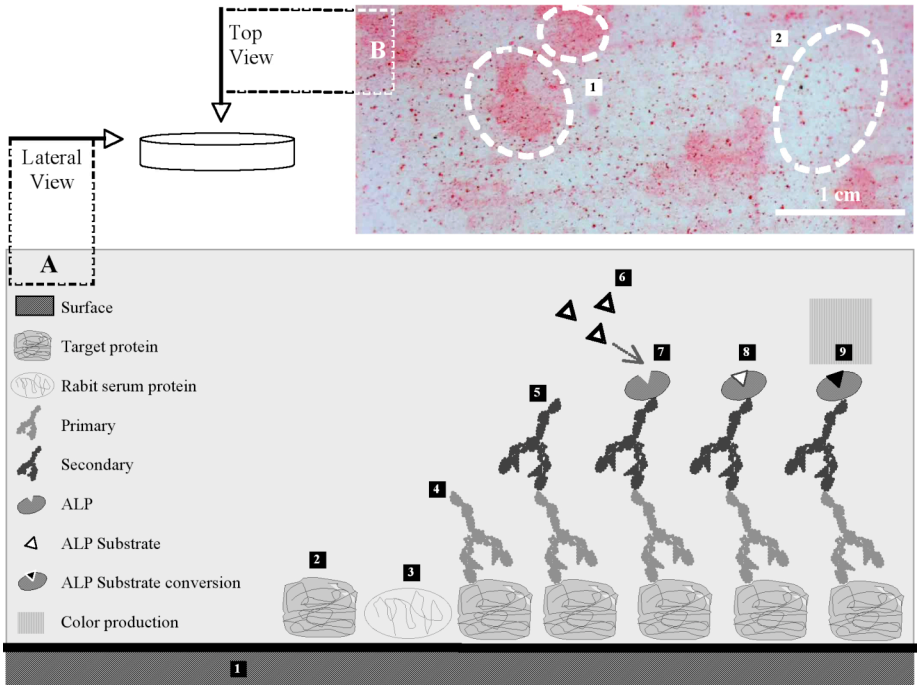

Figure 5. The immunoassay methodology in a simplistic sequence cartoon (A) simulating a lateral view and the final result for a starch based material surface previously coated with human fibronectin and labelled with anti-FN (B).

5. PROTEIN RECOGNITION AND CELL ADHESION MECHANISMS

The recognition that cellular functions at the implant-interface are determinant for the degree of success of a biomedical device, the clinical use of a material, which allows for predicting and to develop beneficial reactions from the implants surrounding cells and tissue, can be easily understood.

In the early 90s, Ratner[246] envisioned and described for the first time the "next generation of biomaterials" as engineered surfaces deliberated to invoke specific cell responses. Furthermore, this concept would include materials able to compensate the complex medical condition of the injured patient, simultaneously improving the healing process.[246]

For the design of biomaterial devices, normal cell mechanisms such as adhesion, migration, proliferation and differentiation of the specific cell populations involved are clearly required to be understood.[247]

Anchorage dependent cells such as fibroblasts, osteoblasts or endothelial cells are so designated due to the need of establishing adhesion mechanisms for normal cell functioning as development, organization and maintenance of tissues.[248,249] Following attachment, an intracellular cascade of events will be developed leading to the regular phenotype and genotype of the specific cell lineage, including spreading, differentiation, secretion, extracellular matrix (ECM) production and migration. Adhesive cells use extracellular matrix proteins to attach and to migrate on substrates.

The primary mode of adhesion and migration is performed by means of integrins, cell transmembranar receptors composed by α and β units that recognize the well-studied RGD peptides.[250-253] Integrins proteins are expressed in several cell types including bone and in bone cells culture.[254] RGD adhesive sequences, so designated due to its ability to bind a specific ligand,[255] are know to be present in several ECM proteins including, bone sialoprotein, collagen, fibronectin, osteopontin, thrombospondin and vitronectin.[256-259] Following the receptor-sequence interaction, integrins cluster together and organize into focal adhesion complexes with mechanical and chemical activity of cell anchoring, generating an intracellular cascade of multiple signalling events.[253,260-262] This subsequently regulates cell migration, proliferation, phenotype, genotype, and thus, cell differentiation.[253,260,263-265] In this context, the behaviour of anchorage-dependent cells seems to depend on the availability/exposition of these adhesion sequences, which in turn is affected by the protein tree-dimensional conformation. Adhesion of a cell to the underlying substratum can be controlled by increasing the ligand density, the affinity of the binding receptor-ligand or by the amount of adhesion receptors expressed on the exterior surface of the cell phospholipids membrane.[266] Several integrins need other peptidic sequences for efficient binding. For instances, $\alpha_5\beta_1$ integrin involved in the control of osteoblast and myoblast cells proliferation and differentiation[267,268] only binds to RGD segment on the 10th type III repeat of fibronectin in the presence of the PHSRN motif to which binding is performed with 9th type III repeat. Adhesion strength is significantly increased by this synergistic association.[269,270]

Intimately connected to the adhesion of cells is the process by which cell movement is triggered.[271-273] Cell migration is a complex dynamic mechanism achieved by the cell transition of cytoplasmic generated forces into tractional forces that will pull the cell itself across the substrate.[274,275] Traction is provided by the interaction between integrins, cell adhesion surface receptors, and specific ligands covalently immobilized on the biomaterial surface.[252,276,277] Following these interaction receptor-ligand, several intracellular processes take place, namely cytoskeleton organization, signalling, force generation, cell body displacement and rupture of the bonding receptor-ligand in the surface area opposite to migration direction.[278-281] This is, migrating cells while protruding and stabilizing leading edges, release the early formed complexes at the rear of the cell.[282] The extent or strength of the adhesion between the cell and the underlying extracellular matrix is critical in determining the efficiency of cell migration.[266,276] Experimental work developed by Dee *et al.*[272] studied the dependence of random migration, also designated as haptokinesis onto RGDS and RDGS peptides which include adhesive and non-adhesive domains, respectively. Results showed significant reduction of migration and enhanced proliferation of osteoblasts over RGDS segments as compared to RDGS-modified surfaces.[272] Thus, the correlation between migration and proliferation, and the contribution of both processes on the surface colonization by osteoblastic cells was shown. It is rather important to realize that migration has an important function in processes as embryogenesis, inflammation and

tumurogenesis,[274] and in the colonization of newly body contacting surfaces as the desired migration of bone related cells to the implanted bone prosthesis.[272]

6. SELECTIVE PROTEIN ADSORPTION: STRATEGIES FOR CONTROLLING AND MODULATING CELL AND TISSUE RESPONSE

Several literature sources[5,9,283] describe the problematic of the non-specific bioadhesion like protein adsorption, as the main cause of biomaterials failure due to uncontrolled accumulation of biological material at the interface. Controlling this non-specific phenomenon have been reflecting the manipulation of the chemical, physical and biochemical properties of an implant surface. Underneath the biomaterials scope, is widely recognized that the first interaction between biomedical devices and the biological environment occurs at the interface, which plays an important role in the biomaterials design. Attempts have been made[147,284-288] in order to control the adsorption of proteins mainly by means of surface modification methodologies as the use of polysaccharides, phospholipids, proteins, fragments and grafting of polymer molecules to the surface of the materials.

For the control of protein adsorption and biomaterials design, surface treatments, different protein species, their concentration and physical properties of the aqueous environment (blood or tears) are to be considered. Moreover, the chemistry and physics of the surface and the application time scale of the device play important roles.

Designing surfaces to control and predict protein adsorption is not a new topic. In this context, during the last 20 years, researchers have conducted experimental and theoretical work[211,289,290] that resulted in a lot of information but low molecular-based comprehension. The understanding of submolecular events of proteins adsorption is fundamental and the emerging of even more powerful characterization techniques is becoming urgent.[147]

6.1. Proteins and peptide sequences

The identification and recognition of the value of adhesive sequences (such as RGD and PHSRN) motivated bio-inspired surface modification techniques as the incorporation of short peptides onto adequate surfaces, which are generally non-adhesive. The ultimate gold of these protein-mimetic surfaces is the reduction of non-specific protein adsorption for obtaining functional surfaces.[291]

In the last years, several groups have been studying, both *in vitro* and *in vivo*, different surfaces incorporated with cell adhesion peptides on the binding properties, attachment, proliferation, differentiation, migration, morphology, and spreading of cell lines and primary cultures.[199,277,292-298]

These approaches offer advantages over the use of entire molecules that include:[299-301] antigenicity decrease, biocompatibility increase by removing domains able of starting adverse reactions as complement activation domains, fibrinogen, collagen and heparin binding domains. Simultaneously, recombinant fragments allow for conferring specific characteristics for enhancing the immobilization

of proteins improving their activity and finally the ratio efficiency/financial cost increases.[302] On the other hand, according to Akiyama *et al.*,[303] the use of small segments instead of higher dimension fragments increases the possibility of defect in activity due to conformational changes, and also represent lack of integrin specificity.

Table 2. Different protein peptide domains and peptide combinations found to control cell response (*used as negative controls), compiled from references 247, 272, 276, 302, 304-309.

Peptide and Aminoacid Composition	Type of Domain	Observed Cellular Effect	Refs.
RGD (arginine-glycine-aspartic acid)	Adhesive	Neonatal rat calvarial osteoblasts presented enhanced attachment, spreading and cytoskeleton organization. The formation of mineralized matrix was stimulated	304
YIGSRG (tyrosine-isoleucine-glycine-serine-arginine-glycine)	Adhesive	Enhanced bovine endothelial cells proliferation and motility	247
CRGD (cysteine-arginine-glycine-aspartic acid), **CREDV** (cysteine-arginine-glutamate-aspartic acid -valine), and **CCRRGDWLC** (cysteine-cysteine-arginine-arginine-glycine- aspartic acid -tryptophan-leucine-cysteine)	Adhesive	CCRRGDWLC enhanced human vascular endothelial cells adhesion and mouse fibroblasts adhered best to CREDV	305
RGDS (RGD-serine)	Adhesive	enhanced mouse fibroblast cells proliferation	306
RGDS (RGD-serine) and **RDGS*** (arginine-aspartic acid-glycine-serine)	Adhesive and Non-adhesive*	Reduction of haptokinesis of neonatal rat calvarial osteoblasts decreased on adhesive peptide presence	272
GRGDSPC (glycine-RGD-serine-proline-cysteine) and **GRGESPC** (glycine-arginine-glycine-glutamic acid-serine-proline-cysteine)	Adhesive and Non-adhesive*	Mouse melanoma cells migration persistence time decreased for increasing adhesiveness	276
RGD and **PHSRN** (proline-histidina-serina-arginina-asparagine)	Both Adhesive and PHSRN is a FN synergy site	Macrophage adhesion, activation and foreign body giant cells formation (FBGC)	307
RGD, **PHSRN** and **PRRARV** (praline-arginine-arginine-alanine-arginine-valine)	All adhesive and PRRARV is a FN C-terminal heparin-binding domain	RGD and PHSRN promoted FBGC in opposition to PRRARV	308
RGDS, **YIGSR**, **VAPG** (valine-alanine-proline-glycine), **VGVAPG** (valine- glycine-**VAPG**), **KQAGDV** (lysine-glutamine-alanine-glycine-aspartic acid-valine), and **RGES** (arginine-glycine-glutamic acid-serine)	All adhesive and RGES non-adhesive*	Adhesion of SHR smooth muscle cells increased for all adhesion peptides	309
RGD, **PHSRN** and **PRRARV** (praline-arginine-arginine-alanine-arginine-valine)	All adhesive and PRRARV is a FN C-terminal heparin-binding domain	RGD and PHSRN promoted FBGC in opposition to PRRARV	308
FNIII7-10 (FN fragment compassing RGD and PHSRN domains)	Both adhesive	Murine immature osteoblast like cells adhered via $\alpha5\beta1$ integrins, spread, displayed cytoskeleton reorganization and assembled robust focal adhesions	302

The use of complete proteins instead of small peptide segments or sequences envisions the study of *in vivo* biology by simulating the molecular structures presented by nature. Several studies reveal the cellular effect of pre-adsorbing different proteins onto polymer surfaces (Table 2). Fibronectin was soon considered the archetypal cell-adhesive protein[259,310] and a regulator of cell behavior,[165,311] generating research interest in studying the effect of this molecule over different polymeric surfaces and in terms of cell adhesion and spreading,[262,312-319] migration,[271,320,321] proliferation[267,322] and signalling pathways.[261,262] Besides fibronectin, other proteins such as fibrinogen[316,323-326], albumin[243,324,327,328] and collagens[317,329,330] have also been extensively studied.

6.2. Protein-resistant surfaces

In the case of blood-contacting biomaterials, the adsorption of plasma proteins is known to occur within seconds of exposure and to trigger numerous adverse effects: coagulation, platelet adhesion and activation, complement activation, and immunological reactions like thrombosis[331-335]. By compromising the normal homeostasis of the particular bioenvironment, the implant can be subjected to biological reactions some how similar to body response to virus attack response but an increased scale. Depending on its composition, the protein layer can trigger adverse biological mechanisms: certain concentration of platelet adhesive proteins, such as fibrinogen and fibronectin, develops mural aggregates and thrombus. Thrombus formation may block smaller diameter vascular grafts and embolisation leading further on to more serious complications. Besides hemocompatibility-related devices, on ophthalmic applications the adsorption of tear proteins is associated with lens fouling. Similarly, the applicability and usage of lenses becomes limited and determinant of patient discomfort.[336] Also undesirable is the protein adsorption effect on accelerating clearing of bare liposomes by the reticuloendothelial system.[337]

The pre-existing know-how on thrombogenesis, foreign body-response and interfacial protein behaviour as natural biological mechanisms of body defence allowed for the development of approaches for its prevention: protein resistant surfaces.

To obtain a non-fouling surface, the combined forces of attraction, such as van de Waals, electrostatic, entropic and hydrogen-bonding forces protein-surface need to be smaller than the entropic and hydrodynamic repulsion due to thermal motions of the flexible molecular chains and solvent molecules.

Recent work[338-340] showed how the ability of a polymer layer in reducing protein adsorption, either kinetically and thermodynamically largely depends on the surface coverage of grafted polymer and in the interaction of these segments and the surface. When the polymer chains are attracted to the surface, proteins in the surroundings are subject of a strong steric repulsion and also competition for the surface between proteins and polymer chains will take place. Both these aspects explain why surfaces

that attract the polymer show lower protein adsorption than surfaces that do not, although an equilibrium is needed for assuring the formation of an efficient long range steric barrier for proteins.[284] Such type of surfaces can be achieved by means of immobilizing neutral and hydrophilic polymers such as poly(ethylene oxide) (PEO).[341-343] In fact, early and recent studies of protein resistant surfaces haves been mainly motivated with PEO and its low molecular weight equivalent, and poly(ethylene glycol) (PEG; M_w < 10000).[344-346] The interactions between PEO and proteins have been widely investigated and modelled. Poly(ethylene oxide) is a neutral polymer soluble in aqueous media because of the formation of hydrogen bonds with the water. Within this system description PEO is a simple polymer while comprising monomers that exist in a single start and a complex polymer due to the number of different interconverting states in which the monomer can exist.[337]

PEO polymers exhibit the minimum of interfacial free energy considering water soluble polymers. Both polymers are hydrophilic presenting unique properties and molecular conformation in water, exhibit high surface mobility and steric stabilization.[345,347-349] In opposition, these polymers are also soluble in organic solvents, a result of their hydrophobicity. This conjugation is a distinguishable property fundamental for these polymers excellent biocompatibility. The mechanisms responsible for protein adsorption reduction and eliminating non-specific adsorption are not fully understood. According to several authors,[49,350] PEO surface density and molecular weight are the decisive characteristics that enable these polymers for protein resistance. In the literature several techniques are described as adequate to generate PEO rich surfaces: physical coating, chemical coupling and graft copolymerization,[321,336,347,351-353] by which several types of surfaces have been modified on the scope of different biomedical application.[56,328,336,347,354-357]

Other polymers have been indicated in the literature as low protein interacting surfaces: 2-methacryloyloxyethyl phosphorylcholine[358,359] and other methacrylates with phosphorylcholine group,[360] poly(L-lysine)-*graft*-poly(ethylene glycol),[361] poly(acrylamide), poly(*N*,N-dimethylacrylamide), poly(vinyl alcohol), ethylene-vinyl alcohol copolymer.[349] The effect of coupling proteins, protein residues and other molecules has also been described.[362,363] Examples are albumin,[364] hirudin,[365] thrombomodulin,[366] He^+ ion implanted collagen[356] and variable saccharides as maltose, maltoheptaose and oligomaltose.[362,363] By means of using substrates able to resist to cells adhesion, likewise PEG hydrogels are considered for years,[342] coupled to cell selective ligands allow for binding a specific cell type. On this knowledge, non-adhesive scaffolds have been incorporated with ligands for selective cell adhesion.[367-370] Hubbell and co-workers used fibronectin-derived REDV (arginine-glutamine-aspartic acid-valine) peptides onto non-cell adhesive biomaterials showing the exclusive adhesion of endothelial cells and resistance to fibroblasts, smooth muscle cells and platelets.[371] Also, Mann *et al.*[309,372] proved to successfully photopolymerise TGFβ-1 (transforming growth factor beta-1) to hydrogel surfaces leaded to improved material mechanical properties and collagen synthesis increase, thus improving cell biological activities for certain biomaterial applications. It is recognized

that the adhesion and proliferation of different types of cells on polymeric materials depend on different surface characteristics.[373] It has been demonstrated that cell adhesion occurred preferentially to water wettable substrates.[374] Starch based blends (with ethylene vinyl alcohol (50/50 wt%) - SEVA-C, with cellulose acetate (50/50 wt%) - SCA, and with polycaprolactone (30/70 wt%) – SPCL) have been surface modified in order to enhance cell adhesion and proliferation on their surfaces. Two different methods have been used – chemical surface modification by potassium permanganate/nitric acid system[375] and surface modification by UV-irradiation.[376] In general, both surface treatments have resulted in higher oxygen content (XPS) and as a consequence in lower water contact angle values. This resulted in an increase of the number of human osteosarcoma cell SaOs-2 onto the modified surfaces, especially higher for the blend with polycaprolactone.

7. FUTURE DIRECTIONS AND CONCLUDING REMARKS

The interdisciplinarity of protein adsorption studies defines a complex field of research, where the drawbacks of current applied techniques limit the perfect understanding of the sensitive protein adsorption microenvironment. Efforts, mainly carried out in the last decades accomplished the present know-how where several voids are still to be filled. Nevertheless, these works allowed for reaching a point where surface manipulation for different biomedical applications cannot typically go further before studying the characteristics of the protein adsorbate. This also explains the development and motivation for applying protein related molecules to different surfaces. These types of studies are carried out in several of the most prominent groups that work on the biomaterials field.

After detecting what is desirable or undesirable in terms of the proteins behaviour over biomaterial surfaces, the motivation for its control is now the state of the art and the goal in this field. The idea is not to disregard cells or cellular structures but, aim exactly for directing cell phenotype and implant success improvement. In this sense, there is still a gap between the protein adsorption behaviour observed in *in vitro* situations and the respective correlation with what happens in the *in vivo* bioenvironment.

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SECTION 2

CHAPTER II

MATERIALS AND METHODS

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MATERIALS AND METHODS

1. TESTED MATERIALS

1.1. Starch Based Biomaterials and HA composites

. Starch-based Biomaterials (SBB) have been investigated for a range of applications in the field of Tissue Engineering including scaffolding,[1, 2] bone[3] and drug delivery systems.[4] The *in vitro* biocompatibility[5] and *in vivo* host response[6] of starch-based biomaterials has been assessed and correlated with protein adsorption behaviour.

Starch-based biomaterials (SBB) were the main materials used as interfaces to analyze protein adsorption. Three different polymeric blends of corn starch with synthetic polymers were selected in order to combine the best properties of each one of the materials.

The materials used in this study were biodegradable polymeric blends of corn starch with *(i)* cellulose acetate, 50/50 wt% (SCA), *(ii)* ethylene vinyl alcohol copolymer, 50/50 wt% (SEVA-C) and *(iii)* polycaprolactone, and 30/70 wt% (SPCL). Moreover *(iv)* SCA reinforced with 10% (wt%) of hydroxyapatite (HA, Plasma Biotal, UK) was prepared using twin-screw extrusion (SCA-10%HA).

Blended materials and HA composites were processed in the facilities of the Department of Polymer Engineering, University of Minho. Using conventional injection moulding technology samples were processed into circular discs (∅ 1cm). Furthermore, SEVA-C was also processed into rectangular-shaped blocks 13 x 10 x 7 mm^3. Before implantation, the edges of the SEVA-C blocks were trimmed and samples were rolled for 1 week in glass flasks to round machined edges and reduce the magnitude of edge effects. More details in the processing conditions and properties of starch-based thermoplastic blends can be found in references 7-9.

Samples were sterilised by ethylene oxide (EtO) as previously described,[8] washed, and all subsequent experimental procedures were performed under sterile or clean conditions.

1.2. Poly(D,L-Lactic Acid)

Poly[D,L-lactic acid] (PDLLA) was modified, characterized and studied in terms of protein adsorption and cell behaviour. Moreover, being the gold standard for biodegradables in biomedical applications PDLLA was used as a biodegradable control material.

Poly[D,L-lactic acid] with an approximate molecular weight (MW) of 91000 g/mol was supplied by Birmingham Polymers, Inc. (USA). PDLLA films were fabricated under clean conditions using acetone (EM Science, Germany) as solvent. The polymer used had an inherent viscosity of 0.67dL/g and was dissolved in acetone at a ratio of about 1:23 or 1.64g/37mL. The polymer solution was cast

directly over 1.5cm diameter glass coverslips (PGC Scientifics, USA) Solvent evaporation was achieved after 24 hours in controlled atmosphere (0ºC).

After solvent evaporation, circular PDLLA film samples (∅ 1.5cm) attached to the glass coverslips were obtained. These films were kept under vacuum for further drying until use. The films were sterilized by UV radiation (8 hours) in a tissue culture hood prior to use.

2. MODIFICATION AND CHARACTERIZATION OF THE SURFACES

2.1. Oxygen-based Plasma Treatment

Plasma surface modification methodologies have been used for biomaterials in a variety of applications attempting to improve surface functionalities, like the modulation of proteins[10] and the cellular response.[11] Surface modification of SBB and PDLLA samples was carried out using oxygen-based plasma treatment under previously optimized conditions.

Surfaces were modified by means of oxygen (O_2) gas plasma in a Radio Frequency Glow Discharge (rfGD) chamber (Harrick Scientific Corporation, USA). For the treatment, the plasma reactor chamber was stabilized at vacuum to approximately $3.8x10^{-3}$psi. O_2 was injected into chamber at a pressure of 15psi for 30sec followed by a waiting period of 30sec before plasma treatment. Plasma treatment was initiated for 180sec using a power of 100W and pulsed frequency of 13.5MHz. Time-related changes of treated surfaces were minimized by using the samples within the following 48 hours.

2.2. Surface Characterization: chemical and microscopy analysis

2.2.1. X-Ray Photoelectron Spectroscopy (XPS)

X-Ray Photoelectron Spectroscopy (XPS) measurements were performed for a detailed analysis of the chemical composition of the treated and non-treated surfaces. The experiments were carried out using a Kratos Axis-Ultra (Kratos Analytical Inc., USA) with monochromatic Al x-ray source. X-rays energy was 1486.6eV and base pressure around $2.9x10^{-11}$psi. Triplicates were prepared and results collected from 5 different points of the surface of SCA samples.

2.2.2. Fourier Transform Infra Red Spectroscopy (FTIR)

Spectra were obtained by Attenuated Total Reflection (ATR) using a Nicolet Spectrometer (Nicolet Instrument Coorporation, USA). Each spectrum was recorded with a total of 32 scans and 4.0 resolution after 20sec of vacuum for chamber stabilization. Original and treated surfaces were analyzed in triplicates in the range $400cm^{-1}$ to $4000cm^{-1}$. Before obtaining the surface spectra, a background of the equipment was performed.

2.2.3. Water Contact Angle

Contact angle measurements were used to investigate the wettability of the original and rfGD modified SBB and PDLLA samples. The relative hydrophilicity of treated and untreated surfaces was assessed. The sessile drop method was selected and data acquired using a Video Contact Angle 2000 System (AST Products, Inc., USA). Ultra-pure water (Pierce, USA) was used to analyse the wettability of the surfaces. Each side of water drops was recorded and averaged; 9 drops and 3 samples per condition were used. Measurements were recorded 10sec after liquid contact with the surface.

2.2.4. Surface Energy and Adhesion Tension of Water

The determination of surface energy (γ) of the original and plasma treated SBB and PDLLA samples was based on the Owens and Went method[12] that discerns between a polar (γ^p) and a disperse or nonpolar (γ^d) component of the surface energy. Water and diiodomethane (Sigma, USA) were used as test liquids for the determination of the surface energy. Reported surface tension values for water and diiodomethane are 72.8 and 50.8dyn/cm at 20ºC, respectively.[13] Furthermore, in this study polar and disperse parts of water were considered to be 51.0dyn/cm and 21.8dyn/cm and for diiodomethane 0.0dyn/cm and 50.8dyn/cm, respectively.[13]

According to Janocha *et al.*,[14] the measurement of the adhesion tension of water is an adequate alternative methodology to the calculation of the surface energy of solid surfaces due to its higher experimental or less assumption-based nature. The contact angle ϑ of water on the surfaces was measured and multiplied by the surface tension γ_l of water (72.8mN/m) to obtain the adhesion tension.

Both the adhesion tension of water and surface energy were based on a sessile drop method. Drop contact angles were measured 10sec after contact with the surface; 9 drops and 3 samples per condition were used.

2.2.5. Scanning Electron Microscopy (SEM)

Sample morphology was analysed by means of SEM. Surfaces were sputter coated (Med-010 Sputter Coater by Balzers-Union, USA) to provide a thin Au-Pd layer and examination was performed using a scanning electron microscope (Leica, UK). Triplicates were prepared for all original and plasma treated surfaces.

3. IN VITRO PROTEIN ADSORPTION

3.1. Studied Proteins

Protein adsorption was investigated in unitary and complex protein systems. The objective was to analyze protein-surface interactions in competitive conditions that mimic the physiological conditions

(Table 1). The relative amounts of these biomolecules in the human body provided for the data to be related to the analysis of the *in vivo* biological response to implanted surfaces.

A full understanding of the properties of the proteins in study is fundamental for reaching the complexity of the adsorption phenomena. In this study proteins were selected for investigation based on their relevance in the biomedical field and their specific properties. The adsorption of albumin, fibronectin, vitronectin and fibrinogen was studied. Albumin is a model molecule in protein adsorption studies due to its high concentration in physiological fluids like blood plasma and interstitial space[15] and to the ability to "passivate" biomaterial surfaces, reducing inflammatory and thrombogenic processes.[16] Members of the family of substrate adhesion molecules fibronectin[17, 18] and vitronectin,[19] were studied for their relevance in physiological processes such as the modulation of the immune system and tissue remodelling. These proteins are components of the extracellular matrix (ECM) and their integrin-binding sequences are responsible for modulating cell adhesion and migration. Finally, fibrinogen was selected considering its prominent role in multi-component processes such as the FBR in which fibrinogen is believed to adsorb immediately to the implanted materials.[20, 21]

Table 1. Typical concentration of serum albumin, fibronectin, vitronectin and fibrinogen in the human plasma.

Proteins	**Typical Concentration in Human Plasma (mg/mL)[22, 23]**
Human Serum Albumin (HSA)	35.0
Human Fibronectin (HFn)	0.4
Human Vitronectin (HVn)	0.3
Human Fibrinogen (HFbg)	0.2

The protein adsorption and desorption was investigated using Human serum albumin (HSA), human fibronectin (HFn), human vitronectin (HVn) and human fibrinogen (HFbg), obtained from Sigma (UK). Human vitronectin was reconstituted to a final concentration of 2μg/mL in tissue-culture-grade water and sterilized by filtration. The studies with bone-related cells were performed with proteins from a bovine source: serum albumin (BSA), plasma fibronectin (Fn), vitronectin (Vn) and fetal bovine serum (FBS), obtained from Pierce (USA), Sigma (USA), Calbiochem (USA) and Atlanta Biologicals (USA), respectively. The saline solution was supplied by Baxter (USA). Single and complex protein solutions were prepared in saline solution (pH 7.4).

3.2. Single and Complex Protein Systems

In the *in vitro* investigation of protein-surface interactions as well as in the study of cell behaviour, single and complex protein solutions were prepared. Initially, different dilutions of the protein solutions were prepared. In the final stages of the work protein solutions were studied to 0.1 and 0.2%

of their concentration in the human blood plasma. The different unitary protein systems studied were the following:

i. 1000, 70 and 35μg/mL of HSA,

ii. 1000, 350, 175, 140, 105, 70 and 35μg/mL of BSA,

iii. 1.0, 0.8 and 0.4μg/mL of HFn,

iv. 100, 4.0μg/mL of Fn,

v. 0.6 and 0.3μg/mL of HVn,

vi. 3.0 and 0.7μg/mL of Vn,

vii. 0.6μg/mL of HFbg.

Besides single protein solutions, competitive systems were studied. Binary solutions were prepared to final concentrations of: (*i*) 1000μg/mL of HSA and 1μg/mL Fn, and (*ii*) 70μg/mL of HSA with 0.8μg/mL Fn. In addition, a ternary system was prepared by combining (*iii*) 350μg/mL of BSA with 4μg/mL of Fn and 3μg/mL of Vn.

Moreover, the complex *in vivo* environment was also studied using human blood serum and human blood plasma. For serum separation, whole blood was collected from healthy un-medicated adult donors, coagulated and centrifuged at 2500rpm during 5min at 4ºC. Protein solutions of different concentrations were prepared by diluting complete serum in phosphate buffered saline (PBS) solution: 0.2%, 1%, 10% and 20% (V/V). In brief, whole blood was collected from healthy un-medicated, adult volunteers, anticoagulated with 0.002% of heparin and centrifuged at 2500rpm for 5min at 4ºC. Human blood plasma and serum were collected and stored at 0ºC. Human blood plasma solutions were prepared at 0.2% (V/V) in PBS solution. Finally, 1% (V/V) of Fetal Bovine Serum (FBS) was also used to mimic complex protein environments.

3.3. Labelling of Proteins with Fluorescent Probes

A commercially available BSA-Alexa Fluor 488 conjugate was used as the model of adsorption studies and also to control the experiments with HSA and HFn labelled according to the manufacturer's specifications (Molecular Probes, Netherlands). For all studies, Alexa Fluor® 488 and Alexa Fluor® 555 Protein Labelling Kits (Molecular Probes, The Netherlands) were used to fluorescently label human serum albumin and human fibronectin. The labelling procedure was conducted following the manufacturer's instructions.[24, 25] Protein solutions at a concentration of 2mg/mL in standard phosphate buffer saline were mixed with 50μL of sodium bicarbonate solution and allowed to react with Alexa-Fluor dye for 1h at room temperature. After reaction, labelled proteins were separated from unincorporated dye using Bio-Rad BioGel P-30 fine size exclusion purification chromatography.

The determination of the molar extinction coefficient of HSA and HFn were determined using the Beer's Law equation:

$$\varepsilon_{percent}\, c\, L = A \quad \textbf{(1)}$$

Where ε is the extinction coefficient, c is the molar concentration, L is the light path length and A is the absorbance at 280nm of 1% (W/V) solutions measured in a 1cm path length. The molar extinction coefficient (ε_{molar}) of the protein is determined using the molecular weight of the protein (Protein MW) according to Equation 2:

$$(\varepsilon_{molar})\ 10 = (\varepsilon_{percent})\ x\ (\text{Protein MW}) \quad \textbf{(2)}$$

The concentration of HSA-Alexa Fluor 488 was calculated by the following equation:

$$[\text{Protein}]\ (M) = \frac{\left[A_{280} - (A_{494}\ x\ 0.11)\right]\ x\ \text{dilution factor}}{\varepsilon} \quad \textbf{(3)}$$

Where A_{280} and A_{494} are the absorbance of the conjugate solution at 280nm and 494nm in a 1cm path length, respectively, ε is the molar extinction coefficient of the protein ($cm^{-1}M^{-1}$) and 0.11 is a correction factor to account for absorption of the dye at 280nm. The concentration of HFn-Alexa Fluor 555 was determined using Equation 3, replacing A_{494} by A_{555} and 0.11 by the correction factor 0.08.

UV-visible spectra of protein-dye conjugates (Bio-Tek Instruments, KC4™ Data Analysis Software, USA) were used to determine the degree of labelling according to the following equation:

$$\text{moles dye per mole protein} = \frac{A_{294}\ x\ \text{dilution factor}}{71,000\ x\ \text{protein concentration (M)}} \quad \textbf{(4)}$$

Where, 71,000$cm^{-1}M^{-1}$ is the approximate molar extinction coefficient of the Alexa Fluor 488 dye at 494nm.

The degree of labelling of HFn with Alexa Fluor 555 was determined using Equation 4, replacing A_{494} by A_{555} and 71,000 $cm^{-1}M^{-1}$ by the value 150,000 $cm^{-1}M^{-1}$, which is the approximate molar extinction coefficient of the Alexa Fluor 555 dye at 555nm. The degree of labelling of HSA and HFn with Alexa Fluor Probes was determined: 4.0 and 2.9, respectively. Labelled proteins were stored at 4ºC and used within 1 week of preparation.

3.4. Protein Incubation Assays: the immersion and the drop methods

3.4.1. Immersion method

In the immersion method, proteins were adsorbed to starch-based surfaces by transferring each sample to polypropylene tubes and immersing them in 2mL of the prepared solution. The experimental procedure was performed in sterile conditions. Samples were incubated in static conditions at 37ºC. Incubation periods ranged from 15min to 7h in the case of Fluorimetry and Laser scanning confocal

microscopy (LSCM) analysis; incubation during 24h was also performed for immunostaining studies. Batches of samples were also immersed in PBS solution without proteins to be used as control surfaces. Tissue Culture Polystyrene Coverslips (TCPS) were used as the control surfaces (Sarstedt, UK). For immunostaining studies, samples were fixed after 7 or 24h in 4% formaldehyde for 5min, immersed in PBS and kept at 2 to 8ºC. Care was taken in order to prevent drying of the protein coated surfaces before further analysis.

3.4.2. Drop method

Using the Drop methodology, proteins and controls were incubated with characterized samples for 15min at 37ºC as described elsewhere.[26] According to this methodology, 300 µl of protein solutions were pipetted onto the surfaces and incubation was performed in a sterile humidified incubator. Nonadherent proteins were removed and samples were washed twice using saline solution. Unintended protein loss from other sources was controlled by means of using positive displacement pipettes, capillaries and pistons purchased from Gilson Medical Electronics S. A. (France).

The final volume of the recovered solutions was the same for all samples. Batches of samples were also immersed in PBS solution without proteins to be used as control surfaces. Glass coverslips (PGC Scientifics, USA) were used as the control surfaces.

4. *IN VITRO* ANALYSIS OF PROTEIN-SURFACE INTERACTIONS

The process of protein adsorption onto a solid surface is considered for years as a complex type of interactions of physical and chemical origin that is established between surface, solvent and proteins.[27] Characterizing the complexity of protein covered surfaces is required[28]demanding advances in surface science instrumentation. The success of protein adsorption studies depends directly on the selected techniques of analysis.

4.1. Colorimetric Assay

Protein adsorption onto the surface of PDLLA films was assessed by coupling a depletion method with a protein assay as follows: after incubation, 100µl aliquot of the initial and removed solution were mixed with 150µl of BCA assay (Pierce, USA) working reagent in a 96 well plate and incubated at 37ºC for 120min. Each protein concentration was calibrated using a standard curve. The degree of adsorption was determined by subtracting the residual protein from the initial added protein. Batches of samples were also immersed in PBS solution without proteins to be used as control surfaces. Glass coverslips were used as control surfaces and control polymer surfaces were prepared using incubation in saline solution.

4.2. Fluorimetry

A commercially available BSA-Alexa Fluor 488 conjugate was used as the model in adsorption studies and also to control the experiments with HSA and HFn that were fluorescently labelled according to manufacturer's specifications. For all studies, Human Serum Albumin was labelled with Alexa Fluor 488 and Human Fibronectin was labelled with Alexa Fluor 555. Samples were incubated as described for the analysis of protein adsorption. For each time point, fluorescence measurements were taken according to the conjugate spectroscopic properties using a Microplate Fluorescence Reader (Version FLx800T, Bio-Tek Instruments, USA). Measurements were automated for top probe detection, using one or two filter sets (488 and 555nm) to assess labelled biomolecule kinetics and competitive adsorption. Acquired data was analyzed using KCJunior Software (Version 1.31.5, Bio-Tek Instruments, USA). Fluorimetry results were calibrated by assessing other contributions to the total fluorescence intensity. Background fluorescence was subtracted and the emission of SBB surfaces at different wavelengths was analyzed. Briefly, the different surfaces were incubated in protein-free PBS solution and the fluorescence intensities measured over time were corrected to rule out artifactual effects, such as normal absorption interferences from the polymer materials. TCPS was used as the control surfaces (Sarstedt, UK)

The data of protein adsorption was presented as arithmetic means/standard deviations of the mean (mean/SD). Standard curves were prepared for the different protein types and for each time point, fluorescence was converted in protein concentration.

4.3. Dynamic Contact Angle (DCA)

4.3.1. Theoretical Principles

DCA measurements were performed based on the Wilhelmy plate method.[29] Wilhelmy-balance tensiometry was performed using a computer-controlled instrument (Camtel CDCA 100F, Royston, UK). The theoretical principles of this methodology are described in the literature.[30, 31] Briefly, a plate was immersed and emersed from a liquid and the forces acting in the specimen were recorded by an electrobalance. According to the procedure, the balance was reset to zero and linear regression was performed to the immersion depth zero. This provided for the elimination of the weight of the sample and the buoyancy forces.

The relation between force and surface tension can be represented by the following equation:

$$\frac{F}{L} = \gamma_{lv} \cos\theta \qquad \textbf{(5)}$$

where F is the force acting on the sample in mN, γ_{lv} the surface tension of the liquid and θ is either the advancing (θ_{adv}) or receding (θ_{rec}) contact angle.

Finally, contact angle hysteresis is the difference between advancing and receding contact angles. This parameter is affected by the distribution of chemistries on the surface with different properties and thus is a measure of the homogeneity of the surface.

4.3.2. Adsorption and Desorption Studies

Adsorption and desorption studies were performed running multiloop DCA at 22ºC. The immersion/emersion speed was a constant at 0.060mm/sec and the immersion depth was 2mm. In all experiments, a single hysteresis loop lasted 3min and therefore a complete 60-loop-experiment ran for approximately 3h. The duration of the experiments and subsequently the number of DCA cycles were selected based on previous studies with starch-based biomaterials, showing that HSA and HFn adsorption plateaux were completed in approximately 90 minutes after incubation of the samples.

Prior to the evaluation of the effect of the different protein conditions on the advancing (adv), receding (rec), dynamic contact angles (DCAs), ultra-pure water and PBS solution were studied. The effect of the different proteins and human blood plasma on the advDCAs and recDCAs of SCA, SEVA-C and SPCL was investigated and hysteresis (H) calculated. In the adsorption study, experiments were performed using each protein solution for 60 loops. In contrast, 30-loop-desorption studies were performed in protein-free buffer (desorption phase) directly after the 30-loop experiment of the adsorption phase.

4.4. Immunostaining of Adsorbed Proteins

4.4.1. Alkaline phosphatase (ALP) procedure

Samples and control surfaces were removed from PBS solution and coated for 20min with rabbit serum (1:10 dilution, Serotec, UK) in order to block non-specific binding. Monoclonal mouse anti-human HSA, Fn or Vn antibodies (Dako, Germany) were used as primary antibodies in the immunostaining procedure; all were diluted to 1:1000. Discs were washed then coated with biotinylated rabbit anti-mouse immunoglobulins (IgG, Dako, Germany) diluted to 1:200. Both primary and secondary antibody solutions were diluted in PBS with 1% BSA and incubated with discs for 30min. The detection of proteins adsorbed onto starch-based surfaces was processed using the alkaline-phosphatase protocol. The incubation of alkaline phosphatase (Vector, UK) and alkaline phosphatase substrate (Vector, UK) solutions was performed for 30min. Between each step of the immunostaining procedure, samples were repeatedly washed with PBS. For each assay, an additional control was prepared consisting of a protein-coated sample submitted to the same procedure but instead of incubating with the primary, PBS was used. Three replicates for each experimental condition were prepared and the procedure repeated three to five times.

4.4.2. Probe-labelled antibodies

Antibody labelling of specific biological molecules was selected to detect the adsorption of albumin, fibronectin, vitronectin and fibrinogen on the different surfaces. For the detection of the biomolecules, samples used in the adsorption and desorption DCA studies were fixed using 4% formaldehyde solution and washed with PBS solution. Initially, the materials were exposed to horse serum for 20min, followed by incubation with primary antibodies for 30min at 37ºC. For the identification of the different biomolecules, antibodies that had been shown to cross-react with rat specie were used: sheep anti-Human Albumin, Fibronectin, Vitronectin, and Fibrinogen (Farnell, UK). After that time, materials were incubated with donkey anti-sheep Alexa Fluor 488 antibody (Molecular Probes, The Netherlands) for 1h at 37ºC. Results were obtained by performing Laser Scanning Confocal Microscopy (LSCM).

4.5. Microscopy Analysis

4.5.1 Light Microscopy

Samples immunostained using the ALP procedure were observed using reflected light microscopy (Axioplan 2 Imaging, Zeiss, Germany) and image acquisition by digital camera equipment (AxioCam, Zeiss, Germany).

4.5.2. Laser Scanning Confocal Microscopy (LSCM)

A Confocal Laser Scanning Microscope (Version LSM 510 Zeiss, UK) was used to visualize the fluorescently labelled proteins adsorbed on the different starch based materials. Alexa 488 probe was used in single protein studies to label HSA and HFn molecules. For the study of binary protein systems, HSA and HFn were labelled with Alexa 488 and Alexa 555 probes, respectively. An argon laser (λ=488nm) and a HeNe laser (λ=543nm) provided the excitation of the protein-probe conjugates. Moreover, samples obtained after adsorption and adsorption/desorption DCA experiments were labelled with fluorescent secondary antibody (Alexa 488). The argon laser was used to detect adsorbed HSA, HFn, HVn and HFbg previously immunostained by probe-conjugated antibodies. Image analysis was performed using KS400 image analysis software (Imaging Associates, UK).

4.6. Adsorption Isotherms: Langmuir and Freundlich models

To develop the fundamental understanding of protein adsorption, two typical adsorption models, Langmuir and Freundlich isotherms,[32] were used. The linearised Langmuir equation can be expressed as:

$$\frac{C}{Q} = \frac{1}{Q_m} C + \frac{1}{bQ_m} \quad \textbf{(6)}$$

Where, C is the BSA concentration at a certain time, Q and Q_m are the adsorption amounts for BSA at a certain time and the maximal adsorption amount respectively, and b is the Langmuir's equilibrium constant that describes the strength of interaction between the protein and the surface.[33]

Other models such as the Freundlich model are more suitable for use with heterogeneous surfaces, but can only describe adsorption data over a restricted range:[34]

$$\ln Q = \frac{1}{n}\ln C + \ln K \quad \textbf{(7)}$$

In this case, C, Q, and Q_m are the same as in equation (1), n and K, constants at a specific condition. The constant K is a measure of the capacity of the adsorption and n is a measure of the intensity of adsorption.[32, 33]

In the low concentration range, changes in the bulk concentration produce large changes in the amount adsorbed, resulting in a roughly linear increase in adsorption. However, as the bulk concentration is further increased, adsorption is reduced and a plateau or maximum adsorption level is reached. This type of adsorption behaviour is referred to as a Langmuir isotherm. In other cases, the increase in adsorption at high bulk concentration does not stop entirely, but presents a slow rise. This type of adsorption behaviour is referred to as a Freundlich isotherm.

5. *IN VITRO* CELL STUDIES

It is known that cellular functions at the material-interface are essential for the degree of success of biomedical devices. Also, to accomplish the characterization of the complexity of protein covered surfaces it is clearly required to understand normal cell mechanisms such as adhesion, proliferation and morphology of the specific cell populations.

Cell studies were performed onto SBB and PDLLA surfaces after protein pre-adsorption. Cell seeding was carefully performed sample by sample and immediately after the protein incubation step to evade surface drying and consequent protein conformational changes or denaturation. Surface rinsing was not performed and any enrichment of the cell culture media that could result from remaining non-adsorbed proteins was considered negligible.

5.1. Attachment of Human Leukocytes

5.1.1. Isolation and culture of human monocytes/macrophages and lymphocytes

Whole blood was collected from healthy un-medicated, adult volunteers and anticoagulated with 0.002% of heparin. Human blood mononuclear cells were isolated by means of using a one-step gradient centrifugation method. 5mL of anticoagulated blood was carefully layered over 3mL of LymphoSep Media (ICN, USA) and centrifuged at 2500rpm for 25min at 4ºC. The interface was

harvested, washed three times in PBS and cells were resuspended in 199-cell culture medium (GibcoBRL, USA) supplemented with 10% foetal bovine serum (FBS).

After isolation, cells were seeded onto the protein pre-coated SEVA-C surfaces at a density of 5 x 10^4cells/mL (in 199 medium with 10% FBS). Samples were incubated for 30min and 24h at 37ºC in 5%CO_2 and 100% humidity. After incubation, surfaces were fixed for 10min in 4% formaldehyde, immersed in PBS and kept in the refrigerator at 4ºC.

5.1.2. Immunostaining and haematoxylin staining

After leukocyte culture, fixed samples and controls were removed from PBS solution, coated with rabbit serum (1:75 dilution, Serotec, UK) for 20min and incubated overnight with purified monoclonal mouse anti-human CD3 or CD14 antibodies (1:1000 dilution, PharMingen, USA). Each disc was then coated with biotinylated rabbit anti-mouse IgG (1:200 dilution, Dako, Germany), followed by phosphatase alkaline (Vector, UK) and PA (Vector, UK) incubation, 30min each. Between every step, samples were rinsed with PBS for 5min. For each assay, an additional control was prepared, by replacing the incubation of the primary by PBS solution. Samples labelled with antibodies were then counterstained with haematoxylin (Sigma, UK). Surfaces were then rinsed in warm water for 1min and analysed using the reflected light microscope and digital camera for image acquisition.

5.1.3. Cell counting

In order to obtain lymphocyte, monocyte/macrophage and total cell numbers, cell counting was performed in a two-step procedure: after labelling leukocytes with CD3 and CD14 primary antibodies and after haematoxylin staining. For each sample, counting was performed from 20 different and arbitrary fields of vision using KS400 3.0 image analysis software coupled to a transmitted light microscope (Zeiss, Germany) and digital camera (JVC, USA).

5.2. Attachment and Proliferation of Fetal Rat Calvaria (FRC) cells

5.2.1. Isolation and culture of FRC cells

Fetal Rat Calvaria (FRC) cells were isolated by sequential enzyme digestions from calvaria of 21 days Sprague-Dawley rat foetuses as described elsewhere.[35] Briefly, calvaria (frontal and parietal bones) were aseptically removed and stripped of the periosteum. The minced fragments underwent 9 sequential digestions in fresh 0.2% collagenase/0.05% trypsin (Sigma, USA) in Hank's Balanced Salt Solution (HBSS) for 20min at 37ºC (Sigma, USA). Cells were resuspended in α–MEM (Gibco, USA) enriched with 10% (V/V) FBS (Atlanta Biomedicals, USA) and 1% (V/V) of Penicillin-Streptomycin Mixture (Gibco, USA), plated in T-75 falcon tissue culture flasks and incubated at 37ºC in 5%CO_2 until confluent. Adherent cells were considered viable. For long culture periods, cells were trypsinized and grown as described by Bellows and co-workers.[36] Populations II–V were seeded

at $4x10^4$cells/mL in α–MEM containing 1mg/mL of β-glycerophosphate (Caliochem, USA), 0.05mg/mL of L-ascorbic acid (Sigma, USA), 1% (V/V) of Penicillin-Streptomycin Mixture and 10% (V/V) FBS onto the different surfaces: PDLLA, plasma treated PDLLA and the same surface batches after carrying out the first protein adsorption procedure described above at: 1000μg/mL BSA, 100μg/mL Fn, 0.7μg/mL Vn and 1000μg/mL of FBS. Incubation was performed for 3 hours, and 7, 9, and 14 days.

5.2.2. Trypsinization and cell counting

After each incubation period, cultured samples were transferred to new wells with fresh media. Attachment (measured at the 3h time point) and proliferation (assessed with the 7, 9 and 14 day time points) measurements were conducted by trypsinization (Trypsin-EDTA, Sigma, USA) of the cultures for 5min, and subsequently by cell counting using a Coulter Zi Dual cell counter (Coulter Corporation, USA). Appropriate controls were used, including tissue culture polystyrene (TCPS).

5.3. MG63 Osteoblast-like Cells

5.3.1. Culture of MG63 cells

Cell response was studied using the MG63 osteoblast-like osteosarcoma cell line that has been well characterized in the literature and consists of a good model for the study of human bone cells. MG63 cells are known to present numerous osteoblastic traits, including increased levels of bone alkaline phosphatase and inhibition of proliferation following treatment with $1,25\text{-}(OH)_2D_3$.[37, 38] Cells (American Type Culture Collection, USA) were seeded on the relevant surfaces at 4 x 10^4cells/mL and incubation was performed for 1, 4 and 7 days in DMEM (CELLGRO, USA) containing 10% FBS (Atlanta Biomedicals, USA) and 1% of Penicillin-Streptomycin Mixture (Gibco, USA). Tissue culture polystyrene (TCPS) was used as maximum control.

5.3.2. WST-1 cell quantification assay

After each incubation period, cultured samples were transferred to new wells with fresh media and analyzed for mitochondrial activity using colorimetric WST-1 tetrazolium conversion assay (TAKARA, Japan).[39] Briefly, 10μL of WST-1 reagent was added per well, and the cells were incubated for an additional 2h. The absorbance of the WST-1-containing cell supernatant was determined at 450nm (Benchmark Microplate Reader, Bio-Rad, USA). To avoid interference from both cell culture media and SBB biodegradable materials, the following controls were prepared and considered as blank samples: fresh media, and SBB samples immersed in fresh media but no cells were seeded.

5.3.3. Scanning Electron Microscopy (SEM)

Cell morphology was evaluated by SEM. The preparation of cell cultured samples for SEM observation was performed using 4% formaldehyde and 1% gluteraldehyde as fixative solution (Electron Microscopy Sciences, USA). Samples were then washed using PBS solution (Sigma Diagnostics, USA) and gradually dehydrated by incubation in crescent ethanol concentrations. Drying was accomplished by means of hexamethyldisilazane (HMDS) solution (Polysciences Inc., USA), as recommended for SEM preparation of soft tissue.

6. *IN VIVO* PROTEIN ADSORPTION

The experimental procedures herein described provided for investigating a critical phase of biomaterials interaction: the analysis of the protein biolayer and tissue surrounding the *in vivo* implanted polymeric blends of corn starch and ethylene vinyl alcohol (SEVA-C). One of the aims of the research was to determine the relationship between key biological molecules and the tissue reactions evoked by SEVA-C in the *in vivo* subcutaneous environment.

6.1. Subcutaneous implantation of SEVA-C materials

The experiments were performed in Wistar rats, anaesthetized using Immobilon as previously described.[6] Briefly, four different materials were implanted subcutaneously in the back, two either side of the spine, for 3, 7 and 14 days, with three repeats for each material per time period. At the end of the implantation period, rats were sacrificed by CO_2 and the tissue surrounding the implant was carefully dissected with the material *in situ* and snap frozen using isopentane in cardice. Explanted samples were stored at -80ºC until resin embedded.

6.2. Preparation of implanted materials

Samples of the SEVA-C material were embedded in Technovit 8100 New®-embedded (Heraeus Kulzer, Wehrheim, Germany) that is a low temperature glycolmethacrylate embedding system that facilitates the preservation of tissue antigenicity. Resin embedding of the implants was performed according to the manufacturer's indications. Fixation was performed in a mixture of paraformaldehyde, lysine and periodate (PLP fixative) for 24h at 4ºC and washed in 50mM ammonium chloride buffer. The implants were dehydrated in a cold acetone bath for 24h at 4ºC. A mixture of 0.6g of Technovit Hardener I in 100ml base-liquid 100% Technovit was used as the infiltration mixture for 24 hours. An embedding mixture was then used (1 part of Technovit Hardener II and 30 parts of infiltration solution); the infiltration of the samples was performed at -55ºC for 4 days followed by 2 days at -20ºC for polymerisation. Sample blocks were then trimmed with a low speed circular saw (IsoMet® Low Speed Saw, Buehler LTD., USA). 7μm thick sections were cut

using a Polycut Microtome (Leica, UK) and then mounted on 3-aminopropyl-triethoxysilane (APES)-coated slides, fixed with acetone for 5min, air-dried and kept short term at 4ºC until staining.

6.3. Immunostaining

Antibody labelling of specific biological molecules was used to determine the adsorption of Albumin (Alb), Fibronectin (Fn), Vitronectin (Vn) and Fibrinogen (Fbg). For the detection of the biomolecules, tissue sections were stained using an avidin-biotin alkaline phosphatase technique, as described elsewhere.[40] In brief, materials were incubated with 0.1% trypsin solution to expose masked epitopes, exposed to horse serum for 20 min and incubated with primary antibodies overnight at 4ºC, for the identification of the different proteins. After that time, materials were incubated with biotinylated rabbit anti-goat IgG antibody (Dako A/S, Denmark) for 1h at room temperature. The avidin and biotinylated horseradish peroxidase complex (Vector Laboratories Ltd., UK) was added to all materials for 30min and the substrate reaction was developed using the Alkaline Phosphatase Substrate Kit (Vector Laboratories Ltd., UK). Each incubation was followed by one wash with PBS solution for 5min. Materials were washed and mounted in permanent aqueous mounting medium (Serotec Ltd, UK). Each time period studied had one sample stained as a control replacing the primary antibody with buffer and parallel isotype reference staining was always conducted.

6.4. Image Analysis and Protein Diffusion

Immunostaining results were observed using transmitted light microscopy (Axioplan 2 Imaging, Zeiss, Germany) and image acquisition by digital camera equipment (AxioCam, Zeiss, Germany). Subsequently, measurements of protein diffusion and biolayer thickness were performed for the assayed molecules. For each staining, three sections per time period were analyzed and twenty repeats per section were used. To quantify protein diffusion KS400 3.0 image analysis software (Zeiss, Germany) was used.

7. STATISTICAL ANALYSIS

Data analysis was presented as arithmetic means/standard deviations of the mean (mean/SD). Different statistical data were evaluated using the bi-tail Students *t*-test and the ANOVA/Tukey multiple comparison tests to detect differences between groups (SPSS 13.0.1, Statistical Analysis Software, USA). In all statistical evaluations, $n>9$ and $p<0.05$ was considered as statistically significant.

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SECTION 3

CHAPTER III

PRELIMINARY STUDY ON HUMAN PROTEIN ADSORPTION AND LEUKOCYTE ADHESION TO STARCH BASED BIOMATERIALS

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PRELIMINARY STUDY ON HUMAN PROTEIN ADSORPTION AND LEUKOCYTE ADHESION TO STARCH BASED BIOMATERIALS*

Alves, CM[1,2], Reis, RL[1,2], Hunt, JA[3]

[1]Department of Polymer Engineering, University of Minho, Campus de Azurém, 4800-058 Guimarães, Portugal
[2]3B's Research Group – Biomaterials, Biodegradables and Biomimetics, Campus de Gualtar, 4710-057 Braga, Portugal
[3]UK Centre for Tissue Engineering, Clinical Engineering, University of Liverpool, P.O. Box 147, Liverpool L69 3QA, United Kingdom

Abstract

In this study, the adsorption of human serum albumin (HSA), fibronectin (FN) and vitronectin (VN) onto the surface of novel biodegradable materials was evaluated by immunostaining. Specifically, polymeric blends of corn starch with cellulose acetate (SCA), ethylene vinyl alcohol copolymer (SEVA-C) and polycaprolactone (SPCL) were immersed in unitary and competitive systems; that is, binary and more complex protein solutions. For binary solutions, different HSA, FN and VN protein distribution patterns were observed depending on the starch-based surface. Furthermore, the relative amount of proteins adsorbed onto starch-based surfaces was clearly affected by protein type: a preferential adsorption of VN and FN as compared to HSA was observed. On tests carried out with unitary, binary and more complex solutions, it was found that vitronectin adsorption ability was enhanced in competitive systems, which was associated with a lower amount of adsorbed albumin. In order to assess the effect of these human proteins on cell behaviour, a mixed population of human lymphocytes and monocytes/macrophages was cultured over pre-coated SEVA-C surfaces. Through anti-CD3 and CD-14 monoclonal antibody labelling and cell counting, leukocyte adhesion onto pre-coated SEVA-C surfaces was analysed. Based on the results, it was possible to detect albumin long-term effects and fibronectin short-term effects on cell adhesion proving that previously adsorbed proteins modulate leukocyte behaviour.

Keywords

Starch-based polymers, biodegradables, protein adsorption, leukocyte adhesion.

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1. INTRODUCTION

Protein adsorption from surrounding tissue fluids is the first very important phenomenon of the host-biomaterial interactions that may lead to implant integration or rejection.[1-3] Immediately upon contact with physiological solutions, many proteins adsorb to the implant surface, subsequently promoting nearby cells to interact with the material.[4-11] It can be said that when implanted, foreign materials interact indirectly with living tissues meaning that cell-surface interactions are mediated by proteins adsorbed from surrounding fluids.[8,9] Together with other interfacial processes, protein adsorption will regulate cell function and the biocompatibility of the implant,[1,12] being responsible for homeostasis and tissue healing but also for adverse immunologic mechanisms[1,13] such as, inflammation[10,13-18] and thrombosis.[10,13-18]

To investigate further the phenomenon of protein adsorption and the effect of the surfaces on it, human proteins were used to coat different starch-based biodegradable polymeric blends. These novel biodegradable materials were recently shown to be biocompatible[19-20] and cytocompatible surfaces,[21] and have been proposed for a range of biomedical applications,[22-25] including tissue-engineering scaffolding.[26]

Proteins conventionally classified as adhesive and non-adhesive were used in this study. Fibronectin, a glycoprotein known to contain both RGD, PHSRN and synergistic sequences,[27,28] influences several processes, such as cell attachment, migration, differentiation and matrix assembly.[27,29,30] Vitronectin, also found in plasma and ECM, comprises an important domain, the RGD peptide that is known for mediating attachment and spreading of cells.[29,31] On the other hand, by binding to plasminogen activation inhibitor-1, this protein can potentially regulate the proteolytic degradation of the extracellular matrix.[29,31,32] The third protein is human serum albumin that, although known to be depleted of adhesion ligands, was selected due to its presence in high concentrations in serum and its ability to bind other molecules, influencing competitive protein adsorption.[33-35] The typical concentration of these proteins in the human serum is 35, 0.4 and 0.3 mg/mL, respectively for HSA, FN and VN.[33]

In this study, antibody labelling of specific biological molecules was selected to detect HSA, VN and FN adsorption on the surface of starch-based materials by means of adapting a previously described technique.[36] The methodology is based on the initial binding of a primary antibody to the surface of the sample where the antigen is adsorbed. Following this, the surface is incubated with a second antibody solution resulting in the formation of a primary-secondary complex. If this secondary is conjugated with an enzyme, such as alkaline phosphatase, the addition of the corresponding substrate will produce a colour change and, consequently, visualise the presence of the antigen on the surface.[37]

Using the immunostaining technique, the relative presence of human albumin, fibronectin and vitronectin on the surface of different starch based polymers was determined. In addition, both distribution patterns and type of proteins that preferentially adsorb from human blood serum were

analysed. Furthermore, the effect of human proteins in modulating the interaction of a mixed population of leukocytes and the materials surfaces was investigated. For this purpose, monoclonal mouse anti-human CD3 and CD14 antibodies were used to label human lymphocytes and monocytes/macrophages, respectively.

2. MATERIALS AND METHODS

2.1. Starch based materials

The materials used in this study were biodegradable polymeric blends of corn starch with: (i) cellulose acetate (SCA), (ii) ethylene vinyl alcohol copolymer (SEVA-C) and (iii) polycaprolactone (SPCL), supplied by Novamont (Italy). The amount of starch is 50% by weight (wt%) on SCA and SEVA-C and 30% wt. on SPCL. Using conventional injection moulding technology samples were processed into 10 mm circular discs. Efforts were made in order to obtain the most reproducible surfaces. Samples were sterilised by ethylene oxide, as described in other works,[38] washed, and all subsequent experimental procedures were performed under sterile conditions.

2.2. Protein adsorption assay

Human serum albumin, human fibronectin and human vitronectin were obtained from Sigma (UK). Human vitronectin was reconstituted to a final concentration of 2μg/mL in tissue-culture-grade water and sterilised by filtration. Whole blood was collected from healthy un-medicated adult donors, coagulated and centrifuged at 2500rpm during 5min for serum separation. Protein solutions of different concentrations were prepared by diluting initial solutions in 0.1M phosphate-buffered saline (PBS) solution without Ca^{2+} and Mg^{2+} and pH 7.4. HSA solutions used in the immersion experiments had concentrations of 1000, 70 and 35μg/mL. The FN solutions had concentrations of 1, 0.8 and 0.4μg/mL and VN solution were diluted to 0.6 and 0.3μg/mL. Besides single protein solutions, competitive systems were studied: (i) binary solutions prepared to final concentrations of 1000μg/mL of HSA and 1μg/mL of FN and (ii) also complex solutions with 1%, 10% and 20% (v/v) of human blood serum. All solutions were sterilised by filtration through 0.2μm of pore size. Proteins were adsorbed to starch-based surfaces by transferring each sample to polypropylene tubes and immersing them in 2mL of the prepared solution for 24h at 37ºC. Batches of samples were also immersed in PBS solution without proteins to be used as control surfaces. After 24h samples were fixed in 4% formaldehyde for 5min, immersed in PBS and kept at 2 to 8ºC. Care was taken in order to prevent drying of the protein-coated surfaces before further analysis.

2.3. Detection of surface adsorbed proteins by immunostaining

Samples and control surfaces were removed from PBS solution and coated for 20min with rabbit serum (1:10 dilution, Serotec, UK) in order to block later non-specific binding. Monoclonal mouse anti-human HSA, FN or VN antibodies (Dako, Germany) used as primaries in the immunostaining procedure were diluted to 1:1000. Discs were washed and, afterwards, coated with biotinylated rabbit anti-mouse immunoglobulins (IgG, Dako, Germany) diluted to 1:200. Both primary and secondary antibody solutions were diluted in PBS with 1%BSA and incubated for 30min. Detection of proteins adsorbed onto starch-based surfaces was processed using the alkaline-phosphatase protocol. The incubation of alkaline phosphatase (Vector, UK) and alkaline phosphatase substrate (Vector, UK) solutions was performed for 30min. Between each step of the immunostaining procedure, samples were repeatedly washed with PBS. For each assay, an additional control was prepared consisting of a protein-coated sample submitted to the same described procedure but instead of incubating with the primary, PBS was used. Three replicates for each experimental condition were prepared and the procedure repeated three to five times. Immunostaining results were observed using reflected light microscopy (Axioplan 2 Imaging, Zeiss, Germany) and image acquisition by digital camera equipment (AxioCam, Zeiss, Germany).

2.4. *In vitro* human monocytes/macrophages and lymphocytes isolation and culture

Whole blood was collected from healthy un-medicated, adult volunteers and anticoagulated with 0.002% of heparin. Human blood mononuclear cells were isolated by means of using a one-step gradient centrifugation method. Five mL of anticoagulated blood was carefully layered over 3mL of LymphoSep Media (ICN, USA) and centrifuged at 2500rpm for 25min at 4ºC. The interface was harvested, washed three times in PBS and cells were resuspended in 199-cell culture medium (GibcoBRL, USA) supplemented with 10% foetal bovine serum (FBS).

After isolation, cells were seeded onto the protein pre-coated SEVA-C surfaces at a density of 5 x 10^4cells/mL (in 199 medium with 10% FBS). Samples were incubated for 30min and 24h at 37ºC in 5%CO_2 and 100% humidity. After incubation, surfaces were fixed for 10min in 4% formaldehyde, immersed in PBS and kept in the refrigerator at 4 ºC.

2.5. Analysis of leukocytes attachment by immunostaining and haematoxylin staining

After leukocyte culture, fixed samples and controls obtained were removed from PBS solution, coated with rabbit serum (1:75 dilution, Serotec, UK) for 20 min and incubated overnight with purified monoclonal mouse anti-human CD3 or CD14 antibodies (1:1000 dilution, PharMingen, USA). Each disc was then coated with biotinylated rabbit anti-mouse IgG (1:200 dilution, Dako, Germany), followed by phosphatase alkaline (Vector, UK) and PA (Vector, UK) incubation, 30 min each. Between every step, samples were rinsed with PBS for 5 min. For each assay, an additional control

was prepared, by replacing the incubation of the primary by PBS solution. Samples labelled with antibodies were then counterstained with haematoxylin (Sigma, UK). Surfaces were then rinsed in warm water for 1min and analysed using the reflected light microscope and digital camera for image acquisition.

2.6. Cell counting

In order to obtain lymphocyte, monocyte/macrophage and total cell numbers, cell counting was performed in a two-step procedure: after labelling leukocytes with CD3 and CD14 primary antibodies and after haematoxylin staining. For each sample, counting was performed from 20 different and arbitrary fields of vision using KS400 3.0 image analysis software coupled to a transmitted light microscope (Zeiss, Germany) and digital camera (JVC, USA).

3. RESULTS AND DISCUSSION

3.1. Immunostaining analysis of protein adsorption

3.1.1. Unitary systems

In this study, immunostaining technique was used to analyse protein adsorption with respect to their proportional amounts in the human serum 0.1% and 0.2% of those concentrations[33] were used. The immunostaining technique allowed qualitative and semi-quantitative analysis of different starch-based blends studied in terms of protein adsorption pattern and intensity (Figures 1, 2 and 3). In the first case, the aim was to detect different protein distribution patterns and to determine whether it was a material or protein dependent phenomenon. For all proteins studied it was observed that SCA and, to some extent, SPCL present a less homogeneous protein coating than SEVA-C. Starch and ethylene vinyl alcohol blends are inter-penetrating networks (INP). SPCL presents some complexion and interaction between the starch and PCL phases, while SCA is a non-miscible blend. Considering the order SCA, SPCL and SEVA-C, we obtain an increase in miscibility and interaction between these natural and synthetic polymeric phases, explaining why proteins form a smooth and homogeneous protein coating at SEVA-C surfaces when compared with SPCL and SCA. By means of analysing Figures 1, 2 and 3 it can be concluded that protein adsorption pattern is determined by the type of material. Whatever the protein used the same staining distribution for a certain starch-based surface was obtained. In terms of intensity of the staining, and comparing Figures 1a, 2a and 3a with Figures 1b, 2b and 3b it is possible to observe that human albumin, fibronectin and vitronectin at 35, 0.8 and 0.4μg/mL are not saturating for almost all surfaces, once the protein concentration is doubled and a more intense staining is achieved. Furthermore, it can be observed that both fibronectin and vitronectin adsorb in higher amounts to the starch-based surfaces and at least 85 lower concentrations are used. These results indicate higher adsorption affinity of FN and VN to the materials studied.

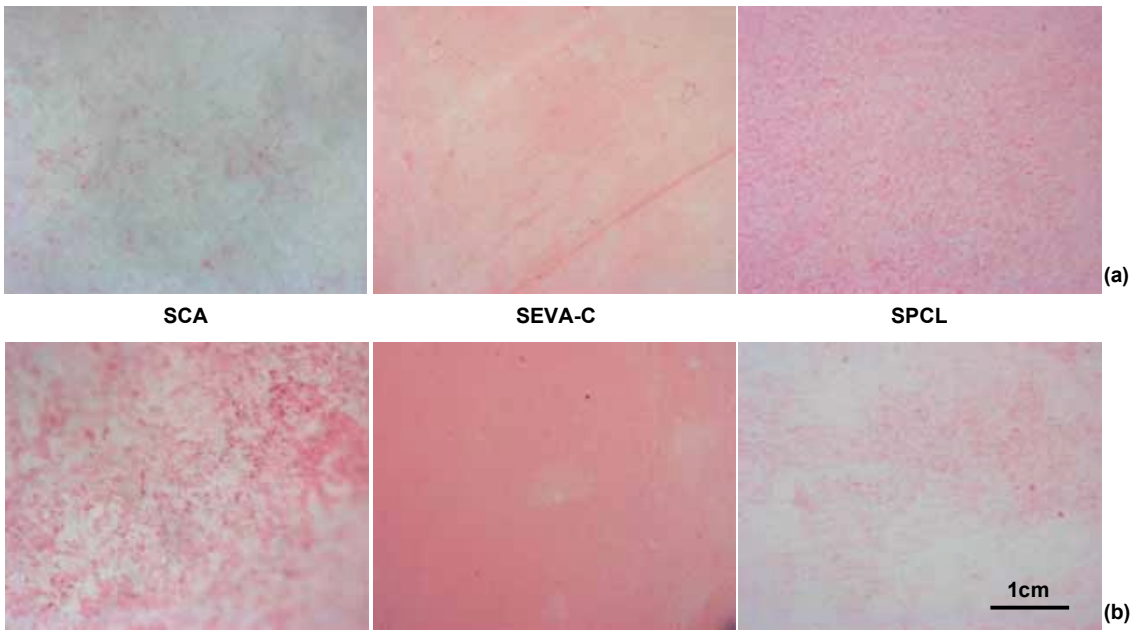

Figure 1. Immunostaining results (5x) of different starch-based materials (SCA, SEVA-C, and SPCL) immersed in two distinct human serum albumin solutions: 35.0μg/mL – 0.1% of the concentration in human serum – (a) and 70.0 μg/mL – 0.2% of the concentration in human serum (b).

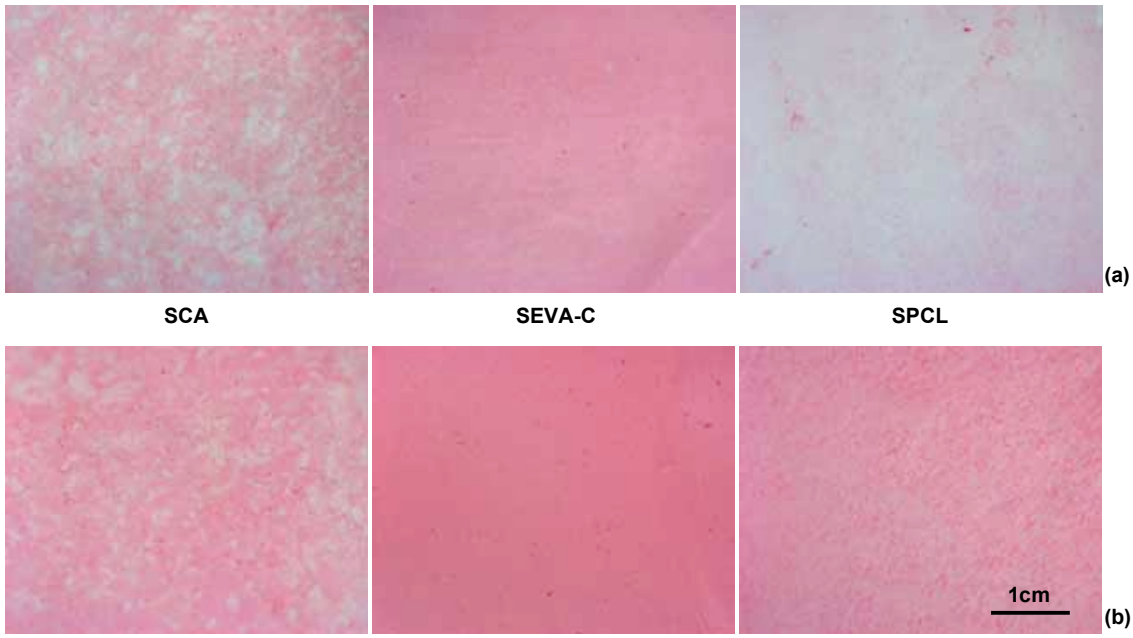

Figure 2. Immunostaining results (5x) of different starch-based materials (SCA, SEVA-C, and SPCL) immersed in two distinct human fibronectin solutions: 0.4 μg/mL – 0.1% of the concentration in human serum – (a) and 0.8 μg/mL – 0.2% of the concentration in human serum (b).

3.1.2. Binary systems

In order to examine protein adsorption in a competitive environment, binary solutions of 1000μg/mL of albumin and 1μg/mL of fibronectin were prepared and samples were immersed in the solutions for

24h at 37ºC. Figure 4 presents the different starch-based surfaces previously immersed in the two-protein solutions and immunoassayed for HSA and FN. It was observed that, in opposition to single protein solutions (see Figure 1 and Figure 2), staining intensities were generally lower although higher albumin and fibronectin concentrations were used. In unitary systems, SEVA-C adsorbed more FN than HSA and showed a more intense staining for HSA. In addition, although SCA and SPCL present a general decrease in staining intensity, higher adsorptions are still obtained for fibronectin. In opposition to the previously discussed results, the binary solutions indicate low adsorption capabilities of fibronectin in competitive environment and, subsequently, a synergistic effect driven by the presence of albumin.

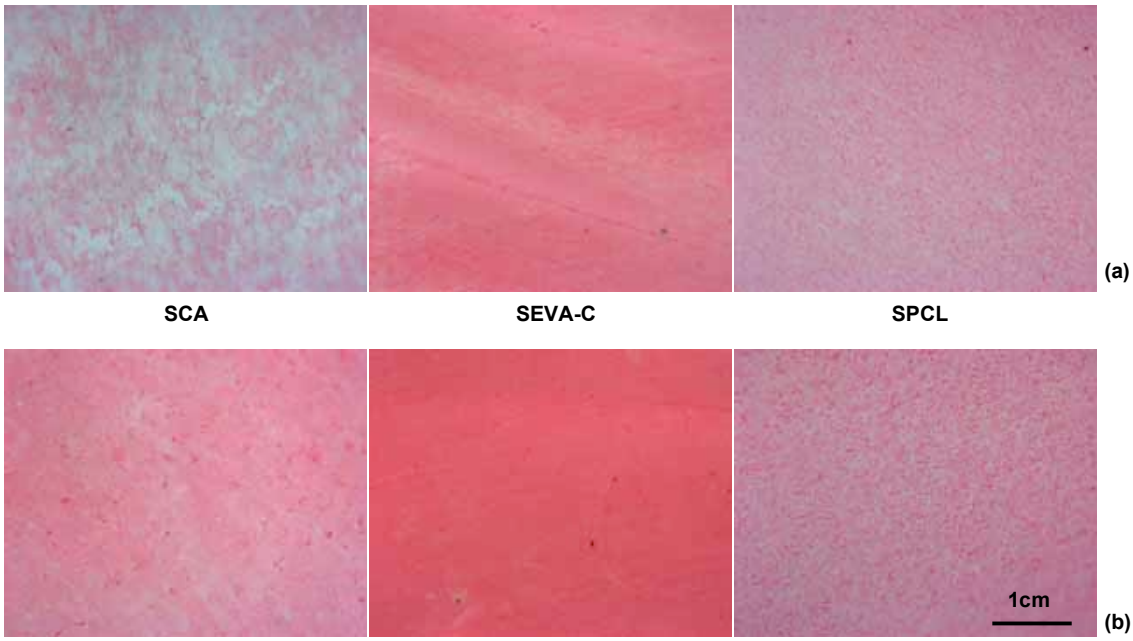

Figure 3. Immunostaining results (5x) of different starch-based materials (SCA, SEVA-C, and SPCL) immersed in two distinct human vitronectin solutions: 0.3 μg/mL – 0.1% of the concentration in human serum – (a) and 0.6 μg/mL – 0.2% of the concentration in human serum (b).

3.1.3. Complex systems

To complement the results obtained for binary systems, more complex protein solutions were also prepared and studied. Collected serum was diluted to 20%, 10% and 1% (v/v) in PBS without Ca^{2+} and Mg^{2+}. After immersion in serum solutions, surfaces were immunoassayed for HSA, FN and VN (Figure 5). Although it was not possible to observe any increase in staining intensity from less to more concentrated solutions, both 10% (results not shown) and 20% human serum solutions seemed, in quantitative terms, to stimulate higher protein adsorption than the 1% solution (results not shown). Analysis of the results leads to the conclusion that in opposition to vitronectin, albumin is the protein that is adsorbed to the smallest extent onto the starch-based materials. Alternatively, from the materials perspective, SPCL and SEVA-C were observed to present higher protein adsorption affinity when compared to starch cellulose acetate (SCA) polymeric blends (see Figure 5).

Figure 4. Immunostaining results (5x) of different starch based materials (SCA, SEVA-C and SPCL) immersed for 24 hours in a solution with both human serum albumin (1000 µg/ml) and human fibronectin (1 µg/mL) and stained for albumin (a) and fibronectin (b).

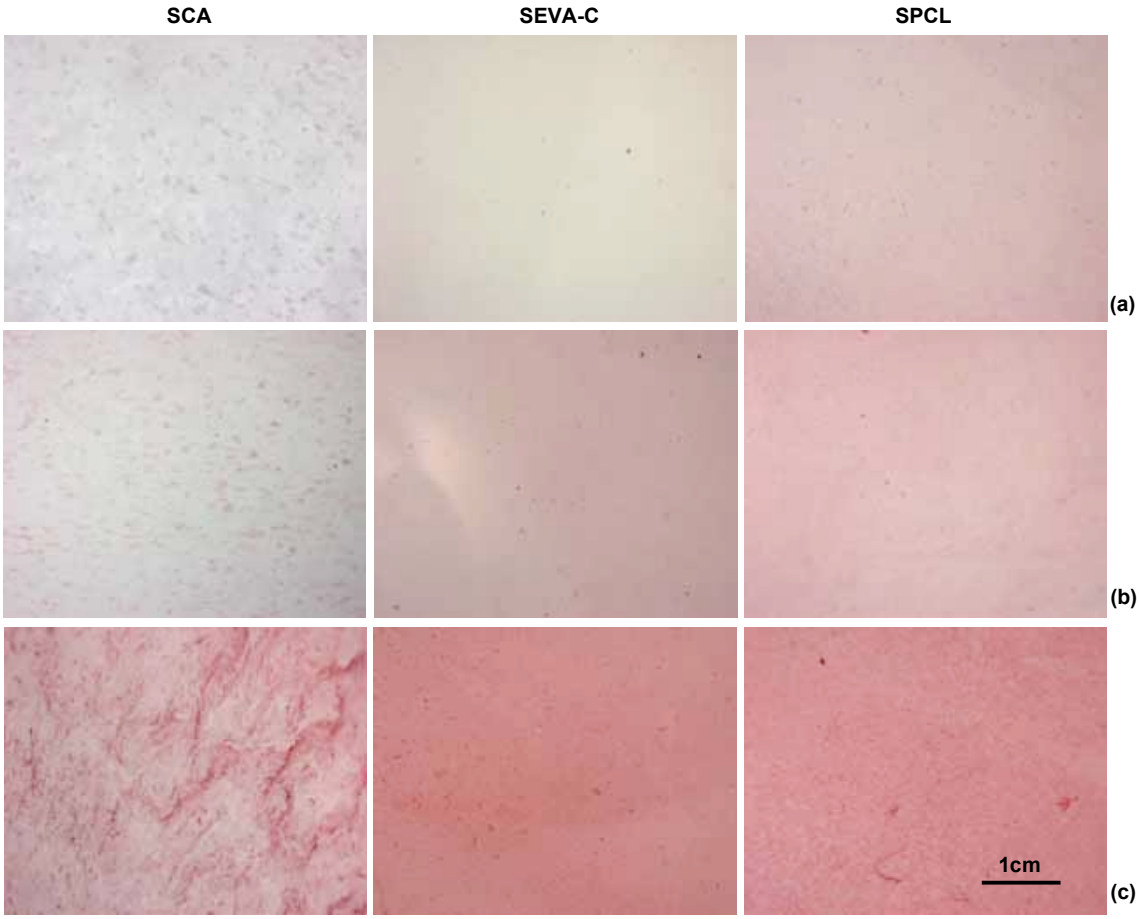

Figure 5. Immunostaining results (5x) of different starch-based materials (SCA, SEVA-C and SPCL) immersed 20% (v/v) of human serum and stained for human serum albumin (a), human fibronectin (b) and human vitronectin (c).

3.2. Immunostaining analysis of leukocyte adhesion to protein coated surfaces

3.2.1. Effect of cell culture media, foetal bovine serum and leukocyte culture onto pre-adsorbed proteins

In order to study cell-protein interactions on starch-based material surfaces the effect of cell culture media and foetal bovine serum were examined. Samples were immersed in protein solutions concentrated at 0.2% of the human serum and then placed in cell culture media with and without bovine serum. The main aim was to study the effect of cell culture media, but also the effect of foetal serum, as this is a highly concentrated complex protein solution where protein-protein interactions are certain to occur. Although no significant differences in staining intensities were observed for SEVA-C pre-coated with FN and VN (see Figure 6), albumin antibody-labelled surfaces showed some loss of this protein after immersion in serum-supplemented media. This change may be a result of protein release from the SEVA-C surface to the surrounding solution or due to the coverage of this substrate with bovine foetal serum proteins.

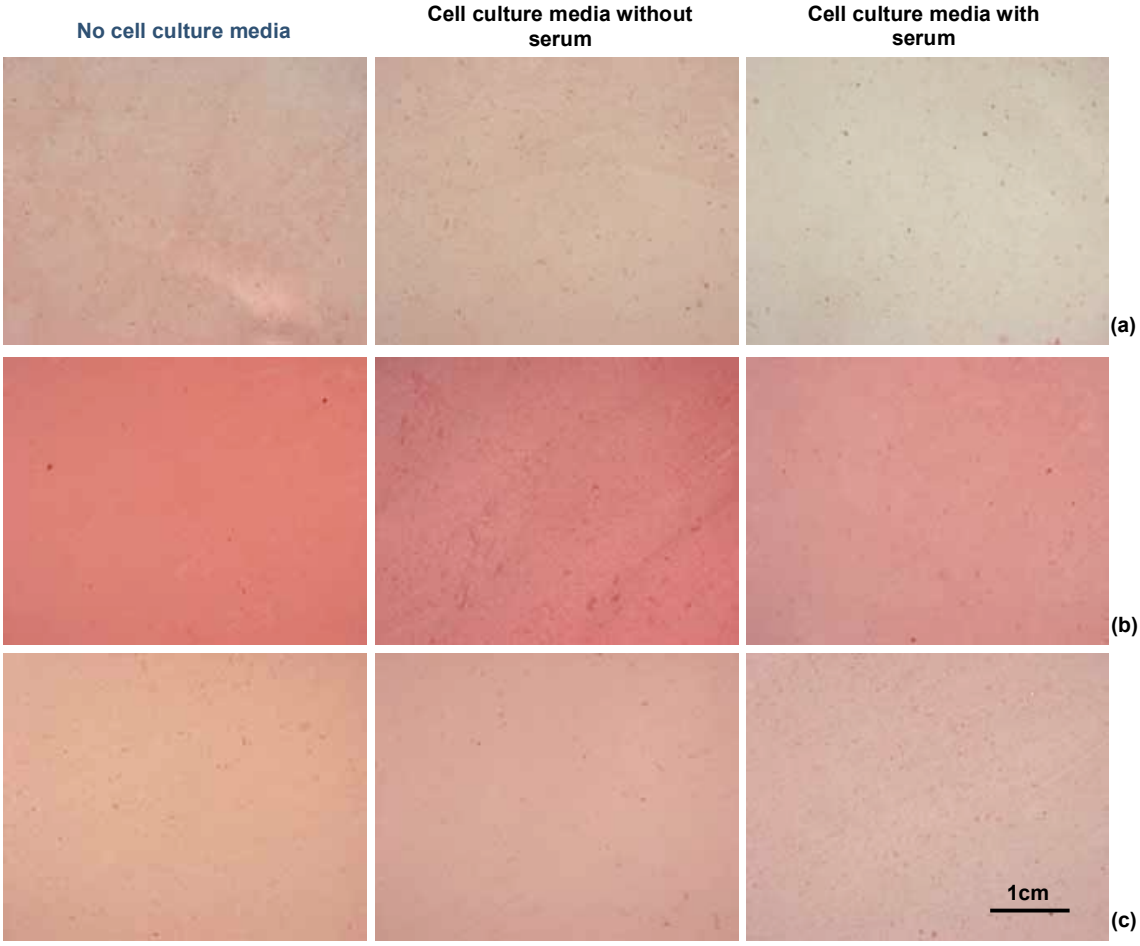

Figure 6. Immunostaining results (5x) of SEVA-C initially immersed in HSA (a), HF (b) and HV(c) at 0.2% of the concentration in the human serum, 70.0, 0.8 and 0.6 mg/mL, respectively, and then exposed 24 hours to the cell culture media with and without foetal bovine serum.

After confirming that albumin, fibronectin and vitronectin are still present on the surface after cell culture immersion, the effect of previously adsorbed proteins on cultured cells was assessed. Figure 7 shows SEVA-C surfaces exposed to 0.2% of protein concentrations, on which cells were seeded and then stained for the presence of HSA, FN and VN. After 30min and 24h of leukocyte culture, fibronectin samples showed no interference determined by cell incubation. With respect to HSA and VN, results indicate protein desorption from the surface leading to the idea that fibronectin might be the more strongly adsorbed protein or at least less affected by cell culture interactions.

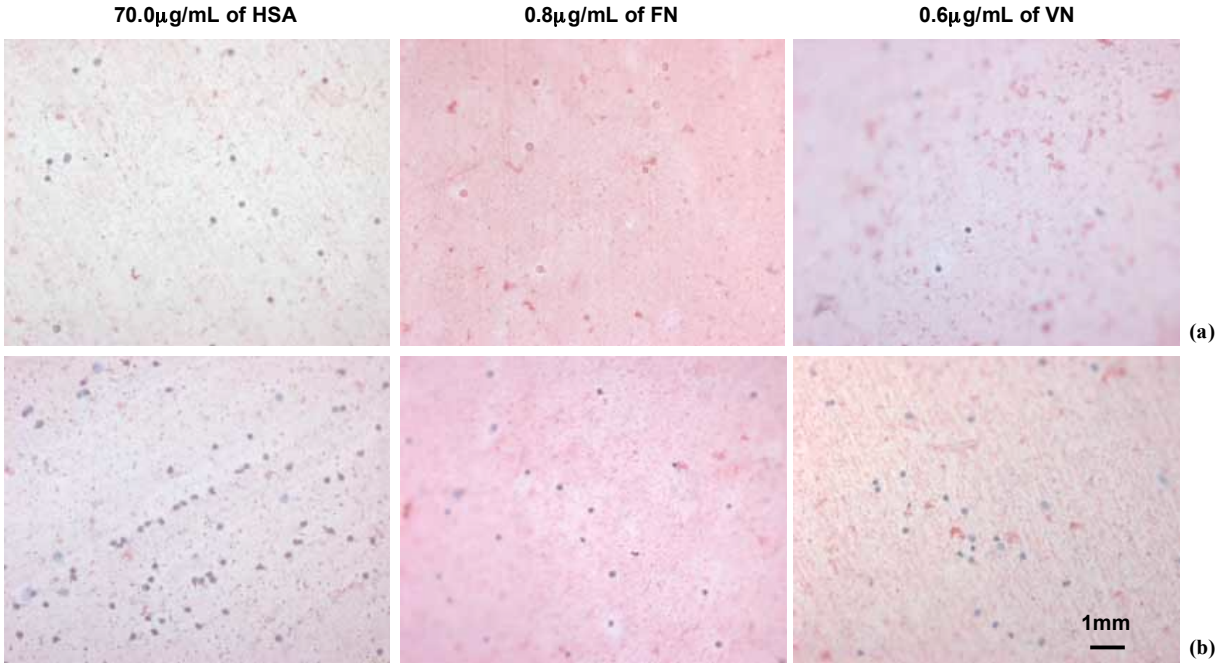

Figure 7. Protein immunostaining samples (20x) of SCA, SEVA-C and SPCL previously immersed in 0.2% of HSA, HF and HV and then submitted to cell culture for 30min (a) and 24h (b).

3.2.2. Immunostaining and cell density analysis of adherent leukocytes

To examine how adsorbed proteins on SEVA-C surface affect cell adhesion, samples were immersed in albumin, fibronectin and vitronectin solutions, concentrated at 0.2% of physiological levels. Surfaces were used as substrates for cell culture and afterwards, antibody-labelling was performed. An example of the immunostaining results is presented in Figure 8 where it is possible to observe lymphocytes and monocytes/macrophages (CD3 and CD14 positive stained cells, respectively) incubated with SEVA-C surfaces previously immersed in vitronectin solution. From the preliminary results obtained, it was not possible to observe inhibition of monocytes/macrophages or lymphocytes adhesion to SEVA-C surface driven by the presence of HSA, FN nor VN.

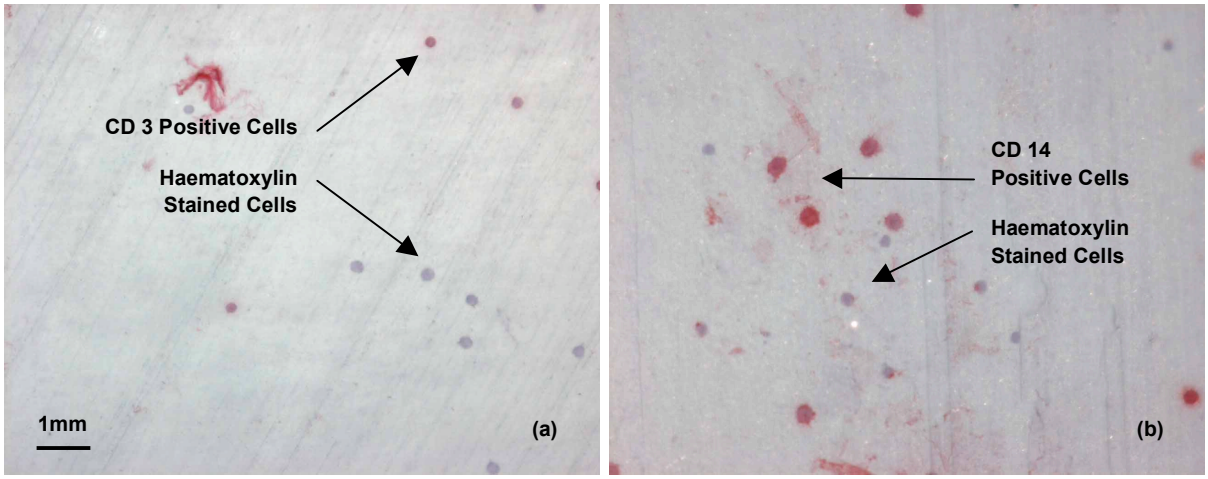

Figure 8. Example of immunoshistochemical detection of human leukocytes cultured on SEVA-C surfaces previously immersed in 0.6mg/mL of VN. Red cells consist of CD3 (a) and CD14 (b) positive cells; blue cells are a result of haematoxylin stain (20x).

To assess quantitatively the effects of albumin, fibronectin and vitronectin on monocyte and lymphocyte adhesion to SEVA-C surfaces, CD3 and CD14 positive cells were first counted and then counterstained with haematoxylin to determine total cell number.

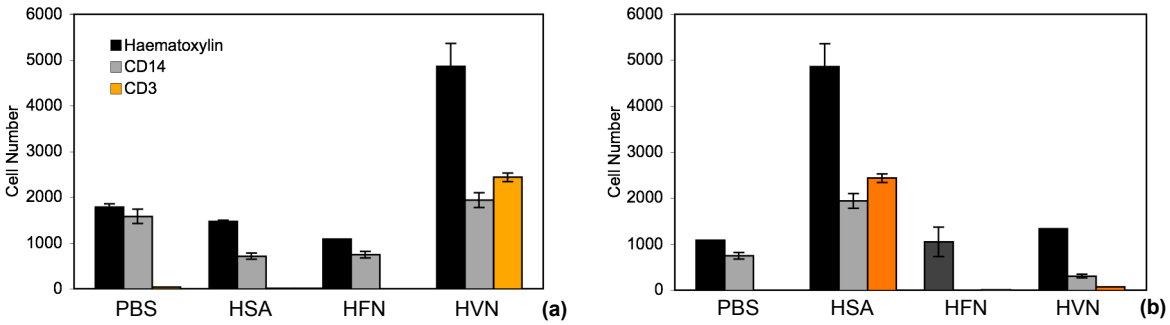

Figure 9: Total cell number, CD3 and CD14 positive cell numbers obtained for each different pre-treatment promoted to the surface of SEVA-C samples. Two different cell incubation periods are presented: 30min (a) and 24h (b).

From Figure 9a and 9b, it is possible to see differences in cell number from 30min and 24h of cell incubation. In terms of specific populations, it is obvious the preference of monocytes/macrophages to adhere to SEVA-C surfaces when compared to CD3 positive cells. These observations conflict with established procedures regularly used to isolate lymphocytes from other leukocytes, which consider these CD3 positively stained cells not capable of adhesion to exposed surfaces.[40] After 24h (Figure 9b), it was possible to observe a cell number decrease for all the studied conditions. This behaviour was not obtained for HSA previously immersed samples that exhibit a clear increase in total cell number, and in CD3 and CD14 positive cells. The decrease in cell number can be explained by these cells ability to detach from surfaces in order to adhere later or begin the apoptotic cycle.[40,41] Once

again, CD3 positive cells seem to adhere less than CD14 positive cells to SEVA-C surfaces (with or without proteins previously adsorbed on it). In summary, it might be said that vitronectin promotes a short-term effect in leukocyte adhesion and albumin was found to endorse long-term effects, subsequently leading to cell number increase after 24h of cell culture.

4. CONCLUSIONS

In this study, the use of antibodies to label human proteins allowed to obtain reproducible results, useful for understanding protein adsorption on biodegradable surfaces. Single protein solutions lead to higher adsorptions of FN and VN followed by HSA. Preference of FN adsorption in comparison to HSA was also detected when studying binary systems, except for SEVA-C, which was the only material on which HSA preferentially adsorbed. When human blood serum was used as the protein source, differences in protein behaviour were observed: in opposition to vitronectin and to fibronectin, the competitive potential of albumin to adsorb onto starch-based surfaces was decreased. Furthermore, SPCL presented the highest protein adsorption levels, even though for the studied serum concentrations surfaces showed, in general, good VN adsorption. These results support the idea that single solution studies are not good simulations of the real situation of the complex bioenviromnent. Consequently, complex systems should be considered in future for protein adsorption studies to be carried out on starch-based biomaterials.

In the second part of this study, the effect of human albumin, fibronectin and vitronectin on modulating leukocyte adhesion gave rise to interesting results. In fact, short and long-term effects in cell adhesion were found to be developed in the presence of vitronectin and albumin, respectively. In addition, it was shown that cell populations adhere to protein coated and non-coated surfaces, although monocytes and/or macrophages were found in higher numbers mainly for shorter incubation periods.

Acknowledgements

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CHAPTER IV

MODULATING BONE CELLS RESPONSE ONTO STARCH-BASED BIOMATERIALS BY SURFACE PLASMA TREATMENT AND PROTEIN ADSORPTION

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MODULATING BONE CELLS RESPONSE ONTO STARCH-BASED BIOMATERIALS BY SURFACE PLASMA TREATMENT AND PROTEIN ADSORPTION*

Alves, CM[1,2], Yang, Y[3], Carnes, DL[3], Ong, JL[5], Sylvia, VL[3], Dean, DD[3,5], Agrawal, CM[3,5], Reis, RL[1,2]

[1]3B´s Research Group – Biomaterials, Biodegradables and Biomimetics, Dept. of Polymer Engineering, University of Minho, Campus Gualtar, 4710-057 Braga, Portugal

[2] IBB – Institute for Biotechnology and Bioengineering, PT Government Associated Laboratory, Braga, Portugal

[3]Center for Clinical Bioengineering, Department of Orthopaedics, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229-3900, USA

[5]Department of Biomedical Engineering, The University of Texas at San Antonio, San Antonio, TX 78249, USA

Abstract

The effect of oxygen-based radio frequency Glow Discharge (rfGD) on the surface of different Starch-based Biomaterials (SBB) and the influence of proteins adsorption on modulating bone-cells behaviour was studied. Bovine serum albumin, fibronectin and vitronectin were used in single and complex protein systems. RfGD-treated surfaces showed to increase in hydrophilicity and surface energy when compared to non-modified SBB. Biodegradable polymeric blends of corn starch with cellulose acetate (SCA; 50/50 wt%), ethylene vinyl alcohol (SEVA-C; 50/50 wt%) and polycaprolactone (SPCL; 30/70 wt%) were studied. SCA and SCA reinforced with 10% hydroxyapatite (HA) showed the highest degree of modification as result of the rfGD treatment. Protein and control solutions were used to incubate with the characterised SBB and, following this, MG63 osteoblast-like osteosarcoma cells were seeded over the surfaces. Cell adhesion and proliferation onto SCA was found to be enhanced for non-treated surfaces and on SCA+10%HA no alteration was brought up by the plasma modification. Onto SCA surfaces, BSA, FN and VN single solutions improved cell adhesion, and this same effect was found upscaled for ternary systems. In addition, plasma treated SEVA-C directed an increase in both adhesion and proliferation comparing to non-treated surfaces. Even though adhesion onto treated and untreated SPCL was quite similar, plasma modification clearly promoted MG63 cells proliferation. Regarding MG63 cells morphology it was shown that onto SEVA-C surfaces the variation of cell shape was primarily defined by the protein system, while onto SPCL it was mainly affected by the plasma treatment.

Keywords

Starch-based materials; Oxygen-based rfGD; Protein adsorption; Cell adhesion and proliferation.

*This chapter is based on the following publication:
C. M. Alves, Y. Yang, D. L. Carnes, J. L. Ong, V. L. Sylvia, D. D. Dean, C. M. Agrawal, R. L. Reis, "Modulating bone cells response onto starch-based biomaterials by surface plasma treatment and protein adsorption"; Biomaterials, 28 (2007), 307-315

1. INTRODUCTION

The strategy of surface modification of biomaterials has been adopted over the years in order to alter the area of the biomaterial that first comes in contact with a biological environment. Surface modifications methodologies have been used in a variety of applications, with many researchers concentrating on the study of different surface stimuli and optimize the short-term and long-term performance of biomaterials. Examples are attempts to prevent or improve adsorption of proteins and adhesion of cells to biomaterial surfaces.[1-3] Plasma surface modification methodologies have been used for biomaterials in a variety of applications such as post-treatment grafting processes[4,5] for altering surfaces functionality, and modulate proteins[6,7] and cell behavior.[8,9]. The ability to retain the bulk properties constant while surfaces are changed is the key to the success of such type of approaches.[10, 11] Radio frequency Glow Discharge (rfGD), has been used for surface modification over the years because it is considered an economical technique and clearly due its reproducibility, flexibility and clean nature.[7,12] The degree of interaction of the plasma with the polymer is partly determined by the chemical structure and composition of the surface and is usually accompanied by roughening of the surface[13,14]. Although, plasma modification can yield instable and irregular surface chemistries,[14] enables the use of a diversity of chemical and thus the production of different special functional groups on the surface.[12] Interestingly both surface roughness[15,16] and chemical features[17] direct the response of osteoblastic cells.

Protein adsorption has been described by different authors[18,19] as the initial and key step following the contact of an artificial surface with blood. Either in *in vivo* or *in vitro* conditions, cells are known not to interact with biospecies free-surfaces.[20,21] Along with a number of other interfacial processes, the amount, type and conformation of adsorbed proteins directs the bio-integration of an implant, thus defining its final outcome: integration or rejection.[22]

Protein adsorption is dependent on the chemistry, wettability, energy and topography of a polymeric surface.[23,24] For instance, studies with chitosan[25] show that an increase in hydrophobicity (water contact angle of ~ 100º) lead to increased protein adsorption compared to the more hydrophilic non-modified surfaces. In this case, hydrophobic interactions govern the protein adsorption and the majority of blood proteins form proteinaceous layers over the surface.[25,26] On the other hand, very hydrophilic surfaces also favour high biocompatibility due to the preferential adsorption of albumin, which firmly binds in high concentration.[26, 27] In high concentrations albumin reaches the surface and binds, leading to a thrombogenicity lowering effect.[28] In contrast, other authors[29, 30] state that strongly hydrophobic or hydrophilic surfaces show a very low ability for protein adsorption.

When contacting a material cell behaviour is dependent on the orientation or three-dimensional conformation and nature of the adsorbed biomolecules. Plasma treatment of different surfaces has for instance shown to up-regulate the expression of adhesion molecules and improve adhesion and growth of endothelial[8] and bone related cells.[31] The biological response of tissues to biomaterials is to a large

extent cell specific and also depends on the subsequent procedures after the initial surface preparation processes.[13]

Starch-based biomaterials are biocompatible and non-cytotoxic materials that have been explored for several applications, including drug delivery systems,[32] bone replacement and regeneration,[33] and tissue engineering scaffolding.[34, 35] In this study, starch-based biomaterials (SBB) modified by oxygen rfGD were analyzed in terms of surface chemical and physical changes and its potential for positively modulate bone-cells behaviour was assessed. The surface modification was characterized by measurement of surface contact angles, surface energy, scanning electron microscopy (SEM), X-ray spectrophotometry (XPS) and Fourier transformed infra-red spectroscopy (FTIR). Subsequently, non-adhesive proteins such as serum albumin (BSA),[36] adhesive proteins like fibronectin (FN)[37] and vitronectin (VN) [38] and complex protein solutions such as fetal bovine serum (FBS) were incubated both with treated and original surfaces. These proteins were chosen on the basis of their importance in a variety of biomedical applications, including drug delivery and tissue engineering; and also on their characteristics: albumin represents highly concentrated, non-adhesive and globular proteins;[36] and FN and VN represent adhesive sequence containing proteins also present in the extracellular matrix (ECM) and known to influence cell attachment, migration, differentiation and matrix assembly.[39] Finally, the influence of these proteins coupled to the effect of surface plasma treatment on the adhesion, growth and morphology of bone-like cells was studied.

2. MATERIALS AND METHODS

2.1. Starch-based Biomaterials (SBB)

The materials used in this study were biodegradable polymeric blends of corn starch with: (i) cellulose acetate (SCA), (ii) ethylene vinyl alcohol copolymer (SEVA-C) and (iii) polycaprolactone (SPCL). The amount of starch was 50% by weight (wt%) for SCA and SEVA-C and 30% wt. for SPCL. Furthermore, a composite of SCA reinforced with 10% (wt%) of hydroxyapatite (HA) was prepared using twin-screw extrusion. Samples were processed into 10 mm circular discs using conventional injection moulding technology. Samples were sterilised by ethylene oxide,[40] washed, and all subsequent experimental procedures were performed under sterile conditions.

2.2. Oxygen-based Plasma Treatment

Surfaces were modified by means of O_2 gas plasma in a rfGD chamber (Harrick Scientific Corporation, USA). The plasma reactor chamber was stabilized at vacuum to approximately 26.7 Pa using a vacuum and O_2 was injected into chamber at a pressure of 15psi for 30sec followed by a waiting period of a 30s before plasma treatment. Plasma treatment was initiated for 180s using a

power of 100W and pulsed frequency of 13.5MHz. Time-related changes of treated surfaces were minimized by using the samples within the following 4h.

2.3. Characterization of SBB Surface Modification

2.3.1. Water Contact Angle

Contact angle measurements were used to investigate the wettability of the surfaces following rfGD modification. The relative hydrophilicity of treated and untreated SBB surfaces was assessed by using the sessile drop method on a Video Contact Angle 2000 System (AST Products, Inc., USA) and ultra-pure water (Pierce, USA). Each side of water drops was recorded and averaged; 9 drops and 3 samples per condition were used. Measurements were recorded 10 sec after liquid contact with the surface.

2.3.2. Surface Energy and Adhesion Tension of Water

The determination of surface energy (γ) of SBB compact samples after plasma treatment was based on the Owens and Went method [41] that discerns between a polar (γ^p) and a disperse or nonpolar (γ^d) component of the surface energy. Water and diiodomethane (Sigma, USA) were used as test liquids for the determination of the surface energy. Reported surface tension values for water and diiodomethane are respectively, 72.8 and 50.8dyn/cm at 20ºC.[42] Furthermore, in this study polar and disperse parts of water were considered to be 51.0 and 21.8dyn/cm and for diiodomethane 0.0 and 50.8dyn/cm, respectively.[42]

According to Janocha et al.,[43] the measurement of the adhesion tension of water is an adequate alternative methodology to the calculation of the surface energy of solid surfaces due to its higher experimental or less assumption-based nature. The contact angle ϑ of water on the surfaces was measured and multiplied by the surface tension γ_1 of water (72.8mN/m) to obtain the adhesion tension of water.

Both adhesion tension of water and surface energy were based on a sessile drop method. Drop contact angles were measured 10s after contact with the surface 9 drops and 3 samples per condition were used.

2.3.3. X-Ray Photoelectron Spectroscopy (XPS)

XPS measurements were performed in order to characterize the surface composition of biodegradable blends of corn starch with cellulose acetate (SCA) following the rfGD treatment. The experiments were carried out using a Kratos Axis-Ultra (Kratos Analytical Inc., USA) with monochromatic Al X-ray source. X-rays energy was 1486.6eV and base pressure approximately 2.9 x 10^{-11}psi. Triplicates were prepared and results collected from 5 different points of the surface of SCA samples.

2.3.4. Fourier Transform Infra Red Spectroscopy (FTIR)

Spectra were obtained by attenuated total reflection (ATR) using a Nicolet Spectrometer (Nicolet Instrument Coorporation, USA). Each spectrum was recorded with a total of 32 scans and 4.0 resolution after 20s of vacuum for chamber stabilization. Original and treated surfaces were analyzed in triplicates 400-4000cm^{-1}.

2.3.5. Scanning Electron Microscopy (SEM)

Samples morphology was analyzed by means of SEM. Surfaces were sputter coated (Med-010 Sputter Coater by Balzers-Union, USA) for obtaining a thin Au-Pd layer and examination was performed using a scanning electron microscope (Leica, UK). Triplicates were prepared for all original and plasma treated starch-based polymeric materials.

2.4. Protein Incubation Assay

Since protein–surface interactions are highly dependent on the experimental system, all assay conditions were previously optimized and kept constant throughout the performed replicates.

Single and complex protein solutions were prepared for the incubation with non-treated and rfGD-treated SBB surfaces. Proteins from bovine source were used: BSA, FN, VN and FBS and obtained from Pierce (USA), Sigma (USA), Calbiochem (USA) and Atlanta Biologicals (USA), respectively. The saline solution was supplied by Baxter (USA). Proteins were incubated with characterized samples for 15min at 37ºC as described elsewhere.[44] To simulate the blood protein environment, protein solutions were prepared at 1% of the concentration of those proteins in human blood plasma:[45] 350μg/mL of BSA, 4μg/mL of FN and 3μg/mL of VN. By combining BSA, FN and VN in the same solution, a ternary protein system was prepared using the same concentrations described for the single protein solutions. Furthermore, 1% (V/V) of FBS was also used to mimic complex protein environments.

Cell seeding was carefully performed sample by sample and immediately after protein adsorption step to evade surface drying and consequent protein conformational changes or denaturation. Surface rinsing was not performed and any enrichment of the cell culture media that could result from remaining non-adsorbed proteins was considered negligible.

2.5. MG63 Osteoblast-like Cells Culture, WST-1 Assay and SEM

Cell response was studied using the MG63 osteoblast-like osteosarcoma cell line that has been well characterized in the literature and consists of a good model for the study of human bone cells. MG63 cells are known to present numerous osteoblastic traits, including increased levels of bone alkaline phosphatase and inhibition of proliferation following treatment with 1,25-$(OH)_2D_3$.[46,47] Cells (American Type Culture Collection, USA) were seeded on the relevant surfaces at 4 x 10^4cells/mL and

incubation was performed for 1, 4 and 7 days in DMEM (CELLGRO, USA) containing 10% FBS (Atlanta Biomedicals, USA). Tissue culture polystyrene (TCPS) was used as maximum control.

After each incubation period, cultured samples were transferred to new wells with fresh media and analyzed for mitochondrial activity using colorimetric WST-1 tetrazolium conversion assay (TAKARA, Japan). Briefly, 10μL of WST-1 reagent was added per well, and the cells were incubated for an additional 2h. The absorbance of the WST-1-containing cell supernatant was determined at 450nm (Benchmark Microplate Reader, Bio-Rad, USA). To avoid interference from both cell culture media and SBB biodegradable materials, the following controls were prepared and considered as blank samples: fresh media, and SBB samples immersed in fresh media but no cells were seeded. Cell morphology was evaluated by SEM. Briefly, preparation of cell cultured samples for SEM observation was performed by using 4% formaldehyde and 1% gluteraldehyde as fixative solution (Electron Microscopy Sciences, USA). Samples were then washed using phosphate buffered saline (PBS) solution (Sigma Diagnostics, USA) and gradually dehydrated by incubation in crescent ethanol concentrations. Drying was accomplished by means of hexamethyldisilazane (HMDS) solution (Polysciences Inc., USA), as recommended for SEM preparation of soft tissue.

2.6. Statistical Analysis

Results of the tests were tabulated as mean ± SD. The effects of plasma treatment on both the surface parameters and cell density values were statistical analyzed by performing the bi-tail Students *t*-test. Significant differences were considered to exist when $p<0.05$.

3. RESULTS AND DISCUSSION

3.1. Results of Plasma Treatment on the Wettability and Surface Energy of Starch-based Biomaterials (SBB)

Oxygen plasma treatment has been described to result in the grafting of atoms or activation of existing chemical groups in the outer surface layer. Literature sources[48, 49] reveal that oxygen plasma exposure can render higher hydrophilicity on most polymer surfaces. Briefly, the active plasma species attack the polymer surface resulting in the increase or incorporation of carbonyl, carboxyl or hydroxyl functional groups.[48-51] The resulting wettability changes are generally due to oxidation effects, unsaturation, electrostatic charges and surface morphological effects.[50, 51]

The relative hydrophilicity of treated and untreated starch-based biomaterial surfaces was assessed by measuring the contact angle using ultra-pure water and the sessile drop method. Contact angle measurements are frequently applied in surface characterization and considered a high sensitive technique to study surface changes within a depth of a few atomic layers.[52] In the technique used a drop of ultra pure water was placed on the surface of the material and allowed to spread for 10sec,

after which images were acquired and analyzed. The results and characteristic drop profiles can be observed in Table 1. In general terms, all analyzed surfaces showed a significant increase in hydrophilicity following oxygen-based plasma treatment (p<0.05) that was confirmed by the higher contact angles for untreated when compared to plasma-treated surfaces (Table 1). For SCA and SCA+10% HA decreases in the contact angle were observed, 40º and 35º respectively. For SEVA-C and SPCL the plasma treatment resulted in moderate enhancement in wettability bringing surfaces contact angles to 54º and 61º - a result of 33% and 23% reduction, respectively.

Table 1. Contact angle and water drop profiles, surface energy and adhesion tension of the water for plasma treated and non-treated starch based biomaterials.

SURFACE		Contact Angle		Surface Energy		Adhesion Tension		Water Drop Profiles	
		Degrees	Change (%)	dyn/cm	Change (%)	mN/m	Change (%)	nt	t
SCA	nt	76.4±3.2	-51%	43.0±2.7	46%	17.0±3.9	239%		
	t	37.5±2.6*		62.6±0.8*		57.7±2.0*			
SCA+10%HA	nt	65.4±3.1	-53%	46.6±2.2	43%	30.3±3.6	106%		
	t	30.5±5.7*		66.5±3.0*		62.4±3.7*			
SEVA-C	nt	80.0±2.6	-33%	36.2±1.8	43%	12.6±3.3	239%		
	t	54.0±1.7*		51.9±1.1*		42.8±1.7*			
SPCL	nt	78.8±1.7	-23%	46.9±1.0	19%	14.1±2.1	152%		
	t	60.7±3.2*		55.8±1.6*		35.6±3.4*			

Leg.: *nt*- non-treated or original SBB surfaces; *t* rfGD-treated SBB.
*Statistically different from non-treated SBB surfaces (t-Test; Bi-tail; p<0.05; n>9).

The effect of plasma treatment was further studied by means of determining the surface energy and adhesion tension of the water. The surface energy of the SBB was determined using the Owens and Went method[41] with water and diiodomethane as test liquids. For the determination of surface energy, this method distinguishes the contribution of the polar (γ^p) and dispersion forces (γ^d), taking in consideration that γ^d are always present regardless of the chemical nature of the system. The results are shown in Table 1. After rfGD treatment, all surfaces showed a significant increase of the polar (γ^p) term compared to the original surfaces (p<0.05). On the other hand, the dispersion (γ^d) term was not statistically different for treated and untreated SBB samples. It was found that the increasing of the surface energy was attributed to the increasing of the polar (γ^p) term following SBB treatment. As the relative increasing ratio of the polar (γ^p) term is significantly larger than the relative increase ratio of the dispersion (γ^d) term, the values of surface energy for treated samples are higher than those untreated ones that resulted from the increasing hydrophilicity of surfaces after hydration. SCA and SCA+10%HA composite presented the highest increase in surface energy values, of approximately

20dyn/cm (46% change), followed by SEVA-C with 16dyn/cm (43% change) and SPCL with 9dyn/cm (19% change).

In parallel, adhesion tension was used as an alternative methodology to calculate surface energy. This approach permits more accurate measurements by being experimental rather than based on assumptions.[43] RfGD-treated SBB surfaces when compared to untreated ones showed higher surface energy and water adhesion tension values.

The results of water contact angle, surface energy and adhesion tension of the water showed that plasma treatment significantly affected the properties of all studied SBB. The herein described treatment introduced higher variations of the studied surfaces when compared to other chemical-based modification methods.[53] In general, SCA surfaces were the most dramatically modified ones in opposition to SPCL that showed higher surface stability. Both the increase in surface hydrophilicity and surface energy changes are due to the density of -OH polar groups on the studied surfaces, which were highly affected by the selected surface modification technology. In the natural form both SCA and SEVA-C present the same -OH density in contrast to SPCL that is composed of less 20% of starch. On the other hand, -OH groups in the synthetic polymeric fraction of the studied SBB are 2:1:0 for SCA:SEVA-C:SPCL. The nature of the different materials, including the decrease of -OH groups from SCA to SEVA-C and finally to SPCL, gives a possible explanation for the variation of contact angle or surface energy values obtained for these surfaces after plasma treatment.

3.2. Effect of Plasma Treatment on Total Oxygen Content of SBB

From the three polymeric blend studied, SCA modified surfaces showed the lowest wettability, and highest surface energy and adhesion tension. Considering the different used surfaces, SCA blends are also characterized by a higher content of hydroxyl groups known to be directly affected by oxygen reactive species as the environment created in the plasma reactor. As a case study, this material was further analyzed by XPS. XPS retrieves detailed chemical information[54] from the nanometer scale, more specifically with a depth up to 50 Å.[50] XPS was used in the present study to characterize the surface composition of SCA following oxygen rfGD plasma treatment. Table 2 shows the results obtained for treated and untreated SBB.

Table 2. XPS analysis of atomic percentages and functional groups for the native and oxygen rfGD modified SCA.

SURFACE	O1 (%)	N 1s (%)	C-H (%)	C=O (%)	C-OOH (%)	Total O (%)	Total C (%)
SCA	35,19 ± 1,07	0,07 ± 0,16	16,83 ± 5,14	33,68 ± 5,14	14,23 ± 0,85	35,19 ± 1,07	64,74 ± 1,12
rfGD-SCA	41,37 ± 1,10*	1,05 ± 0,26*	4,91 ± 1,75*	36,57 ± 2,31	16,09 ± 0,86*	41,37 ± 1,10*	57,57 ± 0,94*

*Statistically different from non-treated SCA (t-Test; Bi-tail; $p<0.05$; n>9).

The oxygen rfGD treatment was found to add significant amounts of C-O-O bonds to the surface but on the other hand C-H and C=O functional groups decreased following plasma surface modification (p<0.05). After plasma treatment, C:O ratio increases from 1:0.57 to 1:0.77, indicating an increase of oxygen on the surface of the SCA blends. Comparing the contact angle and XPS results it is seen that after the plasma treatment the increase in the total oxygen is due to an increase of OH or COOH groups as an increase in C=O group would lead to an increase in oxygen but to a more hydrophobic surface.

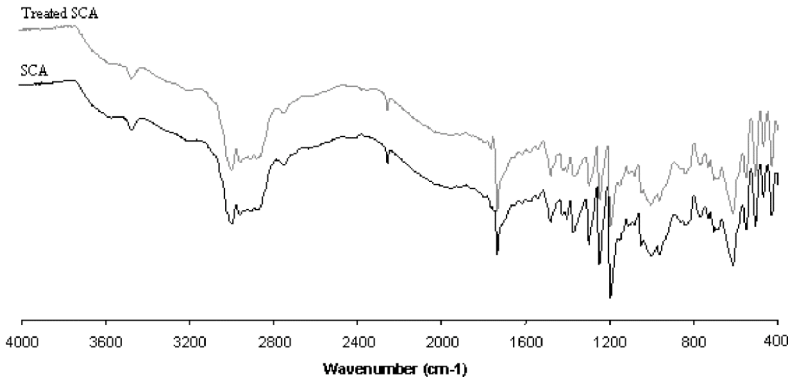

Figure 1. FTIR spectra of original and plasma-treated SCA surfaces.

FTIR spectroscopy with ATR (FTIR-ATR) was also performed to characterize all SBB materials before and after plasma treatment. In contrast to surface methodologies as the wettability analysis described above, the FTIR-ATR spectra results from signals retrieved from up to 100nm thickness, which in practice could mask true surface signals.[52] Spectra analysis did not suggest treatment driven-chemical changes either in the aliphatic, carbonyl or asymmetric stretching regions[55] as can be observed in the example presented in the Figure 1. This indicates that the oxygen plasma surface modification did not affect the properties of the bulk of the material.

3.3. Effects of Plasma Treatments on the Surface Morphology of SBB

SEM was used to perform a qualitative surface analysis of the morphology changes introduced by the plasma treatment (Figure 2). In opposition to SCA+10%HA, non-modified SCA, SEVA-C and SPCL polymeric blends (Figures 2a, 2e and 2g, respectively) presented a smoother surface when compared to treated surfaces. These preliminary results could indicate the ability of oxygen plasma treatment to modify SBB microtopography. The increase in surface heterogeneity may be another factor responsible for changing the hydrophilicity of SBB surfaces since surface micro-features affect wettability.[56]

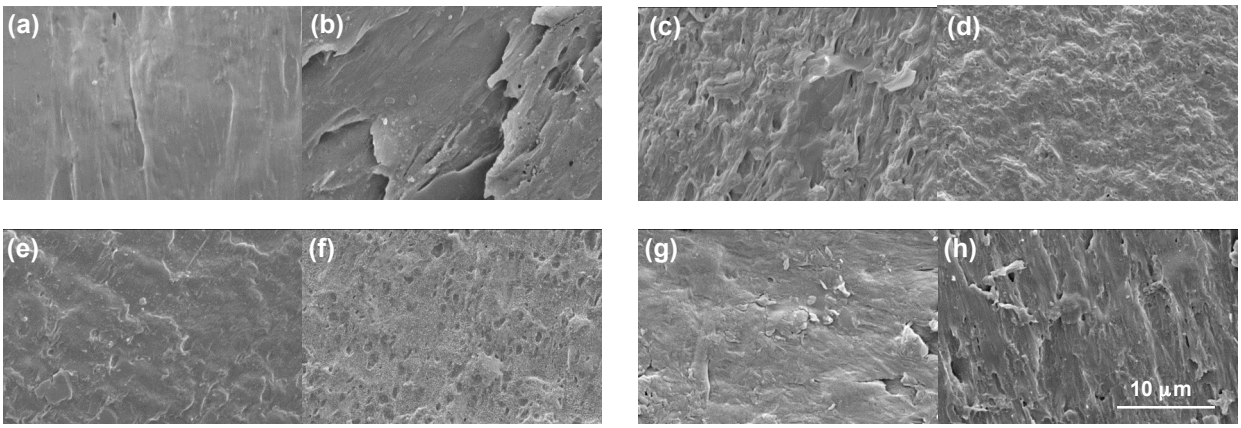

Figure 2. Morphology of original and plasma treated starch-based biomaterials: non-treated (a) and treated SCA (b), non-treated (c) and treated SCA + 10% HA (d), non-treated (e) and treated SEVA-C (f) and non-treated (g) and treated SPCL (h) surfaces.

3.4. Effect of Plasma Treatments and Adsorbed Proteins on the Density and Morphology of Osteoblast-like Cells seeded on SBB

Single and complex protein solutions were prepared for the incubation with non-treated and rfGD-treated SBB surfaces. Proteins were incubated as described elsewhere;[44] to simulate the physiological environment protein solutions were prepared at 1% of the concentration of those proteins in human blood plasma:[45] 350µg/mL of BSA, 4µg/mL of FN and 3µg/mL of VN. By combining BSA, FN and VN in the same solution, a ternary protein system was prepared. Furthermore, in order to mimic complex protein environments, 1% (v/v) of FBS was used. Cell response was studied using MG63 osteoblast-like osteosarcoma cell line. Cells were seeded on the surfaces for 1, 4 and 7 days; cell adhesion and proliferation was assessed in terms of absorbance and cell morphology observed by SEM. WST mitochondria assays measure viability taking in account intact mitochondrial mechanisms, consistent cellular activation and similar ECM interactions. The PreMix WST-1 enables to analyze cell proliferation and cell viability with a colorimetric assay, and is based on the cleavage of tetrazolium salts by mitochondrial dehydrogenase in viable cells.[57]

Cell adhesion and proliferation on SCA (Figure 3a) were found to be enhanced for non-treated surfaces. As for SCA surfaces, proliferation of MG63 cells was promoted on untreated SCA+10%HA (Figure 3b) but no alteration on cell adhesion was introduced by the plasma modification. In addition, plasma-treated polymeric blends of corn starch and ethylene vinyl alcohol showed an increase in both MG63 cells adhesion and proliferation compared to non-treated surfaces (Figure 3c). Even though adhesion on treated and untreated SPCL (Figure 3d) was rather similar, plasma modification promoted MG63 cells proliferation. Between the cells and the surface, proteins are present. In the case of SCA surfaces, gas plasma treatment and subsequent protein incubation revealed to affect MG63 cells adhesion. On SCA surfaces, BSA, FN and VN single solutions improved cell adhesion levels, and this same effect was found for ternary systems.

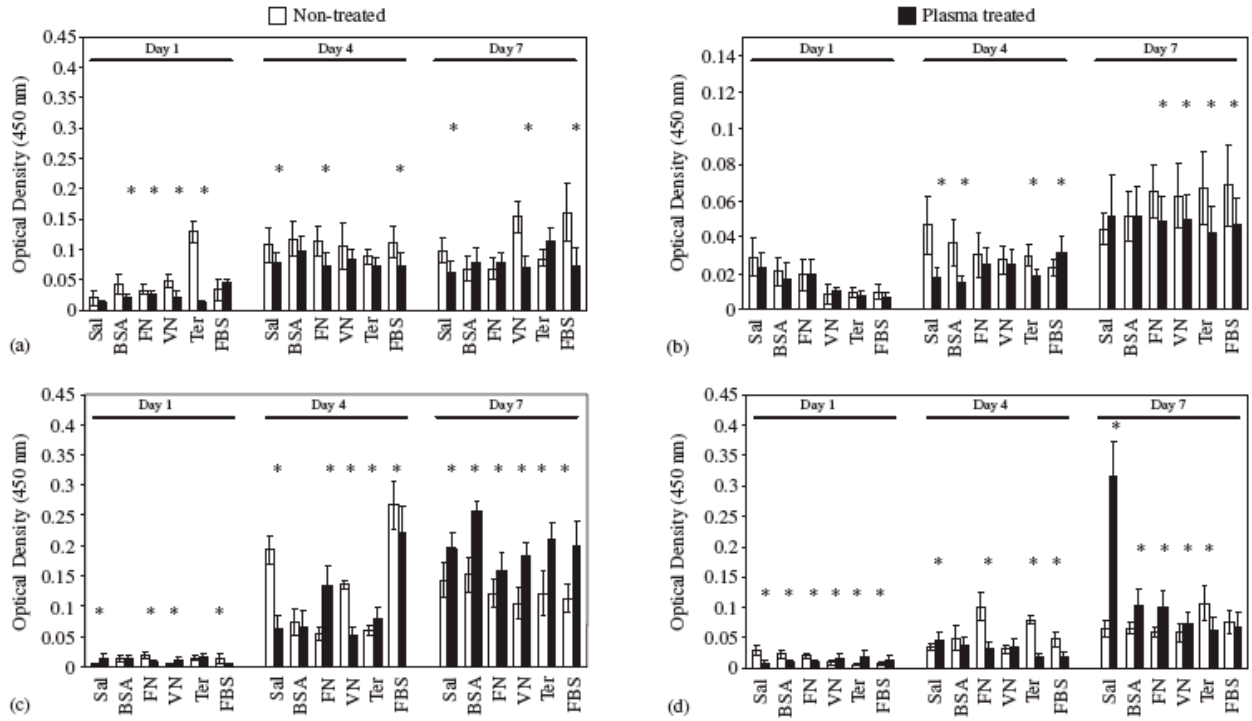

Figure 3. Adhesion and proliferation of MG63 osteoblast-like cells onto the different non-treated and treated starch-based biomaterial surfaces: (a) SCA, (b) SCA + 10% HA, (c) SEVA-C and (d) SPCL. Leg.: *Sal*- saline solution and *Ter*-ternary solution. * Statistically different from non-treated sample (t-Test; Bi-tail; $p<0.05$; n>9).

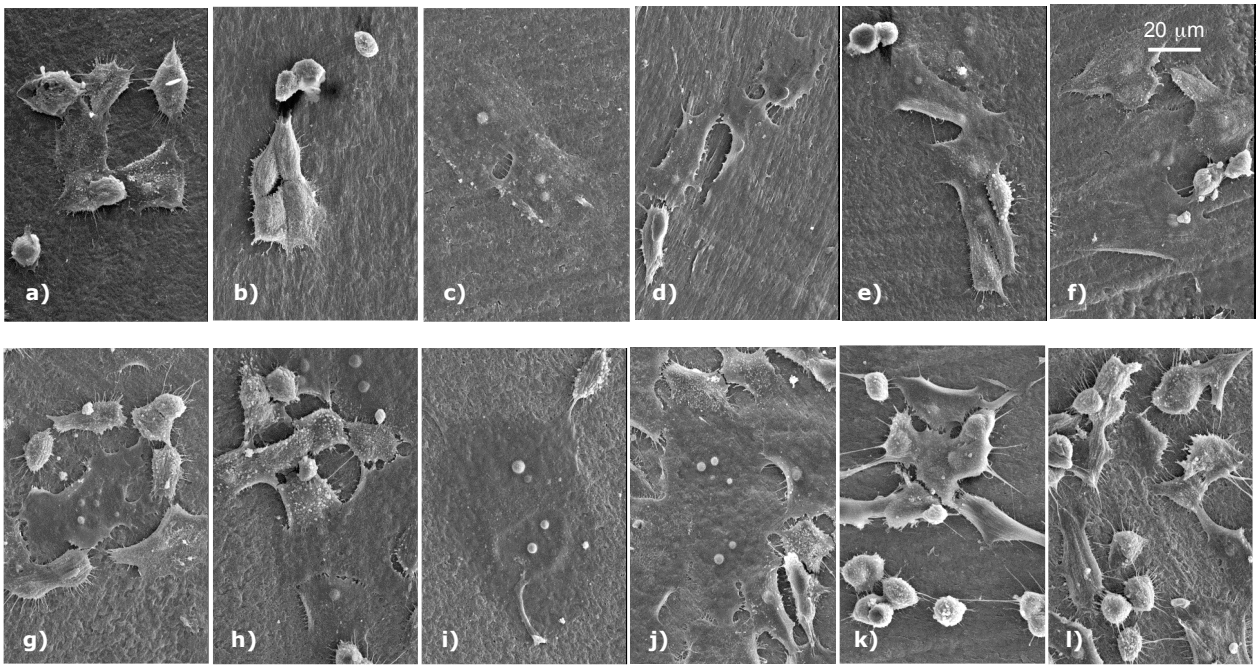

Figure 4. Representative MG63 cells morphology over SEVA-C (a to f) and SEVA-C-treated surfaces (g to l), previously incubated with: saline (a and g), BSA (b and h), FN, (c and i), VN (d and j), ternary (e and k) and FBS (f and l) solutions.

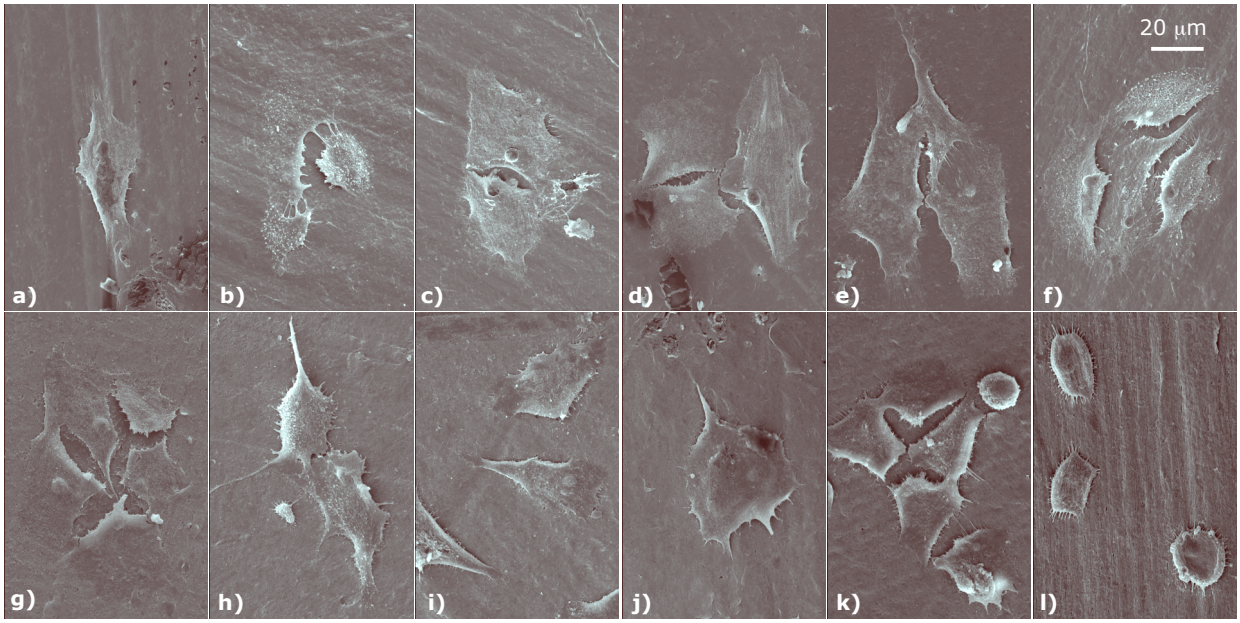

Figure 5. Representative MG63 cells morphology over SPCL (a to f) and SPCL-treated surfaces (g to l), previously incubated with: Saline (a and g), BSA (b and h), FN, (c and i), VN (d and j), Ternary (e and k) and FBS (f and l) solutions.

Regarding MG63 cells morphology it was shown that on SEVA-C surfaces the variation of cell shape was primarily defined by the protein system used (Figure 4). In general, cells presented a similar morphology either for treated or non-treated SEVA-C surfaces. Specific morphological characteristics were observed for the surfaces pre-adsorbed with FN (Figures 4c and 4i) and VN (Figures 4d and 4j), where cell spreading is increased when compared with the surfaces incubated with other protein systems. For SPCL surfaces, cell shape was affected by the plasma treatment (Figure 5).

No cell morphological variation was observed when comparing the different protein systems within the treated or non-treated surfaces. Comparing Figures 5a-f with 5g-l reveals that on non-treated SPCL surfaces cells present lamelipodia structures and on treated surfaces they present preferentially filapodia formation.

4. Conclusion

The surface modification technique used in this study, oxygen-based radio frequency Glow Discharge (rfGD) treatment was successful in changing starch based biomaterials surface properties. Oxygen rfGD was shown to uniformly functionalize/activate the surface of SBB without affecting the bulk properties. The effect of oxygen-based rfGD on the surface of starch-based biomaterials (SBB) was investigated by means of: contact angle, surface energy, adhesion tension of water, scanning electronic microscopy (SEM), X-ray photoelectron spectroscopy (XPS) and Fourier transformed infrared spectroscopy with attenuated total reflection (FTIR-ATR). Both, the effects of plasma modification and the presence of different protein systems on the viability and morphology of MG63 osteoblast-like

cells were also studied. RfGD-treated surfaces showed an increase in the hydrophilicity as well as the adhesion tension and surface energy when compared to non-modified SBB. Biodegradable polymeric blends of corn starch with cellulose acetate (SCA) and SCA with 10% hydroxyapatite (HA) showed the highest change in wettability and surface energy as result of the rfGD treatment. Surface morphological changes were also observed by SEM. XPS analysis of SCA indicated significant differences in the C:O ratio, which increased after treating surfaces by plasma treatment, and may explain the biological response of the different polymeric blends.

In the absence of pre-incubated proteins, the plasma-treated SPCL surfaces showed to highly improve osteoblast-like cells proliferation. Protein types and the presence of other proteins were shown to be the key for cell adhesion and proliferation. In several cases, cell morphology was shown to be related to surface properties created by the plasma treatment. In contrast to SEVA-C surfaces, cell adhesion and proliferation on SCA were found to be enhanced for non-treated surfaces and on SCA+10%HA no significant changes in cell adhesion were introduced by the plasma modification. Even though adhesion on treated and untreated SPCL was very similar, plasma modification clearly promoted MG63 cells proliferation. MG63 cells morphology on SEVA-C surfaces was primarily defined by the protein system used, while on SPCL it was mainly affected by the plasma treatment.

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CHAPTER V

PLASMA SURFACE MODIFICATION OF POLY(D,L-LACTIC ACID) AS A TOOL TO ENHANCE PROTEIN ADSORPTION AND THE ATTACHMENT OF DIFFERENT CELL TYPES

CHAPTER V

PLASMA SURFACE MODIFICATION OF POLY(D,L-LACTIC ACID) AS A TOOL TO ENHANCE PROTEIN ADSORPTION AND THE ATTACHMENT OF DIFFERENT CELL TYPES*

Alves, CM[1,2], Yang, Y[3], Marton, D[4], Carnes, DL[3], Ong, JL[5], Sylvia, VL[3], Dean, DD[3,5], Reis, RL[1,2], Agrawal, CM[3,5]

[1]3B´s Research Group – Biomaterials, Biodegradables and Biomimetics, Dept. of Polymer Engineering, University of Minho, Campus Gualtar, 4710-057 Braga, Portugal

[2] IBB – Institute for Biotechnology and Bioengineering, PT Government Associated Laboratory, Braga, Portugal

[3]Center for Clinical Bioengineering, Department of Orthopaedics, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229-3900, USA

[4]Department of Radiology, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229-3900, USA

[5]Department of Biomedical Engineering, The University of Texas at San Antonio, San Antonio, TX 78249, USA

Abstract

We have studied the influence of oxygen radio frequency glow discharge (rfGD) on the surface and bulk properties of Poly(D,L-lactic acid) (PDLLA) and the effect of this surface modification on both protein adsorption and bone cell behavior. PDLLA films were characterized before and after plasma surface modification by water contact angle, surface energy and adhesion tension of water as well as by scanning electron microscopy (SEM), X-ray electron spectroscopy (XPS) and Fourier transform infra-red (FTIR) spectroscopy. RfGD-films showed an increase in hydrophilicity and surface energy when compared to untreated films. Surface morphological changes were observed by SEM. Chemical analysis indicated significant differences in both atomic percentages and oxygen functional group. Protein adsorption was evaluated by combining solute depletion and spectroscopic techniques. Bovine serum albumin (BSA), fibronectin (FN), vitronectin (VN) and fetal bovine serum (FBS) were used in this study. RfGD-treated surfaces adsorbed more BSA and FN from single specie solutions than FBS that is a more complex, multi-specie solution. MG63 osteoblast-like cells and primary cultures of fetal rat calvarial (FRC) cells were used to assess both the effect of RfGD treatment and protein adsorption on cell attachment and proliferation. In the absence of pre-adsorbed proteins, cells could not distinguish between treated and untreated surfaces, with the exception of MG63 cells cultured for longer periods of time. In contrast, the adsorption of proteins increased the cells' preference for treated surfaces, thus indicating a crucial role for adsorbed proteins in mediating the response of osteogenic cells to the RfGD-treated PDLLA surface.

Keywords

Polylactic acid, Plasma surface modification, Protein adsorption, Cell attachment and proliferation.

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1. INTRODUCTION

Polylactic acid (PLA) is a well know biodegradable aliphatic polymer that has been previously explored for several biomedical applications such as bone fixation devices and tissue engineering scaffolds.[1,2] Although it is known to be biocompatible and is widely used clinically, its low wettability and surface energy have been shown to affect cell attachment and proliferation and remain an issue.[3] In the field of biomaterials it is well established that the surface characteristics of an implant play a more critical role in determining the biologic response compared to the bulk properties of the biomaterial. Thus a wide range of surface modification methodologies have been explored to achieve desirable surface properties, such as chemistry, wettability, surface energy and topography.[4,5] Often the goal of surface modification techniques is to generate a physical and/or chemical modification of the outer molecular layer of the surface, while retaining the bulk properties of the material, including mechanical ones.[4] In this context, plasma treatments are frequently used to modify the chemical functionality of non-reactive biomaterial surfaces because they can be applied on a wide range of implant shapes and sizes.[6] Although it is accepted that plasma modification can yield irregular surface chemistries,[7] this technique presents a major advantage while enables the use of a diversity of chemicals and thus the production of a variety of special functional groups on the surface.[8] Plasma surface activation employs gases, such as oxygen, which dissociate and react with the surface, creating additional functional groups that can be recognized as adhesion sites for surrounding cells.[9]

Cell response to a biomaterial surface is considered one of the major factors in determining the biocompatibility of a material because this step affects subsequent cell proliferation and differentiation pathways.[10] With this in mind, a number of investigators have reported using plasma treatment to improve the behavior of anchorage-dependent cells such as osteoblasts[11] and endothelial cells.[12,13] It is known that interfacial reactions occurring when a material contacts a biological environment are modulated by both the surface and the biomolecules, such as proteins, that interact with it. The outcome of these interactions subsequently affect the cellular response.[14,15] In the past, studies have tried to assess the effect of surface properties on the adsorption of proteins with the goal of creating biomimetic surfaces, such as those that mimic extracellular matrix (ECM) properties, to improve surface recognition by cells.[16,17] Along these lines, in the present study we aim to provide insight into the interaction of two different types of osteogenic cells with poly(D,L-lactic acid) surfaces with differences in wettability, surface energy, chemistry and roughness.

An oxygen-based plasma treatment of PDLLA was used to modify surface properties and alter the adsorption of proteins. Characterization of the surface was performed by measuring the surface contact angle and surface energy, and the use of scanning electron microscopy (SEM), X-ray photoelectron spectroscopy (XPS) and Fourier transform infra-red spectroscopy (FTIR). The adsorption of various proteins was studied as a function of gas plasma treatment; these included non-adhesive proteins such as bovine serum albumin (BSA),[18-20] adhesive proteins such as fibronectin (FN)[21-24] and vitronectin

(VN)[25-27] and the complex protein solution fetal bovine serum (FBS). The influence of these proteins on cell attachment and proliferation was analyzed. Two types of cells were used: MG63 cells as a prototype of an established line of bone-like cells, and fetal rat calvarial (FRC) cells as an example of primary osteoprogenitor cells.

2. MATERIALS AND METHODS

2.1. Materials and Chemicals

Poly[D,L-Lactide] (PDLLA) was purchased from Birmingham Polymers, Inc. (USA) and acetone from EM Science (Germany). Glass coverslips were obtained from PGC Scientifics (USA). Ultra pure water for contact angle was purchased from Pierce (USA) and diiodomethane was supplied from Sigma (USA). The proteins used were from bovine sources; serum albumin (BSA), plasma fibronectin (FN), vitronectin (VN) and fetal bovine serum (FBS) were obtained from Pierce (USA), Sigma (USA), Calbiochem (USA) and Atlanta Biologicals (USA), respectively. Saline solution was purchased from Baxter (USA) and Bicinchronic Acid (BCA) reagents were obtained from Pierce (USA). The enzyme mixture of 0.2% collagenase/0.05% trypsin and Hank's Balanced Salt Solution (HBSS) were obtained from Sigma (USA). Cell culture media αMEM and Penicillin-Streptomycin Mixture were obtained from Gibco, DMEM from CELLGRO, β-glycerophosphate from Caliochem, L-ascorbic acid from Sigma and Fetal Bovine Serum (FBS) from Atlanta Biomedicals (USA). WST-1 (TAKARA, Japan) and trypsin-EDTA (Sigma, USA) were used for cell number quantification.

2.2. Poly(D,L-Lactic Acid) Films Fabrication

Poly[D,L-Lactide] (PDLLA) films were fabricated under clean conditions using acetone as solvent. The polymer used had an inherent viscosity of 0.67dL/g and was dissolved in acetone at a ratio of about 1:23 or 1.64g/37mL. The polymer solution was cast directly over 1.5cm diameter glass coverslips. In this fashion, after solvent evaporation, circular PDLLA film samples attached to the glass coverslips were obtained. These were kept under vacuum for further drying until use. The films were sterilized by UV radiation (8 hours) in a tissue culture hood prior to use.

2.3. Plasma Treatment and Surface Sterilization

The PDLLA film sample surfaces were modified by means of O_2 gas plasma in a Radio Frequency Glow Discharge (RfGD) chamber (Harrick Scientific Corporation, USA). The plasma reactor chamber was stabilized under vacuum to approximately 26.7Pa and then O_2 was injected into chamber at a pressure of 15psi for 30sec followed by a waiting period of 30sec before plasma treatment. Plasma treatment was initiated for 180sec using a power of 100W and pulsed frequency of 13.5MHz. Time-

related changes on treated surfaces were minimized by testing the samples within the following 48 hours. Sterilization of plasma modified PDLLA was performed as described for original surfaces.

2.4. Characterization of PDLLA Surfaces

2.4.1. Water Contact Angle

The relative hydrophilicity of gas plasma treated and non-treated PDLLA surfaces was assessed by water contact angle measurements. Contact angles were measured using the sessile drop method on a Video Contact Angle 2000 System (AST Products, Inc., USA) and ultra-pure water. Both side of the water drops were recorded and averaged; 9 drops and 3 samples per condition were used and the results averaged. Measurements were recorded 10 sec after liquid contact with the surface.

Contact angle measurements were used to investigate the wettability of the surfaces following both RfGD modification and UV sterilization experimental steps.

2.4.2. Surface Energy and Adhesion Tension of Water

Surface energy measurements on control and plasma treated PDLLA films were performed in accordance with the Owens and Went method[28] that distinguishes between polar (γ^p) and disperse or nonpolar (γ^d) components of the surface energy. Literature sources report surface tension values for water and diiodomethane as 72.8 and 50.8dyne/cm at 20ºC, respectively.[29] Moreover, polar and disperse parts for water were considered to be 51.0dyne/cm and 21.8dyne/cm and for diiodomethane 0.0dyne/cm and 50.8dyne/cm, respectively.[29]

Water adhesion tension was determined by multiplying the contact angle (ϑ) of water on the surfaces by the surface tension (γ_1) of water (72.8mN/m).[29] Thus, both the adhesion tension of water and surface energy determinations were based on the sessile drop method.

2.4.3. X-Ray Photoelectron Spectroscopy (XPS)

X-Ray Photoelectron Spectroscopy (XPS) measurements were performed before and following plasma treatment in order to determine the surface composition of the PDLLA films. Measurements were taken at 5 different points on each surface. These experiments were carried out using a Kratos Axis-Ultra (Kratos Analytical Inc.UK) with monochromatic Al x-ray source. The x-ray energy was 1486.6eV and the base pressure was approximately 2.9 x 10^{-11}Pa.

2.4.4. Fourier Transform Infra Red Spectroscopy (FTIR)

FTIR spectra were obtained in the Attenuated Total Reflection (ATR) mode using a Nicolet Spectrometer (Nicolet Instrument Coorporation, USA). Each spectrum was recorded with a total of 32 scans and 4.0 cm^{-1} resolution after 20 sec of vacuum chamber stabilization. Original and treated surfaces were analyzed in triplicates in the range 400 cm^{-1} to 4000 cm^{-1}.

2.4.5. Scanning Electron Microscopy (SEM)

Samples were sputter coated (Med-010 Sputter Coater by Balzers-Union, USA) with a thin Au-Pd layer and examination was performed using a scanning electron microscope (Leica, UK). Triplicates were prepared for all control and plasma treated PDLLA surfaces.

2.5. Protein Adsorption Assay

Two different protein adsorption studies were performed. In both cases, glass coverslips were used as control surfaces. Single and complex protein solutions were prepared for incubation with PDLLA surfaces before and after plasma treatment with the goal to assess the effect on individual molecules and to mimic more complex protein environments.

For the first protein adsorption study the following solutions were prepared: 1000μg/mL of bovine serum albumin (BSA), 100μg/mL of fibronectin (FN), 0.7μg/mL of vitronectin (VN) and 1000μg/mL of fetal bovine serum (FBS). Proteins and controls were individually incubated with characterized samples for 15min at 37ºC as described elsewhere.[30] Protein adsorption for BSA and FN was assessed by coupling a depletion method with a protein assay as follows: after incubation, the amount of non-adsorbed protein in the solution was quantified using the Micro-BCA assay and reading visible emission (570nm) in a Micro Plate Reader (BIO-RAD, USA). Glass coverslips were used as control surfaces and control polymer surfaces were prepared using incubation in saline solution. Unintended protein loss from other sources was controlled by means of using positive displacement pipettes, capillaries and pistons purchased from Gilson Medical Electronics S. A. (France).

In a second set of experiments, protein solutions were prepared at 1% of the concentration of those proteins in human blood plasma:[18] 350μg/mL of BSA, 4μg/mL of FN, 3μg/mL of VN and 1% (V/V) of FBS. As previously stated, protein incubation followed a method described elsewhere.[30]

2.6. Attachment and Proliferation of FRC and MG63 Cells

Cell seeding was performed immediately after the protein adsorption step in order to avoid surface drying and consequent protein conformational changes or denaturation. Surface rinsing was not performed and any enrichment of the cell culture media that could result from remaining non-adherent proteins were assumed negligible.

2.6.1. Primary Cell Culture

Fetal Rat Calvaria (FRC) cells were isolated by sequential enzyme digestions from calvaria of 21 days Sprague-Dawley rat fetuses as described elsewhere.[31] Briefly, calvaria (frontal and parietal bones) were aseptically removed and stripped of the periosteum. The minced fragments underwent 9 sequential digestions in fresh 0.2% collagenase/0.05% trypsin in Hank's Balanced Salt Solution (HBSS) for 20min at 37ºC. Cells were resuspended in α-MEM enriched with 10% FBS, plated in T-75

falcon tissue culture flasks and incubated at 37ºC in 5%CO_2 until confluent. Adherent cells were considered viable. For long culture periods, cells were trypsinized and grown as described by Bellows and co-workers.[32] Populations II–V were seeded at 4 x 10^4 cells/mL in α–MEM containing 1 mg/mL of β-glycerophosphate, 0.05mg/mL of L-ascorbic acid, and 10% (V/V) FBS onto the different surfaces: PDLLA, plasma treated PDLLA and the same surface batches after carrying out the first protein adsorption procedure described above at: 1000μg/mL BSA, 100μg/mL FN, 0.7μg/mL VN and 1000μg/mL of FBS. Incubation was performed for 3 hours, and 7, 9, and 14 days. Attachment (measured at the 3h time point) and proliferation (assessed with the 7, 9 and 14 day time points) measurements were conducted by trypsinization of the cultures and then cell counting using a Coulter Zi Dual cell counter (Coulter Corporation, USA). Appropriate controls were used, including tissue culture polystyrene (TCPS).

2.6.2. Cell Line culture

Cell response was also studied using human MG63 osteoblast-like osteosarcoma cells (American Type Culture Collection, Rockville, MD) that have been well characterized in the literature and provide a good model for the study of human bone cells. Cells were incubated for 1, 4 and 7 days in DMEM containing 10% FBS and 1% penicillin-streptomycin mixture. In this study, the plasma protein environment was simulated by preparing protein solutions at 1% of their concentration in human blood plasma: 350μg/mL of BSA, 4μg/mL of FN, 3μg/mL of VN and 1% (V/V) of FBS.[18] The films were seeded at 4 x 10^4 cells/mL and cell proliferation was assessed by WST-1 assay. After each incubation period, cultured samples were transferred to new wells with fresh media and analyzed for mitochondrial activity using the colorimetric WST-1 tetrazolium conversion assay (TAKARA, Japan). Briefly, 10μL of WST-1 reagent was added per well, and the cells were incubated for an additional 2h. The absorbance of the WST-1-containing cell supernatant was determined at 450nm (Benchmark Microplate Reader, Bio-Rad, USA). To avoid interference from both cell culture media and PDLLA surfaces, the following controls were prepared and considered as blank samples: fresh media and PDLLA samples immersed in fresh media but with no cells seeded.

2.7. Statistical Analysis

Results of the tests were tabulated as mean ± SD. The effects of plasma treatment on both the surface parameters and cell density values were statistically analyzed by two-tail Students *t*-test and differences were considered significant at $p<0.05$.

3. RESULTS

3.1. Characterization of PDLLA and Plasma Treated PDLLA Films

The effect of oxygen based RfGD treatment on PDLLA films was assessed by several surface analytical techniques: water contact angle, surface energy, adhesion tension of water, SEM, XPS and FTIR-ATR.

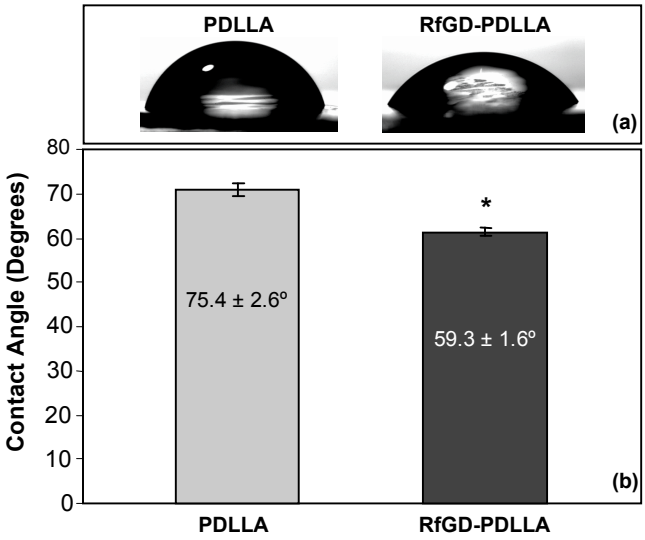

Figure 1. Water drop profiles (a) and contact angle measurements (b) for PDLLA and plasma treated PDLLA films. *Statistically different from PDLLA (t-Test; Bi-tail; $p<0.05$; n>9).

In Figure 1, surface wettability obtained by the sessile drop method is presented as a function of plasma treatment. Contact angle measurements are frequently used because of their sensitivity and because they allow for studying surface changes within the top few atomic layers.[33] As seen in Figure 1a, the characteristic water drop profiles show lower spreading for the control PDLLA surfaces when compared to plasma treated surfaces. As a consequence of the oxygen-based plasma surface modification, contact angles decreased from 75.4 ± 2.6º to 59.3 ± 1.6º (Figure 1b), which comprises a hysteresis of 6º. Thus the results indicate an increase in the hydrophilicity of the RfGD-PDLLA surface ($p<0.05$). Contact angle measurements were also performed after UV sterilization on both treated and control samples because UV radiation is a physic-chemical modification methodology frequently used for photo-activation of polymers and could possibly alter the surface properties.[4] However, measurements of contact angles obtained before and after sterilization by UV do not reflect any statistical differences between PDLLA and RfGD-PDLLA (data not shown).

Table 1. Adhesion tension of water and surface energy measurements for non-treated and RfGD-PDLLA.

	Surface Energy (dyne/cm)	Adhesion Tension (mN/m)
PDLLA	42.7 ± 2.3	18.3 ± 3.2
RfGD-PDLLA	50.0 ± 1.8 *	37.2 ± 1.8 *

*Statistically different from PDLLA (t-Test; Bi-tail; $p<0.05$; n>9).

The results for surface energy and adhesion tension of water are presented in Table 1. The determination of surface energy was based on the Owens and Went method[28] and performed using water and diiodomethane test liquids. In parallel, adhesion tension was also calculated as an alternative indicator of surface energy. RfGD-PDLLA films, when compared to control PDLLA films, showed significantly higher surface energy and water adhesion tension values. PDLLA surface energy significantly increased from 42.7 ± 2.3dyne/cm to 50.0 ± 1.8dyne/cm; the adhesion tension of water increased approximately 19mN/m (from 18.3 ± 3.2mN/m to 37.2 ± 1.8mN/m). These results suggest the incorporation of polar groups as a consequence of oxygen plasma surface treatment.

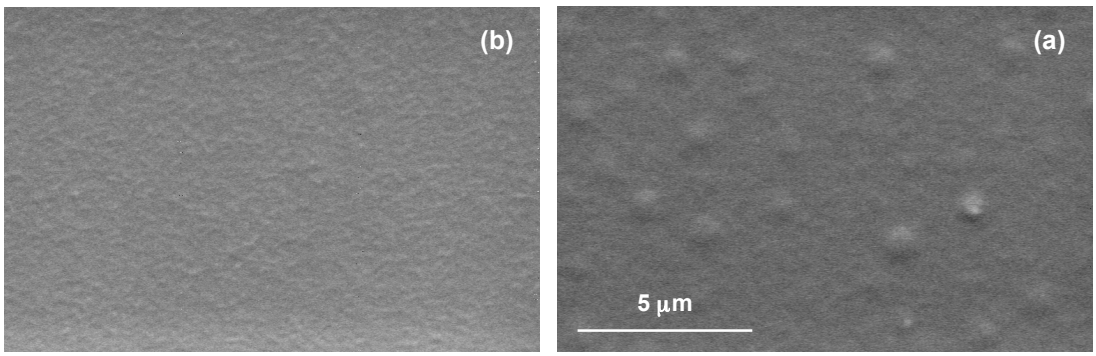

Figure 2. SEM characterization of surface morphology of PDLLA films: (a) before treatment; (b) after plasma treatment.

SEM was used as a qualitative tool to detect any changes in morphology introduced by the plasma treatment (Figure 2). The control surface (non-treated PDLLA) exhibited a uniform texture (Figure 2a), while treated surfaces presented small and irregularly distributed protrusions of less than 1 μm diameter. Although these features were sparsely distributed, it is clear that oxygen plasma treatment can modify the microtopography of PDLLA films.

XPS was used for more detailed chemical analysis of the surface.[34] During the RfGD treatment the active plasma species attack the polymer surface resulting in the incorporation of additional carbonyl, carboxyl or hydroxyl functional groups.[35-38] X-ray photoelectron spectroscopy retrieves information on the nanometer scale, more specifically with up to a depth close to 50 Å.[33] XPS analysis results for treated and non-treated PDLLA films are shown in Table 2. Following plasma surface modification there was a significant increase in the C-O-O bonds while the C-H and C=O functional groups

decreased (p<0.05). Also the total oxygen content exhibited a 4% increase, in contrast to carbon content, which decreased approximately 4%.

Table 2. XPS analysis data: atomic percentages of elements and functional groups for the untreated and oxygen RfGD modified PDLLA.

	O1 (%)	O2 (%)	N 1s (%)	C-H (%)	C=O (%)	C-O-O (%)	Total O (%)	Total C (%)
PDLLA	14.4±0.2	17.4±0.3	1.2±0.1	23.9±0.5	23.2±0.5	19.9±0.1	31.8±0.5	67.0±1.1
RfGD-PDLLA	18.7±0.5 *	17.0±0.3	1.1±0.1	20.7±0.2 *	20.6±0.1 *	21.7±0.4 *	35.8±0.8 *	63.1±0.7 *

*Statistically different from PDLLA (t-Test; Bi-tail; p<0.05; n>3).

In this study FTIR-ATR was performed on non-treated and plasma-treated PDLLA films to detect any changes in chemical composition. Analysis of the spectra did not suggest treatment-driven chemical changes either in the aliphatic, carbonyl or asymmetric stretching regions[39,40] (data not shown). This may be because in contrast to surface sensitive methodologies such as contact angle or XPS, the FTIR-ATR collects spectra from a depth up to 100nm below the surface.[33] Thus changes limited to the top few atomic layers (as detected by XPS) may become indistinguishable.

3.2. Protein Adsorption onto PDLLA and RfGD Treated PDLLA

Several surface properties can influence protein adsorption, such as morphology, chemistry and hydrophobicity.[41] In this study, the potential of oxygen-based RfGD treatment to increase protein adsorption onto PDLLA was assessed. The degree of protein adsorption was carried out using an indirect method by coupling a solution depletion technique and a colorimetric protein (BCA) assay as described earlier. As shown in Figure 3, after RfGD treatment PDLLA surfaces adsorbed a significantly higher percentage of BSA and FN. The adsorption increased approximately 6% and 15% for albumin and fibronectin, respectively, which corresponds to 5.83 μg/mL of BSA and 15.3 μg/mL of FN. In contrast, FBS did not show any preference for either surface. The results for VN were inconclusive.

3.3. Behavior of Bone-related Cells on PDLLA Films

The attachment and proliferation of FRC cells onto RfGD modified PDLLA was compared to the control TCPS and non-treated PDLLA surfaces. Cells were seeded on the surfaces for 3 hours, and 7, 9, and 14 days followed by trypsinization and subsequent cell counting. Figure 4 show the results of cell numbers for the different post-seeding time points. The results indicate that the number of FRC cells on PDLLA was similar to the one observed for plasma modified surfaces. In contrast, BSA and

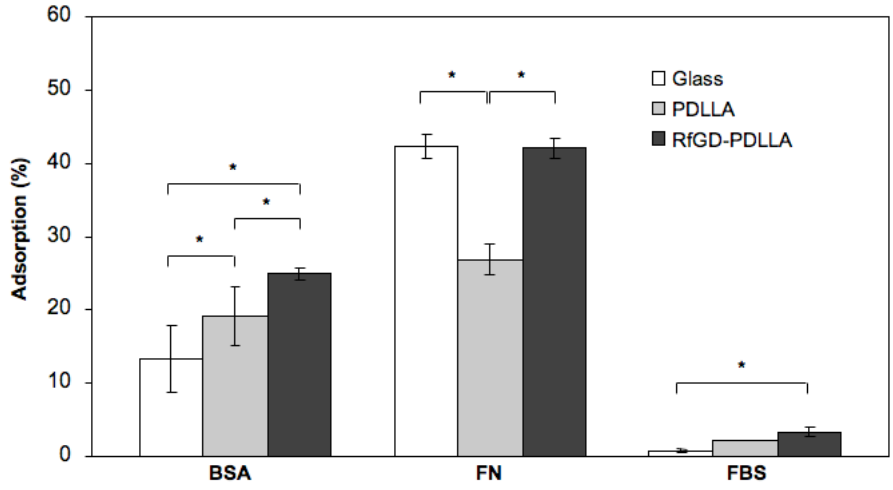

Figure 3. Percentage of adsorbed proteins, BSA, FN and FBS on PDLLA non-treated and treated by oxygen based RfGD. *Statistically different from PDLLA (t-Test; Bi-tail; p<0.05; n>3).

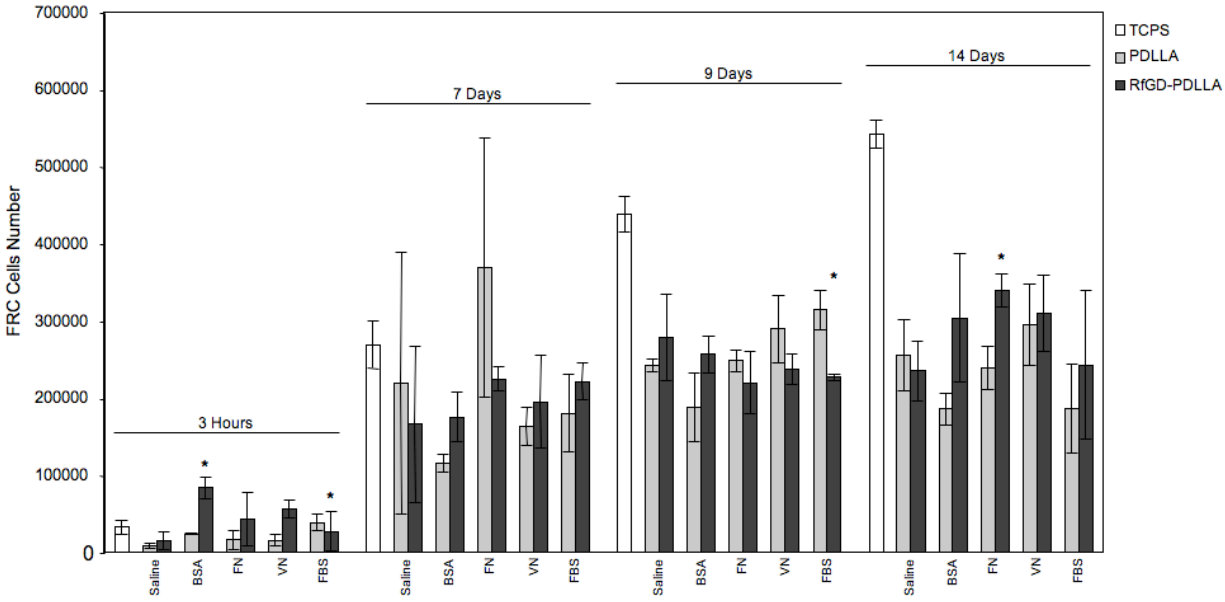

Figure 4. FRC cells attachment and proliferation after 3 hours, 7, 9 and 14 days of culture on PDLLA and RfGD treated PDLLA films, previously incubated with different protein systems. *Statistically different from PDLLA (t-Test; Bi-tail; p<0.05; n>3).

VN had a positive influence on FRC cell attachment to gas plasma treated films as shown by the significantly higher cell numbers at 3 hours (Figure 4). This can be attributed to the combination of plasma treatment and pre-incubation of the films with proteins. By day 14, however, the RfGD treated films with pre-incubated with FN showed a higher average cell number compared to the non-treated PDLLA. Thus, although BSA and VN appeared to cause an increase in cell attachment this effect did

not translate into an advantage by day 14. Furthermore, cell proliferation was similar at days 7 to 14 in both controls and protein-exposed surfaces. Interestingly, this is the time when an increase in alkaline phosphatase expression has been reported previously.[42] Studies on gene expression and cell differentiation could be helpful for further understanding of the observed cell behavior.

In the study with MG63 osteoblast-like cells, the relative proportions of BSA, FN and VN found in human blood were used and FBS was diluted to 1%. WST-1 mitochondria assay was performed for measuring the viability of MG63 cells after 1, 4 and 7 days of culture, taking into account intact mitochondrial mechanisms, consistent cellular activation and similar extracellular matrix interactions.[43]

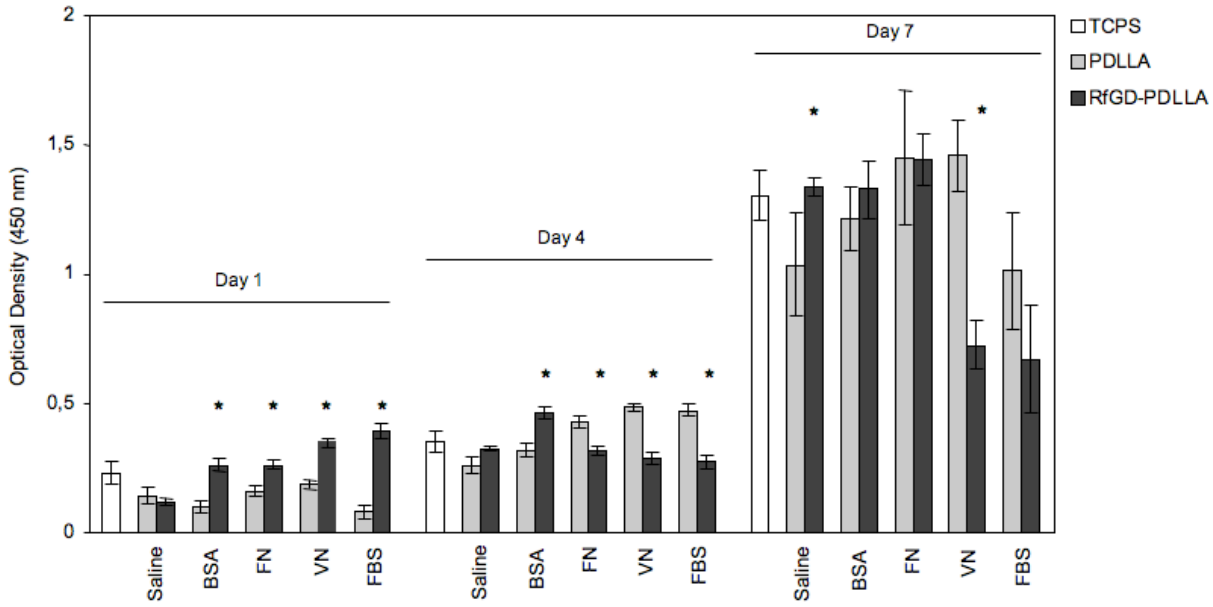

Figure 5. MG63 cells density for 1, 4 and 7 days of culture on control PDLLA and RfGD treated PDLLA pre-coated with proteins. *Statistically different from PDLLA (t-Test; Bi-tail; $p<0.05$; n>3).

Cell attachment and proliferation results for MG63 cells are shown in Figure 5. The test films performed as well as the culture standard TCPS. At day 1, there is an increase in cell attachment on RfGD treated PDLLA films pre-coated with BSA, FN, VN, FBS. However, by day 4 these trends were reversed except for BSA. By day 7 there was significant difference in cell numbers between the treated and non-treated PDLLA films as for the case of VN where the non-treated surfaces showed more cells. For days 4 and 7 of culture, there was a reversal in the relative density (OD) for treated and non-treated PDLLA films with pre-adsorbed VN and FBS compared to day 1. Also, as can be observed in Figure 5, only gas plasma treatment followed by saline pretreatment increased MG63 cell attachment by day 7.

4. DISCUSSION

The herein presented study aimed at assessing the influence of oxygen radio frequency glow discharge on the surface and bulk properties of Poly(D,L-lactic acid) and the effect of this surface modification on both biomolecules adsorption and bone cell behavior. The main purpose of these experiments was to determine whether oxygen-based RfGD could improve the adsorption of various proteins, as BSA, FN, VN and complex protein solutions as FBS and ultimately model cell attachment and proliferation of MG63 and FRC cells.

The surfaces were characterized by evaluating different key parameters such as the surface contact angle, surface energy and adhesion tension of the water. Oxygen based RfGD treatment on PDLLA films was shown to significantly decrease surface contact angle to approximately 60º (Figure 1b), thus indicating an increase in the hydrophilicity of the RfGD-PDLLA surface. Furthermore, surface energy and the adhesion tension of water significantly increased to approximately 50.0dyne/cm and 37mN/m, respectively. These results suggest the incorporation of polar groups as a consequence of oxygen plasma surface treatment. Multiple studies in the past have shown that oxygen plasma exposure can render higher hydrophilicity on polymer surfaces.[36,37] The changes in wettability are generally due to effects of oxidation, unsaturation, electrostatic charges or surface morphology.[35,38] Thus, in this study we used a variety of techniques to further interrogate the test PDLLA surfaces. SEM analysis of PDLLA films indicated that RfGD introduced some surface morphological changes. More precisely, changes in microtopography were evidenced by the increase in surface roughness. This factor has been related to the improve of hydrophilicity since surface micro-features are known to affect wettability.[3] Chemical changes showed an increase in total oxygen atomic percentage ascribed to an increase in the oxygen-containing functional group C-O-O. The FTIR analysis, compared to the XPS results, suggests that the effects of the oxygen plasma surface modification were limited to the surface and did not affect the bulk properties of the material.

The potential of surface properties in influencing protein adsorption, such as morphology, chemistry and hydrophobicity was been evaluated.[41] In this study, the capacity of oxygen-based RfGD treatment to increase protein adsorption onto PDLLA was assessed. The degree of protein adsorption was carried out using an indirect method by coupling a solution depletion technique and a colorimetric protein (BCA) assay as described earlier. The amounts of adsorbed proteins in single systems (BSA, FN) were shown to increase after plasma treatment ($p<0.05$), which modified PDLLA wettability, surface energy and adhesion tension of water as discussed earlier (see Figure 1 and Table 1). The relation between protein adsorption and contact angle or surface energy is controversial in the literature; it has been shown that decreasing surface hydrophilicity leads to lower protein adsorption[44] and, in contrast, it has also been reported that increasing surface energy leads to lower adsorption.[45] It must be stated that in this study, RfGD of PDLLA films did not result in extremely high or low contact angle or surface energy. According to Ikada,[46] intermediate values seem to be the most favorable for

cell adhesion. The increase in O-containing functional group C-O-O and the increase in total O% of treated over non-treated samples as determined by XPS may promote protein-surface interactions. The introduction of oxygen functionalities creates sites for binding proteins by polar interactions or hydrogen bonding. Also, the simultaneous increase in surface micro-heterogeneity may play a role in the increased protein adsorption. However, at the same time, the degree of protein adsorption from multi-protein solutions was not affected by the change in surface properties. This could perhaps be explained by protein competition and the resulting protein conformations taking place on the surface. These factors could be further explored using other approaches, such as extrinsic fluorescent probes.

MG63 osteoblast-like cells and primary cultures of fetal rat calvarial (FRC) cells were used to assess both the effect of RfGD treatment and protein adsorption on cell attachment and proliferation.

In the absence of previously adsorbed proteins, neither the attachment of MG63 nor that of FRC cells showed significant changes resulting from the treatment. Also, the proliferation of FRC cells up to 14 days was not affected by the RfGD treatment. In contrast, proliferative rates of MG63 osteoblast-like cells were higher for plasma treated PDLLA surfaces, showing a direct effect of the oxygen based plasma technique.

5. CONCLUSION

The surface modification technique selected for this study, oxygen-based RfGD treatment, was shown to functionalize/activate the surface of PDLLA films without affecting the bulk properties. After treatment under the described conditions, PDLLA films exhibited presented increased wettability, surface energy and water adhesion tension. Chemically, changes showed an increase in total oxygen atomic percentage ascribed to an increase in the oxygen-containing functional group C-O-O. Moreover, RfGD introduced some surface morphological changes. Regarding protein adsorption studies, oxygen gas plasma treatment of PDLLA films was shown to improve BSA and FN adsorption from single protein solutions. On the other hand, adsorption from complex protein solutions (e.g. FBS) was unaffected by the material treatment. Thus, oxygen RfGD treatment resulted in PDLLA surfaces with preferred adsorption characteristics.

In the absence of pre-adsorbed proteins, neither MG63 nor FRC cells could distinguish between treated and untreated surfaces. However, MG63 osteoblast-like cells showed higher proliferation rates for plasma treated PDLLA surfaces, indicating a direct effect of the oxygen based plasma technique. In turn, gas plasma treatment, by influencing protein adsorption on the surfaces, was shown to affect cell response to the surfaces. The effect on the treatment over the cultured cells was only observed by combining gas plasma modification of the surface with the protein adsorption, thus indicating a crucial role for adsorbed proteins in mediating the response of osteogenic cells to the RfGD-treated PDLLA surface.

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CHAPTER VI

THE COMPETITIVE ADSORPTION OF HUMAN PROTEINS ONTO NATURAL-BASED BIOMATERIALS

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THE COMPETITIVE ADSORPTION OF HUMAN PROTEINS ONTO NATURAL-BASED BIOMATERIALS*

Alves, CM[1,2], Reis, RL[1,2], Hunt, JA[3]

[1] 3B´s Research Group – Biomaterials, Biodegradables and Biomimetics, Dept. of Polymer Engineering, University of Minho, Campus Gualtar, 4710-057 Braga, Portugal

[2] IBB – Institute for Biotechnology and Bioengineering, PT Government Associated Laboratory, Braga, Portugal

[3] Clinical Engineering, UKCTE, University of Liverpool, P.O. Box 147, Liverpool L69 3QA, United Kingdom

Abstract

The correlation between the surface characteristics and protein adsorption from unitary and complex protein systems was investigated with respect to altering the bulk chemistry of the substrate material. Starch-based materials were the biomaterials used as interfaces to analyze the *in vitro* protein adsorption isotherms. To further investigate protein-surface interactions, isotherm data were treated according to Langmuir and Freundlich models. Distribution, quantification and competition profiles of Human Serum Albumin (HSA) and Human Fibronectin (HFN) were measured by fluorescence and visualised by Laser Scanning Confocal Microscopy (LSCM). The adsorption isotherms for albumin fit the Freundlich model. The analysis of unitary systems demonstrated that the adsorption of HSA was increased for SCA and SPCL surfaces (18% and 24%, respectively). The adsorption of fibronectin was higher onto SEVA-C and SPCL corresponding to 89% and 97% of adsorption. In studying the coadsorption of proteins, an increase of both HSA and HFN adsorption for SEVA-C surfaces was observed. On the SCA surfaces, the presence of HFN decreased in binary conditions while an increase in HSA adsorption occurred in comparison to the unitary systems. In contrast, SPCL showed no substantial increase in the adsorption of the proteins in competitive conditions.

Keywords

Protein adsorption, isotherms, competition, albumin, fibronectin, biodegradable materials.

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C. M. Alves, R. L. Reis, J. A. Hunt, "The competitive adsorption of human proteins onto natural-based biomaterials", Biomacromolecules, 2007, *submitted*

1. INTRODUCTION

Analyzing the interaction of proteins with the surfaces of materials intended for biomedical applications is fundamental for understanding cellular events and the overall host response. Furthermore, the importance of a variety of molecules in the biomedical field is known for several applications, including drug delivery, biomaterials, extracorporeal therapy and solid phase diagnostics.[1,2] To improve the understanding of the fundamentals of protein adsorption, many protein adsorption-modelling approaches have been successfully utilized.[3,4] Most approaches treat the electrostatic and van der Waals interactions between the protein and the surface, and thus consider the effects of surface charge, protein size, or solution ionic strength.[5] Although the correlation between the adsorbed protein and bulk concentration of protein solution has been dealt with using many adsorption isotherm equations, the Langmuir equation, however, has been frequently applied due to its lower complexity and broader applicability to various adsorption data.[4] The Langmuir isotherm performs a dynamic adsorption process with the reciprocation between adsorbed and unabsorbed BSA molecules, but disregards the interaction between these adsorbed molecules. On the contrary, the Freundlich isotherm can validate many adsorption processes facilitating the modelling of one or more interactions between adsorbed molecules or adsorbed molecules and the surfaces involved in the dynamic process.[6] The adsorption of two factors from the human blood matrix,[7] fibronectin and albumin, onto the surface of polymeric blends was studied. These proteins were chosen on the basis of their biological effect and importance in a variety of biomedical applications. Furthermore, they represent two different types of proteins, small globular proteins (albumin) and large glycoproteins (fibronectin).[8, 9] Human Fibronectin (HFN) is known to present RGD and PHSRN sequences with synergistic action responsible for substantial modulation of the biological activity subsequent to protein adsorption processes.[9] Fibronectin has been considered for some time to facilitate and precede cell attachment to artificial and natural surfaces when adsorbed in a favourable conformation,[10] while Human Serum Albumin (HSA) was selected considering its high concentration in the human blood plasma.[7] It is therefore likely that the results obtained for HSA and HFN will be representative of a large number of serum proteins. Moreover, authors have reported the ability of albumin, to “rescue” fibronectin molecules influencing it’s molecular conformation on hydrophobic surfaces[11] which was also reported to facilitate cell adhesion dependent processes.[12]

Starch-based Biomaterials (SBB) have been increasingly studied for applications in the field of Tissue Engineering, including their use as scaffolds for bone related applications[13] and drug delivery systems.[14] The starch based materials selected are known to present different properties in terms of bulk chemistry, surface energy, wettability and phase interconnectivity.[15-18] As part of previous research their *in vitro*[19] and *in vivo* biological response[20] has been assessed. In these studies the effect of the adsorption of different molecules onto the surface of starch based materials was evaluated and the behaviour of immunological cells[16] and osteoblast-like cells[17] was investigated as a function of the

pre-adsorbed proteins. Although *in vitro* protein adsorption and competition studies were previously conducted,[16] the adsorption behaviour of biomolecules relevant for the cell-biomaterial interactions has yet to be understood regarding SBB materials. The present work aimed to determine further the relationship between substrate properties and protein competition using Fluorimetry and Laser Scanning Confocal Microscopy (LSCM) in both single and competitive protein adsorption studies. To further understand the observed *in vitro* and *in vivo* cell behaviour, the direct assessment of the adsorptive potential of HSA and HFN onto starch-based biomaterials was evaluated using single molecule solutions. Subsequently, the competitive effectiveness of fibronectin against albumin was studied using binary mixtures that mimic the proportional amounts of both proteins in the human blood plasma.

2. MATERIALS AND METHODS

2.1. Materials and Chemicals

Bovine Serum Albumin (BSA)-Alexa Fluor® 488 conjugates were obtained from Molecular Probes (The Netherlands) and purified Human Serum Albumin (HSA) and Human Fibronectin (HFN) were purchased from Sigma-Aldrich (UK). Alexa Fluor® 488 and Alexa Fluor® 555 Protein Labelling Kits (Molecular Probes, The Netherlands) were used to fluorescently label proteins of human origin. The labelling procedure was conducted following the manufacturer's instructions, using Bio-Rad BioGel P-30 fine size exclusion purification resin to separate uncoupled dye from labelled proteins. All aqueous solutions were prepared using purified deionised water and Phosphate Buffered Saline (PBS, Sigma-Aldrich, UK). Clear-bottomed black 24-well plates were used for fluorescence measurements (Biosera, UK) and Tissue Culture Polystyrene Coverslips (TCPS) were used as the control surfaces (Sarstedt, UK).

2.2. Starch Based Biomaterials (SBB)

Different natural-based polymers were investigated. Biodegradable polymeric blends of corn starch with: (i) cellulose acetate (SCA), (ii) ethylene vinyl alcohol copolymer (SEVA-C) and (iii) polycaprolactone (SPCL). The amount of starch was 50% by weight (wt%) on SCA and SEVA-C and 30% wt. on SPCL. By means of using a conventional injection moulding technology samples were processed into 10 mm circular discs. Samples were sterilized by ethylene oxide in optimised conditions,[21] washed, and all subsequent experimental procedures were performed under sterile conditions.

2.3. Protein Adsorption Studies

The physiological proportion of albumin and fibronectin in the human serum was considered for the preparation of the different solutions and used in the present study according to literature data: 35 mg/mL of albumin and 0,4 mg/mL of fibronectin.[7] Accounting for the relative amounts of these biomolecules in the human body provided for the data to be related to the analysis of the *in vivo* biological response to implanted surfaces.

A commercially available BSA-Alexa Fluor 488 conjugate was used as the model of adsorption studies and also to control the experiments with HSA and HFN labelled according to manufacturers specifications. In brief, HSA and HFN protein solutions at a concentration of 2mg/mL in standard phosphate buffer saline were mixed with 50μL of sodium bicarbonate solution and allowed to react with Alexa-Fluor dye for 1h at room temperature. Subsequently, labelled proteins were separated from unincorporated dye using fine size exclusion chromatography. The concentration and degree of labelling (moles of dye per mole protein) were calculated following supplier indications. UV-visible spectra of protein-dye conjugates (Bio-Tek Instruments, KC4™ Data Analysis Software, USA) were used and the degree of labelling was calculated: 4.0 for HSA and 2.9 for HFN. Labelled proteins were stored at 4ºC and used within 1 week of preparation.

Probe-labelled proteins were used to study the surface concentration and protein distribution by fluorimetry and LSCM. For all studies, Human Serum Albumin was labelled with Alexa Fluor 488 and Human Fibronectin was labelled with Alexa Fluor 555, except for confocal image analysis of HFN adsorption from unitary protein solutions, which was performed using Alexa Fluor 488 labelling.

2.3.1 Study of Unitary Protein Systems

BSA was used for kinetic and isotherm studies. Adsorption studies were performed for the surface of starch-based biomaterials using commercially available BSA-Alexa 488 conjugates, which was also utilised as the control for the experiments using proteins of human origin. The following solutions of BSA conjugates were prepared: 35, 70, 105, 140 and 175μg/mL (pH 7.4), which corresponded to 0.1, 0.2, 0.3, 0.4 and 0.5% (W/V) of albumin concentration in blood.[7]

Human protein adsorption onto the surface of SBB surfaces was studied using purified HSA labelled with Alexa Fluor 488 and HFN labelled with Alexa Fluor 555. HSA-probe conjugates were studied at 2 different concentrations, 35 and 70μg/mL, while HFN-Alexa Fluor 555 solutions were prepared at 0.4 and 0.8μg/mL in PBS solution.

Saline solution was used as the control. Proteins and controls were incubated with samples for different time periods: 15, 30, 60, 120, 180, 240 and 420 minutes at 37ºC. Experiments were performed at least 3 times under the same conditions and in triplicate (n>9).

2.3.2 Protein Competition: study of binary protein systems

Competition of proteins for the different starch-based surfaces was analyzed using two-protein solutions: HSA and HFN fluorescently labelled with Alexa Fluor 488 and Alexa Fluor 555, respectively. Labelling and characterization of the conjugates was accomplished as previously described. A mixture of 70μg/mL of HSA conjugate and 0.8μg/mL of HFN conjugated with Alexa Fluor probes was used. The described solution corresponds to 0.2% (W/V) of these molecules in the human blood serum.

Saline solution was used as the control. Proteins and controls were incubated with samples for different time periods: 15, 30, 60, 120, 180, 240 and 420 minutes at 37ºC. As for unitary protein systems, binary protein adsorption studies were repeated and triplicates were prepared (n>9).

2.4. Fluorimetry

Samples were incubated as described for the analysis of protein adsorption. For each time point, fluorescence measurements were taken according to the conjugate spectroscopic properties using a Microplate Fluorescence Reader (Version FLx800T, Bio-Tek Instruments, USA). Measurements were automated for top probe detection, using one or two filter sets (488 and 555nm) to assess labelled biomolecule kinetics and competitive adsorption. Acquired data was analyzed using KCJunior Software (Version 1.31.5, Bio-Tek Instruments, USA).

Fluorimetry results were calibrated by assessing other contributions to the total fluorescence intensity. Background fluorescence was subtracted and the emission of SBB surfaces at the different wavelengths studied was analyzed. Briefly, the different surfaces were incubated in protein-free PBS solution and the fluorescence intensities measured over time were corrected to rule out artifactual effects, such as normal absorption interferences from the polymer materials.

The data of protein adsorption was presented as arithmetic means/standard deviations of the mean (mean/SD). Standard curves were prepared for the different protein types and for each time point, fluorescence was converted in protein concentration.

2.5. Laser Scanning Confocal Microscopy (LSCM)

A Confocal Laser Scanning Microscope (Version LSM 510 Zeiss, UK) was used to visualize the fluorescently labelled proteins adsorbed on the different starch based materials. Alexa 488 probe was used in single protein studies to label HSA and HFN molecules. For the study of binary protein systems, HSA and HFN were labelled with Alexa 488 and Alexa 555 probes, respectively. An argon laser (λ=488 nm) and a HeNe laser (λ=543 nm) provided the excitation of the protein-probe conjugates. Image analysis was performed using KS400 image analysis software (Imaging Associates, UK).

3. RESULTS AND DISCUSSION

3.1. BSA Adsorption

The interaction of proteins from single systems with different surfaces was determined. SCA and SPCL profiles (Figure 1A and B) reflect a fast burst of adsorption over the initial 60 min of incubation after which a pseudo-steady level is maintained. This could indicate that the adsorption can reach equilibrium within 60 min. In contrast, the shape of the kinetic curves indicated a slower adsorption of this molecule onto the SEVA-C surfaces (Figure 1C).

The different shapes of the adsorption curves could be related to the heterogeneity that characterises the different polymeric blends. The heterogeneity of the materials is known to vary in the order SCA>SPCL>SEVA-C as a result of the increasing miscibility and interaction between the synthetic and natural polymeric phases.[16] SCA is a non-miscible blend, while SPCL presents some interaction between the different phases. SEVA-C consists of the most homogeneous mixture presenting inter-penetrating networks (INP) that could unease the reorientation of the polymer chains at the liquid interface, and thus slower the adaptation of the outermost region of the materials to the environment. This effect could explain the curve shape observed for SEVA-C characterized by a smoother slope than that of SCA and SPCL.

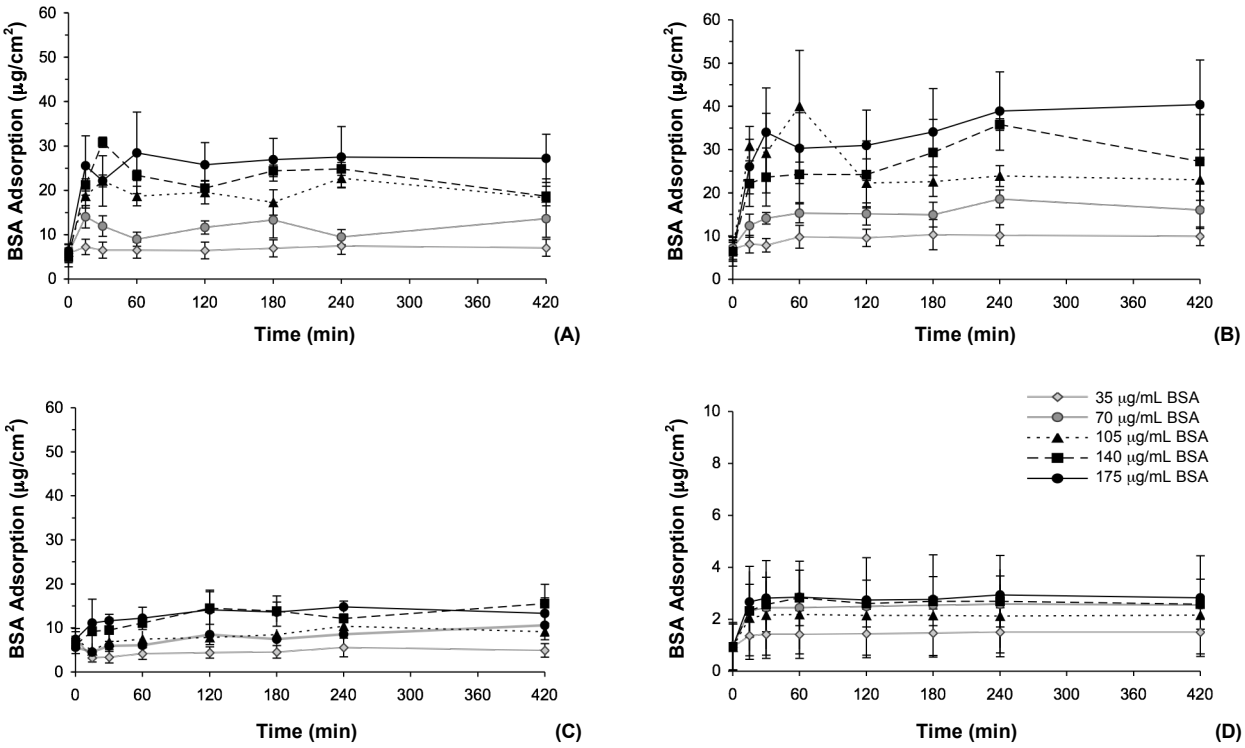

Figure 1. BSA adsorption onto SCA (A), SPCL (B), SEVA-C (C) and TCPS (D) surfaces. The different samples were incubated for 7 hours in solutions of BSA ranging from 35µg/mL to 175µg/mL.

The analysis of Figure 1 indicated that BSA adsorption per unit area was higher for SCA and SPCL polymeric blends than that of SEVA-C surfaces. After 7 hours, SCA and SPCL adsorbed 27μg/cm^2 and 40μg/cm^2 of protein from the more concentrated solution of BSA-Alexa 455 (175 μg/mL), and SEVA-C adsorbed 13μg/cm^2. Adsorption onto TCPS was substantially reduced when compared to the SBB surfaces.

The amounts of BSA adsorbed onto SCA, SPCL and TCPS at 60 min presented no obvious difference from that at 420min. Figure 2 shows the adsorption isotherms calculated from the data obtained at 420min. The results indicated that the fractional coverage was strongly dependent on the bulk concentration of proteins; the adsorbed amount gradually increased with the protein solution concentration. The adsorption isotherms (Figure 2) further indicated the affinity of BSA for the different surfaces; SPCL and SCA demonstrated the highest adsorption for increasing concentrations of BSA solutions. After 7 hours of incubation with solutions of 35 and 175μg/mL of BSA, adsorption onto SCA and SPCL increased respectively 20 and 30μg/cm^2 while for SEVA-C this variation was around 8μg/cm^2.

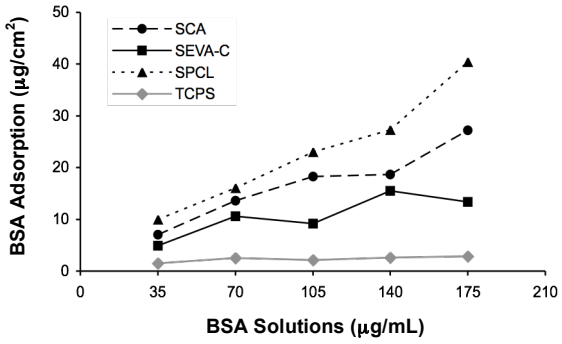

Figure 2. Isotherms of BSA adsorbed onto the different starch based polymeric blends (equilibration time 7 hours).

In previous studies performed to characterize the different polymeric blends, both wettability and surface energy were analysed. Contact angles were shown to progressively decline with decreasing content of OH groups, indicating SCA < SPCL < SEVA-C ranking in terms of hydrophobicity; with contact angles of 76.4±3.3º, 78.80±1.7º and 80.0±2.6º respectively.[17] The contact angle variation between the different materials was low for all surfaces and values were above 65º, which is considered to be the theoretical limit between hydrophobic and hydrophilic properties.[22] Moreover, SEVA-C presented the lowest value for surface energy that reached 36.2±1.8 Dyn/cm in opposition to SCA and SPCL that showed 43.0±2.7 and 46.9±1.0Dyn/cm, correspondingly.[17] Considering previous results it can be stated that the higher the surface energy of the materials, the greater the level of albumin adsorption.

Previous research is contradictory concerning the effect of wettability on the adsorption of different molecules. Some authors reported an increased protein adsorption onto hydrophilic substrates.[23] Whilst the majority found that proteins tended to adsorb more extensively onto hydrophobic surfaces,[6, 24] such as SBB. Surface energy has also been related to the affinity of proteins to surfaces, although there have been publications indicative of increasing surface energy leading to lower adsorption.[25] The results of surface energy previously obtained for SBB surfaces are in good agreement with published data. Michiardi *et al.*[26] compared surface energy and protein adsorption behaviour for untreated and oxidized surfaces and showed that higher surface energy triggered albumin and fibronectin adsorption. Similarly, studies with PDLLA indicated an increase in albumin adsorption onto treated surfaces of higher surface energy.[27]

3.2. BSA Adsorption Isotherms: Langmuir and Freundlich models

To develop the fundamental understanding of protein adsorption, two typical adsorption models, Langmuir and Freundlich isotherms,[4] were used. The linearised Langmuir equation can be expressed as:

$$\frac{C}{Q} = \frac{1}{Q_m} C + \frac{1}{bQ_m} \quad \textbf{(1)}$$

where, C is the BSA concentration at a certain time, Q and Q_m are the adsorption amounts for BSA at a certain time and the maximal adsorption amount respectively, and b is the Langmuir's equilibrium constant that describes the strength of interaction between the protein and the surface.[6]

Other models such as the Freundlich model are more suitable for use with heterogeneous surfaces, but can only describe adsorption data over a restricted range:[28]

$$\ln Q = \frac{1}{n} \ln C + \ln K \quad \textbf{(2)}$$

In this case, C, Q, and Q_m are the same as in equation (1), n and K, constants at a specific condition. The constant K is a measure of the capacity of the adsorption and n is a measure of the intensity of adsorption.[4, 6]

In the low concentration range, changes in the bulk concentration produce large changes in the amount adsorbed, resulting in a roughly linear increase in adsorption. However, as the bulk concentration is further increased, adsorption is reduced and a plateau or maximum adsorption level is reached. This type of adsorption behaviour is referred to as a Langmuir isotherm. In other cases, the increase in adsorption at high bulk concentration does not stop entirely, but presents a slow rise. This type of adsorption behaviour is referred to as a Freundlich isotherm.

Table 1. Langmuir and Freundlich parameters for BSA adsorption onto starch based materials.

Materials	Langmuir Parameters			Freundlich Parameters		
	R^2	Q_m (mg/g)	b (L/mg)	R^2	n	K (L/g)
SCA	0,6538	66,14	0,01516	0,9651	1,27	0,46
SEVA-C	0,6678	24,19	0,00472	0,8263	1,58	0,57
SPCL	0,4276	144,90	0,03963	0,9757	1,21	0,50
TCPS	0,9446	3,36	0,00029	0,7595	2,94	0,49

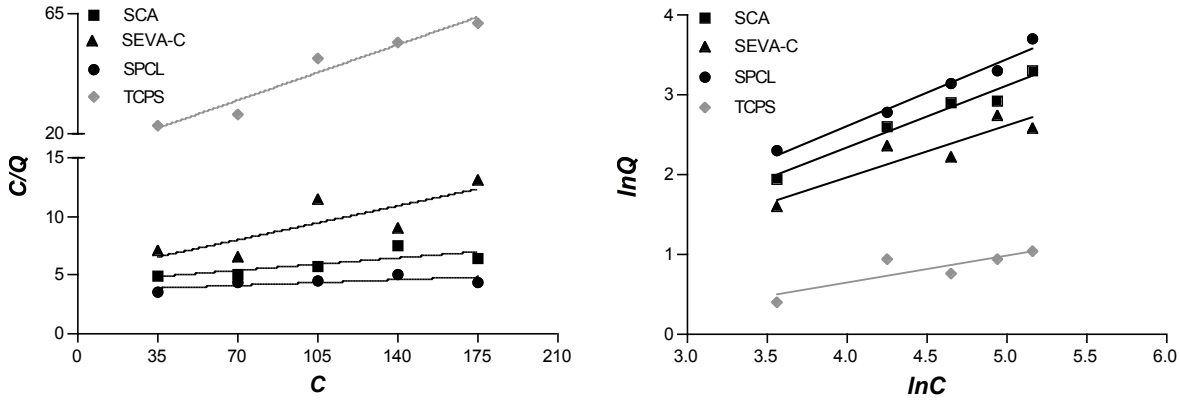

Figure 3. Langmuir (A) and Freundlich (B) fitting curves.

The fit of the different models to the experimental data was analysed taking in account the coefficient of determination (r^2, Table 1). All surfaces, except TCPS, showed higher correlation for Freundlich fitting (Figure 3B) than for Langmuir isotherms (Figure 3A). The fact that albumin adsorption onto TCPS surfaces was reduced could reduce protein-protein interactions and result in a better fit to the Langmuir model. The process of BSA adsorption onto SCA, SEVA-C and SPCL was more complicated than that of ideal adsorption. Possibly interactions between adsorbed BSA molecules and the type of surface played an essential role in influencing these interactions. Clearly, if there were no interactions among adsorbed BSA molecules, the Langmuir's fitting for SBB surfaces would be characterised by a higher r^2. Similarly, if the different surfaces did not affect the interaction, the correlation between both models should be the same.

It is known that the Langmuir model, disregards interactions between adsorbed proteins. The Freundlich isotherm, empirical in origin, is suitable for use with heterogeneous surfaces. This model can fit many adsorption processes considering further the dynamic while taking into account interactions between adsorbed molecules or between adsorbed molecules and the surfaces studied.[6] This could explain the improved correlations from the Freundlich fit for SBB surfaces. Freundlich's n parameter correlates with the averaged energies of adsorption; the lower the n the higher the affinity

between solid and adsorbates.[29] From the protein adsorption data (Figure 2), the values of the constant *n* (Table 1) are higher for SPCL and SCA.

3.3. Adsorption of Human Fibronectin and Human Serum Albumin

The adsorption of Alexa Fluor 488-labelled human serum albumin onto SBB surfaces was measured as a function of the incubation time (Figure 4). The results obtained for HSA were similar to those presented for BSA. For SCA and SPCL, HSA curves presented similar shapes to that of BSA and showed an initial fast adsorption that was reduced to a semi-steady state after 60 minutes. SEVA-C showed a gradual increase in HSA adsorption and did not show a defined plateau over the time period and concentration studied, suggesting that maximum surface coverage was not reached. The increase in protein bulk concentration from 35 to 70μg/mL induced an increase in adsorption. After 7 hours, SCA and SPCL adsorbed around 16μg/cm^2 and 21μg/cm^2 of protein when using the more concentrated solution of HSA (70μg/mL), while SEVA-C adsorption was 8μg/cm^2 and 3μg/cm^2 onto TCPS. In terms of percentage of adsorption, SPCL, SCA, SEVA-C and TCPS adsorbed 24%, 18%, 9% and 3% (W/W) respectively after incubation in 70μg/mL of HSA.

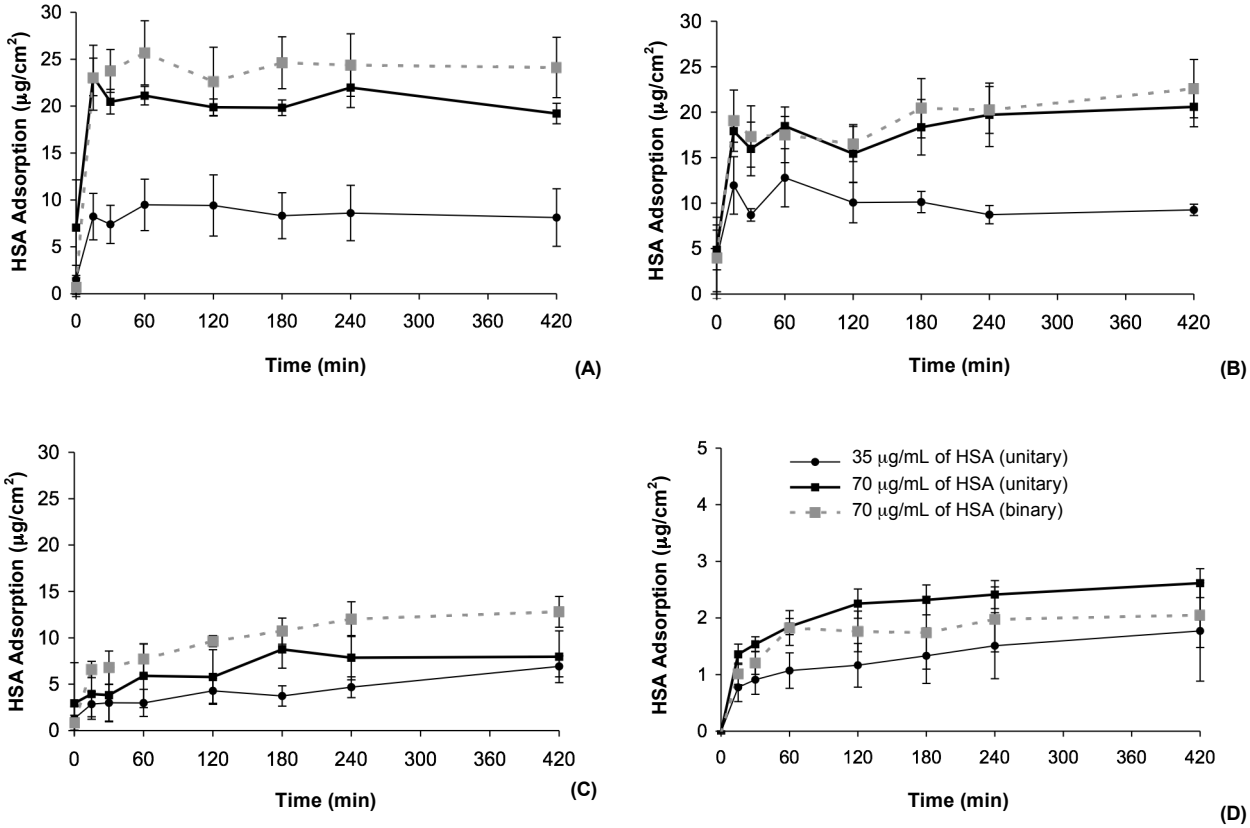

Figure 4. Adsorption of HSA in single and competitive conditions on the surface of SCA (A), SPCL (B), SEVA-C (C) and TCPS (D). Continuous lines (—■—) correspond to unitary protein systems of 35μg/mL and 140μg/ml of HSA and dashed line (- -■- -) represent the adsorption of HSA in the presence of 0,8μg/mL of fibronectin.

In addition, protein coverage of the different surfaces was visualised (Figure 5) and demonstrated heterogeneous HSA distribution on SCA, SPCL and SEVA-C surfaces.

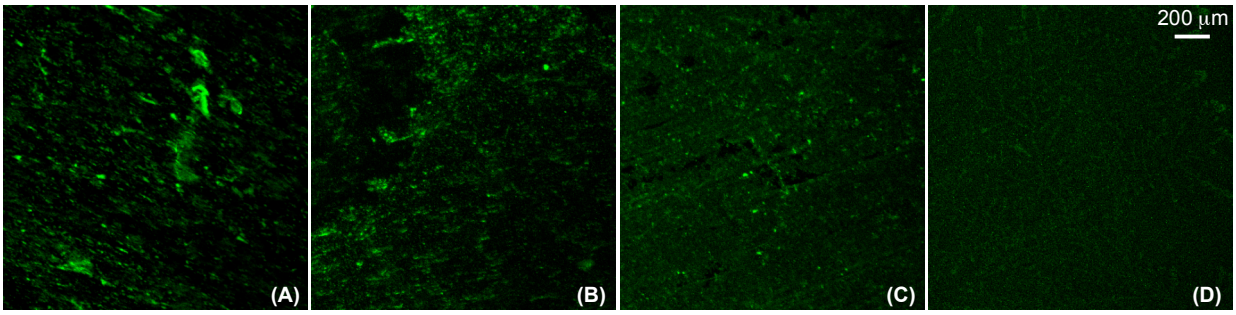

Figure 5. Confocal images of HSA-Alexa488 adsorbed onto the surface of SCA (A), SPCL (B), SEVA-C (C) and TCPS (D) after 7h incubation in 70µg/mL of HSA-Alexa Fluor 488.

For all materials except SCA, fibronectin adsorption curves (Figure 6) were similar to those observed for HSA demonstrating that the molecule specie did not affect the shape of adsorption curves onto SEVA-C, SPCL and TCPS.

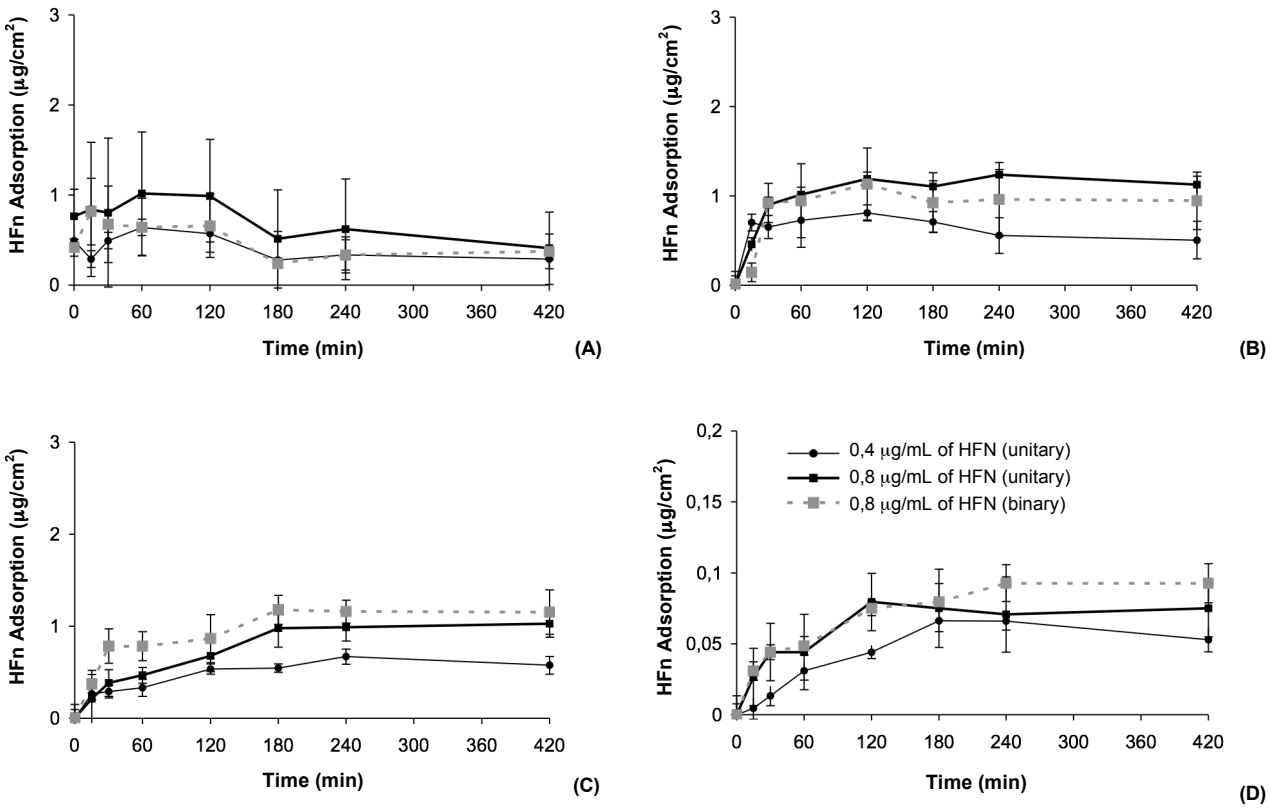

Figure 6. Adsorption of HFN in single and competition conditions on the surface of SCA (A), SPCL (B), SEVA-C (C) and TCPS (D). Continuous lines (—■—) correspond to unitary protein systems of 0,4µg/mL and 0,8µg/mL of Hfn and dashed line (- -■- -) represent the adsorption of HFn in the presence of 70µg/mL of albumin.

Different to the trend observed for HSA adsorption, higher levels of HFN adsorption were obtained for SEVA-C and SPCL polymeric blends. After 7 hours of incubation in 0.8μg/mL solution, SEVA-C and SPCL adsorbed approximately 1.02μg/cm^2 and 1.12μg/cm^2 of HFN, which corresponded to around 89% and 97%, respectively. Fibronectin adsorption onto SCA was lower and reached values of around 0.29μg/cm^2 that corresponded to 36%.

Fluorescence measurements were in good agreement with the results obtained by LSCM. Figure 7 demonstrated that the confocal method was applicable for protein observation and was in agreement with the fluorescence measurements that demonstrated higher intensities for SPCL and SEVA-C polymeric blends.

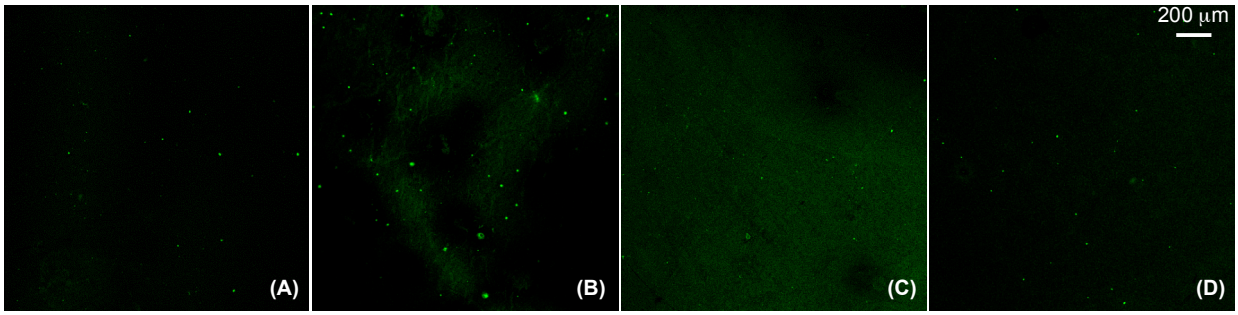

Figure 7. Confocal images of HFN-Alexa488 adsorbed onto the surface of SCA (A), SPCL (B), SEVA-C (C) and TCPS (D) after 7h incubation in 0.8μg/mL of HFN-Alexa Fluor 488.

An important result of the present study is the indication that the protein specie did not affect the adsorption curves. In contrast, adsorption onto SBB was clearly surface-regulated. In fact, using BSA, HSA or HFN resulted in a similar curve shape for the same SBB surface. The adsorption of HSA and HFN was in good agreement with a previously developed *in vitro* protein adsorption study using the same surfaces, proteins and solution concentrations.[16] In this study adsorption has been determined by immunostaining revealing that onto SEVA-C and SPCL surfaces, the adsorption of fibronectin was higher than that of albumin. In general, SPCL surfaces presented the highest affinity of all the studied molecules. Moreover, the reduced adsorption of albumin onto SEVA-C indicated low levels of non-specific protein adsorption onto this blend when compared to SPCL and SCA surfaces. On the contrary, fibronectin adsorption was highest onto SEVA-C, evidence of the higher affinity of this molecule for the SEVA-C surface.

3.4. Protein Behaviour in Competitive Conditions: binary systems

The single adsorption studies were investigated further in two additional experiments: (i) adsorption of HSA-488 Alexa Fluor from a solution containing unlabeled HFN and (ii) adsorption of HFN-555 Alexa Fluor from a binary solution containing unlabeled HSA. The weight ratio of fibronectin to albumin was approximately 1:90, typical of that found in normal human blood serum.[7] The adsorption of albumin and fibronectin from binary mixtures was investigated as a function of time, indicated by

the dashed lines in Figures 4 and 6, respectively. In addition the adsorption of molecules from HSA/HFN binary systems was assessed by LSCM and presented in Figure 8.

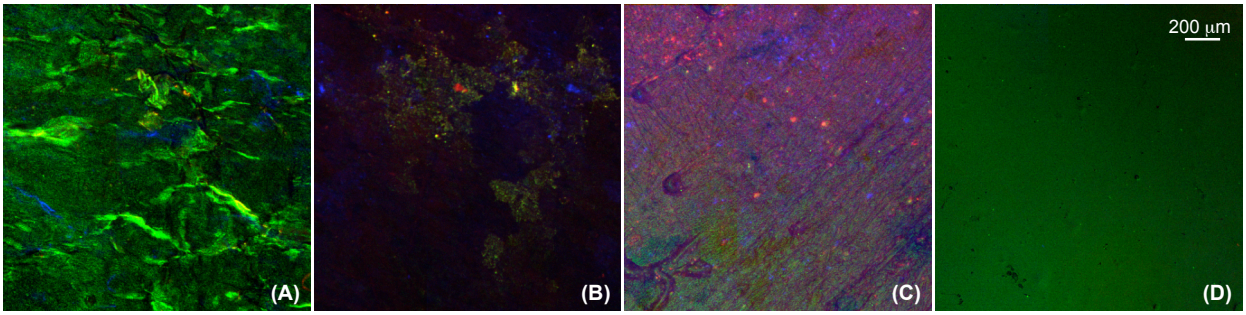

Figure 8. Confocal images of HSA/HFN adsorbed onto the surface of SCA (A), SPCL (B), SEVA-C (C) and TCPS (D) after 7h incubation in binary solutions of 70µg/mL of HSA-Alexa Fluor 488 and 0,8µg/mL of HFN-Alexa Fluor 555.

In competition conditions adsorption profiles shifted from the single protein conditions. A clear difference in HFN/HSA adsorption from the single protein solution versus the binary mixtures containing HFN and HSA was evident for some of the SBB surfaces.

On the SCA surfaces the adsorption of HSA was higher in competitive conditions (Figure 6A) than that observed in unitary systems. Furthermore, in HSA-HFN solutions albumin substantially decreased fibronectin adsorption. The outcome of the competitive behaviour onto the SCA surface has been previously observed using other proteins. Brash's group[30] showed a significant reduction of fibrinogen adsorption on different surfaces when high-molecular-weight kininogen was co-adsorbed. In the present study, results indicated that the presence of HSA had a negative effect on the level of adsorbed HFN. This effect could be brought about by the synergy of two mechanisms: first, by displacing HFN from the surface due to the high affinity of HSA for this surface, and second by a molecular weight and concentration effect favourable to the adsorption of HSA. In contrast to SCA, the fluorescence intensities for single *vs*. binary indicated that adsorbed HFN was not displaced by albumin for the SPCL interface, even at the much higher bulk albumin concentration. However for SPCL, there was little discernible increase or decrease in the fluorescence of both proteins, suggesting that while there may be adsorption/desorption of the same species, there was no visible change in the total quantity of HFN at the interfaces (Figures 4B and 6B). HSA adsorption on SEVA-C from single-protein solutions as well as from competitive systems was lower than that of the SCA and SPCL (Figure 4C), while HFN reached the highest adsorption percentages (Figure 6C). For binary systems (dashed lines) an increase of both HSA and HFN adsorption for SEVA-C surfaces was observed.

Modulation of the biological activity of adsorbed fibronectin has been shown in several studies in which the ability of fibronectin adsorbed to various surfaces to support cell attachment or spreading was found to differ. Grinnell's group showed that fibronectin biological activity was strongly affected by the type of surface to which it was adsorbed as well as by whether albumin was coadsorbed.[11, 31] In

these studies, fibronectin adsorbed to tissue culture grade polystyrene was able to support cell attachment and spreading, whereas fibronectin adsorbed to ordinary polystyrene did not support spreading unless some albumin was added to the fibronectin solution. Addition of BSA to 2μg/ml fibronectin solutions was reported to enhance the fibronectin binding to both bacteriologic and tissue culture grade polystyrene.[31] Lewandowska *et al*., demonstrated that the conformation of fibronectin molecules can vary due to the chemical nature of the substrates. On hydrophobic surfaces preadsorbed with fibronectin, cell spreading was improved only when albumin was coadsorbed, so called "albumin rescuing".[12] The "rescuing" phenomenon is similar to the effect Grinnell reported on polystyrene. This could explain the increase in HFN adsorption observed in binary conditions onto the SEVA-C and TCPS surfaces. In a later extension of this work, BSA-mediated modulation of the ability of the adsorbed fibronectin to induce neurite formation in neuroblastoma cells was noted.[32] The authors concluded that the cellular response was altered due to a change in the conformation of fibronectin molecules as they interacted with the different chemical end groups. This rearrangement of fibronectin onto the surfaces indirectly detected by the analysis of the cellular response could also hinder an increased adsorbed amount of this protein determined in this study by fluorescence measurements.

4. CONCLUSIONS

The adsorption isotherms and the competition of plasma proteins on starch-based biomaterials has been elucidated. In the present work, the type of adsorption obtained onto SBB surfaces was characteristic of a Freundlich type. For SCA and SPCL the equilibrium of adsorption was achieved within the first hour of incubation while for SEVA-C the adsorption was slower. Albumin adsorption onto starch based biomaterials was affected by the material composition as well as by the concentration of the protein solution, preferentially adsorbing onto SCA and SPCL. Fibronectin adsorption reached higher values on SEVA-C and SPCL. There was no effect on the adsorption of HSA and HFN onto SPCL in competitive conditions. Fibronectin adsorption was reduced on SCA in the presence of albumin, for which adsorption simultaneously increased, while the opposite situation was observed for TCPS. Competitive conditions were favourable to the affinity of both molecules by improving the affinity of albumin and fibronectin onto SEVA-C surfaces. Fibronectin demonstrated a different adsorption activity for the different materials as assessed by single and competitive adsorption with albumin.

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CHAPTER VII

THE REVERSIBILITY OF PROTEIN ADSORPTION ONTO BIODEGRADABLE MATERIALS

CHAPTER VII

THE REVERSIBILITY OF PROTEIN ADSORPTION ONTO BIODEGRADABLE MATERIALS*

Alves, CM[1,2], Reis, RL[1,2], Hunt, JA[3]

[1] 3B´s Research Group – Biomaterials, Biodegradables and Biomimetics, Dept. of Polymer Engineering, University of Minho, Campus Gualtar, 4710-057 Braga, Portugal

[2] IBB – Institute for Biotechnology and Bioengineering, PT Government Associated Laboratory, Braga, Portugal

[3] Clinical Engineering, UKCTE, University of Liverpool, P.O. Box 147, Liverpool L69 3QA, United Kingdom

Abstract

The characterisation and quantification of the adsorption and desorption of plasma proteins on different natural based materials was performed by multiloop Dynamic Contact Angle (DCA) measurements based on the Wilhelmy method. Starch-based materials were the interfaces used to analyze the influence different surfaces had on the adsorption, desorption and changes in protein configuration. The studied protein systems included single protein solutions of Human Serum Albumin (HSA), Fibronectin (HFn), Vitronectin (HVn) and Fibrinogen (HFbg). Human blood plasma was used as the competitive protein solution. In the adsorption studies, the DCA loops observed on all the materials were reduced and the decrease in hysteresis was demonstrated. This effect was more significant for SCA surfaces than SEVA-C and SPCL, which had similar hysteresis loops. The effect of protein concentration was assessed and demonstrated to substantially affect the DCA wetting forces of SEVA-C and SPCL surfaces. In the desorption study, during the rinsing phase with phosphate buffered saline (PBS) solution, the DCA loops became larger than that observed for the adsorption phase. The hysteresis of SCA and SPCL surfaces irreversibly changed through the desorption phase. The results indicated that adsorbed proteins could desorb more readily on SCA and SPCL than on SEVA-C. In the later case, stronger interactions such as hydrophobic forces were established and it is likely the rearrangement of protein conformation had occurred.

Keywords

Protein adsorption, desorption, conformation, competition, plasma proteins, biodegradable materials, DCA.

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C. M. Alves, R. L. Reis, J. A. Hunt, "The reversibility of protein adsorption onto biodegradable materials", Advantional Functional Materials, 2007, *submitted*

1. INTRODUCTION

The paradigm of cell material interactions, which holds that protein adsorption is the first event following contact and determines the later interactions of the cell, could be central to the design of new strategies for biocompatibility[1] and tissue engineering[2, 3] From a functional point of view, it is accepted that qualitative and quantitative assessment of the affinity of proteins to surfaces is essential to evaluate cell mechanisms upon attachment to the surfaces and thus develop improvements in the properties of implanted materials.

Plasma proteins are immediately adsorbed onto the surface of biomaterials and are the key factors determining the subsequent cell related events.[4] Proteins such as fibronectin and vitronectin are known to have an early interaction with surfaces and are considered of central importance in the regulation of cell adhesion.[5, 6] Different substrates interact differently with these molecules resulting in conformational changes of the protein structures, thus defining the binding quality of specific cell receptors.[7] Therefore, in the field of surface science the study of adsorption-desorption kinetics or adsorption reversibility related to the folding-unfolding events, have been investigated.

Several methodologies are available for the study of protein interactions with macromolecules providing information on the amount of protein or molecular conformation.[8-10] Yet, protein-polymer interactions are not directly evaluated.[11] Moreover, proteins are essentially large hydrocarbons with complex structures and as such their identification is highly complex. Detection problems are even more apparent at submonolayer coverage, where the substrate adds greater complexity to the analytical spectrum. Proteins are difficult to distinguish from “contamination” by other hydrocarbon species that can be present on the surfaces; but also the intensity of spectral features that can be used for identification are often so weak that detection limits are low and sensitivity is poor.[11] Useful information could be provided by the study of the dynamics of interfacial reactions such as the one that is triggered by the contact of a surface with a biological medium.

Although the adsorptive characteristics of a surface are determined by its wetting tension and wettability,[12] the real time interference of these parameters in protein adsorption have been hardly investigated. Dynamic Contact Angle (DCA) analysis, developed by Andrade *et al.*,[4] has proven to be a useful technique for a first-order interpretation of these dynamic interfacial interactions. DCA is sensitive to the outermost few angstrons of the surface and thus provides a powerful means of monitoring submonolayer changes of the substrate.[4, 13] Currently, the tensiometric DCA has proven to be extremely useful for biomaterial characterization.[14, 15] This technique is generally used to measure the advancing and receding contact angle of water on material surfaces and can therefore be used to determine surface changes by the measurement of the variation in the contact angles (hysteresis). From a thermodynamic perspective, advancing and receding contact angles should be equal. Yet, in experimental systems hysteresis is detected and two types are generally considered: thermodynamic and kinetic hysteresis.[4, 16, 17] DCA provides information from the material side that allows the detection

of quick changes in the surface configuration.[18] The method yields data on a multitude of surface characteristics, such as the presence of chemical and physical heterogeneities, changes in the surface configuration and adsorption-desorption processes. DCA allows the study of sample surfaces while in contact with the biological model fluid, providing a continuous, non-destructive monitoring technique[19] that detects changes over increasing time.[4] The formation of a biofilm from a protein solution also takes place on the same timescale.[20] In protein adsorption studies, changes in DCA hysteresis can reflect the adsorption of proteins onto surfaces forming a biofilm. Upon adsorption, proteins may undergo conformational changes that allow hydrophobic residues to contact a hydrophobic surface exposing hydrophilic residues towards the solution. This results in a stronger bond between the protein and the surface and can lead to a more uniform and hydrophilic surface chemistry.[21]

In the present study, changes in the homogeneity of Starch based Biomaterials (SBB) exposed to protein solutions were determined using DCA measurements. Starch-based Biomaterials have been increasingly studied for applications in the field of Tissue Engineering,[22, 23] including their use as scaffolds for bone related applications[24] and drug delivery systems.[25] The work disclosed herein focused on obtaining the DCAs of different surfaces for single solutions of human proteins: serum albumin (HSA), fibronectin (HFn), vitronectin (HVn), fibrinogen (HFbg); and for complex solutions as human blood plasma (HBP). The DCA technique was used to determine (1) the adsorption of macromolecules, (2) protein concentration effects, (3) the adsorption and desorption rates and (4) partial denaturation effects driven by the surface characteristics. Moreover, visualization of the final surfaces was analysed by Laser Scanning Confocal Microscopy (LSCM).

2. MATERIALS AND METHODS

2.1. Starch-based Biomaterials (SBB)

Different biodegradable polymeric blends of corn starch with: (i) cellulose acetate (SCA), (ii) ethylene vinyl alcohol copolymer (SEVA-C) and (iii) polycaprolactone (SPCL) were studied. The amount of starch was 50% by weight (wt%) on SCA and SEVA-C and 30% wt. on SPCL. By means of using conventional injection moulding technology, samples were processed into 10 mm circular discs. Samples were sterilized by ethylene oxide under optimised conditions[26], washed, and subsequent experimental procedures were performed under clean conditions.

2.2. Proteins and Human Blood Plasma Collection

Dynamic contact angle studies were performed using different commercially available human origin biomolecules: HSA, HFn, HVn and HFbg (Sigma-Aldrich, UK). Single and complex protein solutions were prepared in Phosphate Buffered Saline (PBS; Sigma-Aldrich, UK) solution (pH 7.4). Concentrations of HFn, HVn and HFbg solutions were prepared at 0.2% of their amount in the human

blood: 0.8μg/mL, 0.6μg/mL and 0.4μg/mL, respectively. To assess the effect of protein concentration in DCA hysteresis, 35μg/mL and 70μg/mL HSA solutions were prepared, which corresponded to 0.1% and 0.2% of its physiologic concentration. Furthermore, to analyse the effect of complex protein solutions on DCA hysteresis, human blood plasma was used. In brief, whole blood was collected from healthy un-medicated, adult volunteers, anticoagulated with 0.002% of heparin and centrifuged at 2500rpm for 5min at 4ºC. Human Blood Plasma (HBP) was collected and stored at 0ºC. HBP solutions were prepared at 0.2% (V/V) in PBS solution.

2.3. Dynamic Contact Angle: protein adsorption and desorption

2.3.1. Theoretical Principles

DCA measurements were performed based on the Wilhelmy plate method.[27] Wilhelmy-balance tensiometry was performed using a computer-controlled instrument (Camtel CDCA 100F, Royston, UK). The theoretical principles of this methodology are described in the literature.[11, 17] Briefly, a plate was immersed and emersed from a model liquid and the forces acting in the specimen were recorded by electrobalance. According to the procedure, the balance was reset to zero and linear regression was performed to the immersion depth zero. This provided for the elimination of the weight of the sample and buoyancy forces. The relation between force and surface tension was represented by the following equation:

$$\frac{F}{L} = \gamma_{lv} \cos\theta \qquad \textbf{(1)}$$

where F is the force in mN, γ_{lv} the surface tension of the liquid and θ is either the advancing (θ_{adv}) or receding (θ_{rec}) contact angle.

Finally, contact angle hysteresis is the difference between advancing and receding contact angles. This parameter is affected by the distribution of different chemistries on the surface with different properties and thus is a measure of the homogeneity of the surface.

2.3.2. Adsorption and Desorption Studies

Adsorption and desorption studies were performed running multiloop DCA at a controlled temperature (22ºC). For the experimental set up immersion/emersion speed was a constant set to 0.060mm/sec and the immersion depth was 2mm. In all experiments, a single hysteresis loop lasted 3min and therefore a complete 60 loop experiment ran for approximately 3h. The duration of the experiments and subsequently the number of DCA cycles was selected based on previous studies with starch-based biomaterials showing that HSA and HFn adsorption was completed in approximately 90min after incubation.[28]

Prior to the evaluation of the effect of the different protein conditions on the advancing (adv) and receding (rec) dynamic contact angles (DCAs), ultra-pure water and PBS solution were studied. The

effect of the different proteins and human blood plasma on the advDCAs and recDCAs of SCA, SEVA-C and SPCL was investigated and hysteresis (H) calculated. In the adsorption study, experiments were performed using each protein solution for 60 loops. In contrast 30 loop desorption studies were performed in protein free buffer (desorption phase) directly after the 60 loop experiment of the adsorption phase.

2.4. Protein Labelling

Antibody labelling of specific biological molecules was selected to determine the adsorption of albumin, fibronectin, vitronectin and fibrinogen on the different surfaces. For the detection of the biomolecules, samples used in the adsorption and desorption DCA studies were fixed using 4% formaldehyde solution and washed with PBS solution. Initially, the materials were exposed to horse serum for 20min, followed by incubation with primary antibodies for 30min at 37ºC. For the identification of the different biomolecules, antibodies that have been shown to cross react with rat specie were used: sheep anti-Human Albumin, Fibronectin, Vitronectin, and Fibrinogen (Farnell, UK). After the primary antibody, materials were incubated with donkey anti-sheep Alexa Fluor 488 antibody (Molecular Probes, The Netherlands) for 1 h at 37 ºC.

2.5. Confocal Laser Scanning Microscopy

Samples obtained after adsorption and adsorption/desorption experiments were analysed by Confocal Laser Scanning Microscope (LSM 510 Zeiss, UK) using an argon laser (λ=488 nm) for the excitation of the probe-conjugated antibodies used to detect adsorbed HSA, HFn, HVn and HFbg. Image analysis was performed using KS400 image analysis software (Imaging Associates, UK).

3. RESULTS AND DISCUSSION

3.1. Characterization of SBB Surfaces by DCA Measurements

The dynamics of the advancing and receding wetting tension were detected by means of force loops. The force loops using PBS and water were similar although a slight decrease in hysteresis was observed due to the influence of the phosphate buffer in the homogenization of the surface. DCA loops of the SBB surfaces after immersion in PBS were obtained (Figure 1). The fitting of the advancing arm, the transition from advancing to receding mode and the receding arm were determined. For each cycle, the DCA loops for the surfaces of SEVA-C and SPCL were similar (Figure 1b and 1c) with no reduction in hysteresis after the initial change from dry to wetted state, around the second loop. In contrast, control measurements using PBS and SCA surfaces, indicated an increase in the advancing contact angle with increasing cycles. The maximum hysteresis was obtained at loop 10, indicative that a steady state was achieved (Figure 1a).

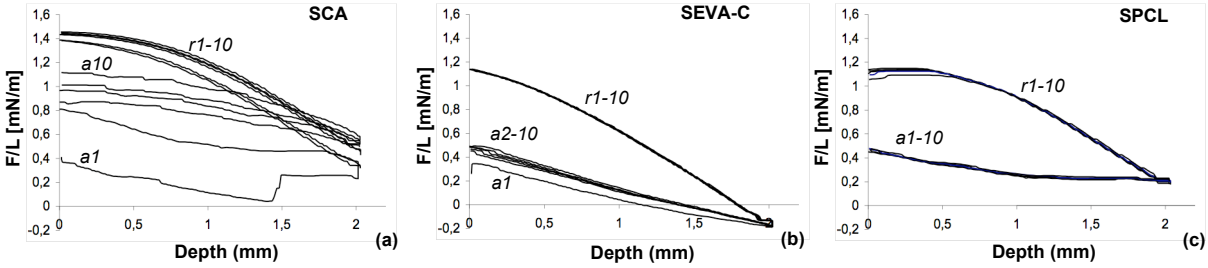

Figure 1. DCA loops of SCA (A), SEVA-C (B) and SPCL (C) immersed in PBS solution. The advancing (*a*) and receding (*r*) loop numbers (1-5 and 10) were indicated.

In theory, the occurrence of contact angle hysteresis is not predicted to occur for "ideal" surfaces.[4] Yet, surfaces generally present either time invariant hysteresis (thermodynamic or true hysteresis) or time-dependent hysteresis that results from the re-equilibration phenomenon taking place in the surface-liquid interface (kinetic hysteresis).[4, 16, 17] The results demonstrated examples of kinetic (Figure 1a) and thermodynamic hysteresis (Figure 1b and 1c). The increase in hydrophilicity was the fundamental difference between SCA, SEVA-C and SPCL polymeric blends. SCA changed completely into a wettable surface at loop 10 after the initial hydrophobic loops. Time-dependent contact angle hysteresis resulted from the changes of the advancing angles, as the variation of receding contact angles was very small.

According to the literature,[17, 21] the kinetic hysteresis observed for SCA could be due to swelling, surface mobility or to changes in surface configuration. In opposition, the hysteresis observed for SEVA-C and SPCL could be caused by surface roughness and chemical inhomogeneity, which was absent in the so called "ideal" surfaces.[17] The results can be further interpreted by considering the miscibility and interaction between the synthetic and natural phases of the materials. Previous studies reported the heterogeneity of SCA surfaces in opposition to the higher homogeneity of SPCL and SEVA-C.[29, 30] The more homogeneous a material is the lower the degree of freedom at the molecular level and thus changes in the surface configuration are limited. This could explain the invariant thermodynamic hysteresis observed for SEVA-C and SPCL. DCA analysis of hysteresis proved to be a sensitive method to assess surface configuration changes. Furthermore, Johnson & Detre derived an empirical model to explain the correlation between surface in homogeneity and contact angles.[31, 32] According to this model, advancing DCAs of the macroscopically heterogeneous surfaces were influenced by 10% hydrophobic surface patches, while the receding DCAs were affected by 10% hydrophilic areas in a hydrophobic surface. Developing from this, it could be stated that SCA presents hydrophobic patches that represent over 10% of the surface.

3.2. DCA and Protein Adsorption: effect of solution concentration

The 10 loop DCA in 70mg/mL HSA for SCA, SEVA-C and SPCL (Figure 2) indicated differences between the advancing angles while receding angles remained constant. The shape of the DCA curves observed for all surfaces substantially changed when compared to the protein-free solution (Figure 1). This variation was more rapid for the SCA surface, indicative of total wetting.

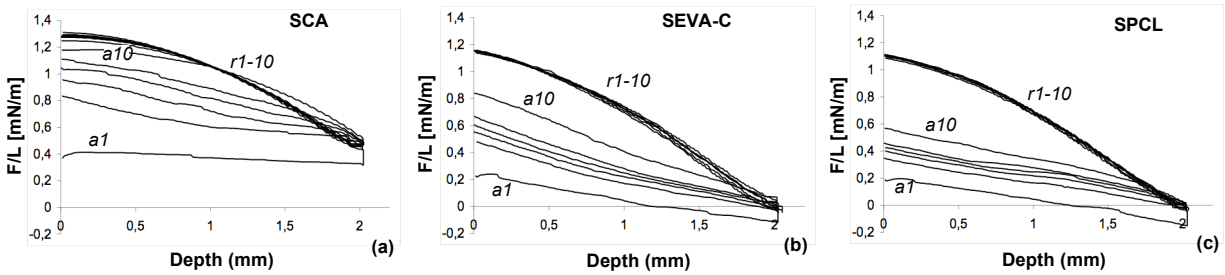

Figure 2. DCA loops of SCA (A), SEVA-C (B) and SPCL (C) immersed in a solution of 70µg/mL of HSA (adsorption phase). The advancing (*a*) and receding (*r*) loop numbers (1-5 and 10) were indicated.

To analyse the effect of protein concentration on hysteresis, the data from the 30 loop experiments were presented as a function of time or cycle number (Figure 3). Albumin solutions of 35µg/mL and 70µg/mL were used as test solutions. For all materials, the data showed that an increase in solution concentration resulted in a decreased hysteresis. These results were in agreement with other studies[19, 21] that indicated the reduction of hysteresis with increasing concentration of Bovine Serum Albumin (BSA) solutions. The hysteresis shifts of SCA, SEVA-C and SPCL were primarily caused by changes in the advancing arms of DCA force loops (Figure 3). This effect was more pronounced for SEVA-C and SPCL than for SCA. Moreover, SCA hysteresis approached a steady state close to zero during the initial 10 cycles. It was determined that higher concentrations did not yield different or further information, which could result from the formation of a monolayer. For SEVA-C and SPCL, the equilibrium between proteins in solution and those on the surface layer was observed around loop 30.

Despite of the lower hydrophilicity of SEVA-C and SPCL than that of SCA, the hysteresis was affected by the protein solution and changed from a thermodynamic to a kinetic type. According to the literature, a cause of kinetic hysteresis is the adsorption of macromolecules from the liquid phase. [4, 16, 17] When compared with the surface state before adsorption, the initial heterogeneity of a surface is affected by the onset of protein-surface interactions and kinetic hysteresis is a consequence of adsorption.[4, 21] For all materials the DCA experiments in PBS contrasted with DCA studies in albumin solution (Figure 1 and 2). Time dependent shifts in immersing and/or emerging forces have been demonstrated. Due to protein adsorption, the increase in hydrophilicity and the homogenization of the surfaces was demonstrated. Proteins were able to undergo conformational rearrangement to adsorb on both hydrophobic and hydrophilic surfaces. The decrease in hysteresis and hydrophilicity showed that

the adsorption of albumin reduced the hydrophobic areas of the surface. In the present study, an energetically favourable system resulted from the rearrangement of the protein structure that exposed the hydrophilic regions towards the solution.

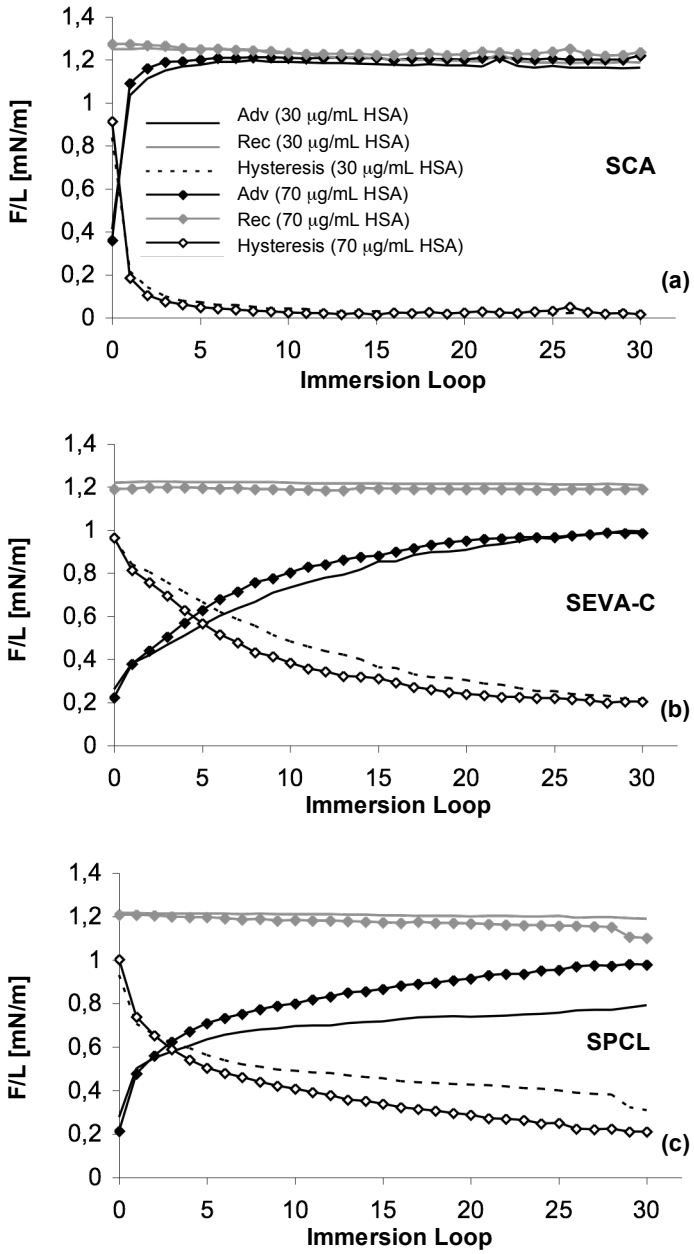

Figure 3. 30-loop DCA experiments showing hysteresis, advancing and receding wetting tensions obtained for 35μg/mL and 70μg/mL HSA solutions. SCA (a), SEVA-C (b) and SPCL (c) surfaces were analysed.

3.3. DCA Hysteresis of Protein Adsorption and Desorption

3.3.1. Single Protein Solutions

The adsorption/desorption studies were performed to analyse the reversibility of the wettability status, which may have changed during adsorption. Desorption studies were performed by replacing the protein solutions (adsorption phase) with PBS (Figure 4).

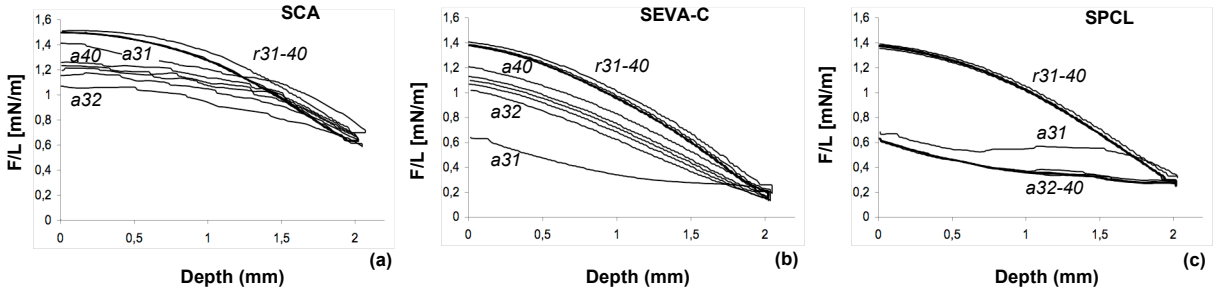

Figure 4. DCA loops of SCA (A), SEVA-C (B) and SPCL (C) immersed in PBS solution (desorption phase) subsequently to the HSA adsorption experiment. The advancing (*a*) and receding (*r*) loop numbers (31-35 and 40) were indicated.

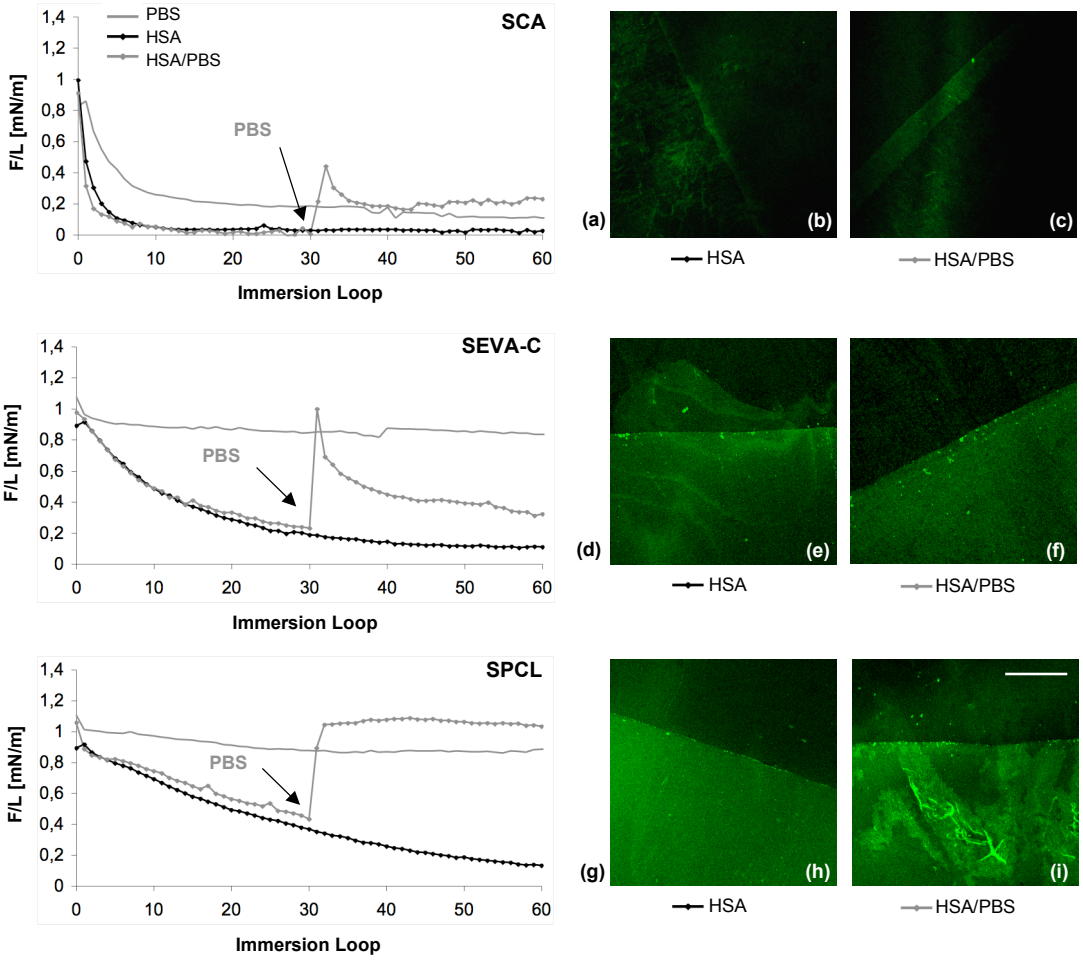

Figure 5. 60-loop DCA adsorption/desorption hysteresis of SCA (a), SEVA-C (b) and SPCL (c) surfaces using 70 μg/mL HSA solutions. Adsorbed albumin at loop 30 (b, e and h) and at loop 60 (c, f and i) can be observed. Bar corresponds to 500 μm.

DCA results for SCA and SPCL surfaces demonstrated that the advancing arms of the contact angle loops irreversibly changed towards an increase in hysteresis. Similar trends were obtained for SEVA-C surfaces during the first cycles of the desorption phase. Nevertheless, at the end of the study, SEVA-C hysteresis returned to the levels observed for the adsorption phase (Figures 2B and 4B). The results of the adsorption/desorption experiments from the 60-loop Wilhelmy measurements are shown in Figure 5.

The equilibrium wetting tensions at loop 60 of the adsorption study were nearly identical for SCA, SEVA-C and SPCL. In the study of protein desorption (loops 31-60), drastic changes could be observed and the influence of surface identity on the desorption kinetics was clear. Conditioning of SCA and SPCL with PBS during loops 31-60 showed a kinetic enhancing effect. The advancing and receding wetting tensions showed an immediate increase in hysteresis at the 31st loop, the first loop of the desorption phase (Figure 4b). The advancing data partially shifted back in the hydrophobic direction, whereas the receding wetting tension showed an irreversible behaviour, the original small hysteresis increased irreversibly. According to these DCA results, the adsorption of HSA on SPCL and SCA was reversible. In contrast, the experiments with protein-free solution during desorption that indicated reversibility of the SEVA-C hysteresis shifts. As for SCA and SEVA-C, the advancing wetting tension moved in the hydrophobic direction. However at loop 60, hysteresis values were similar to those obtained at the end of the adsorption experiment. It can be concluded that HSA adsorption on SEVA-C was irreversible.

DCA experiments were also performed using HFn, HVn and HFbg at much lower concentrations. The results for SEVA-C are shown in Figure 6. The trends observed for fibronectin, vitronectin and fibrinogen were similar to that of albumin, including a fast increase in hysteresis at loop 31. Also, at loop 60 the adsorption profiles were partially recovered for HVn and HFbg, and totally recovered in the case of HFn. This showed that, in approximately 90 minutes (loop 31-60), the proteins adsorbed on SEVA-C rearranged their conformation back to the structure adopted during the adsorption stage (loop 1-30).

DCA was also performed on the surface of SCA and SPCL using HFn, HVn and HFbg test solutions. Yet, in adsorption and desorption experiments no changes in hysteresis were observed (Figure 7a). Although protein adsorption was not detected by DCA analysis, it was observed by confocal microscopy performed after the adsorption (Figure 7b) and desorption studies (Figure 7c).

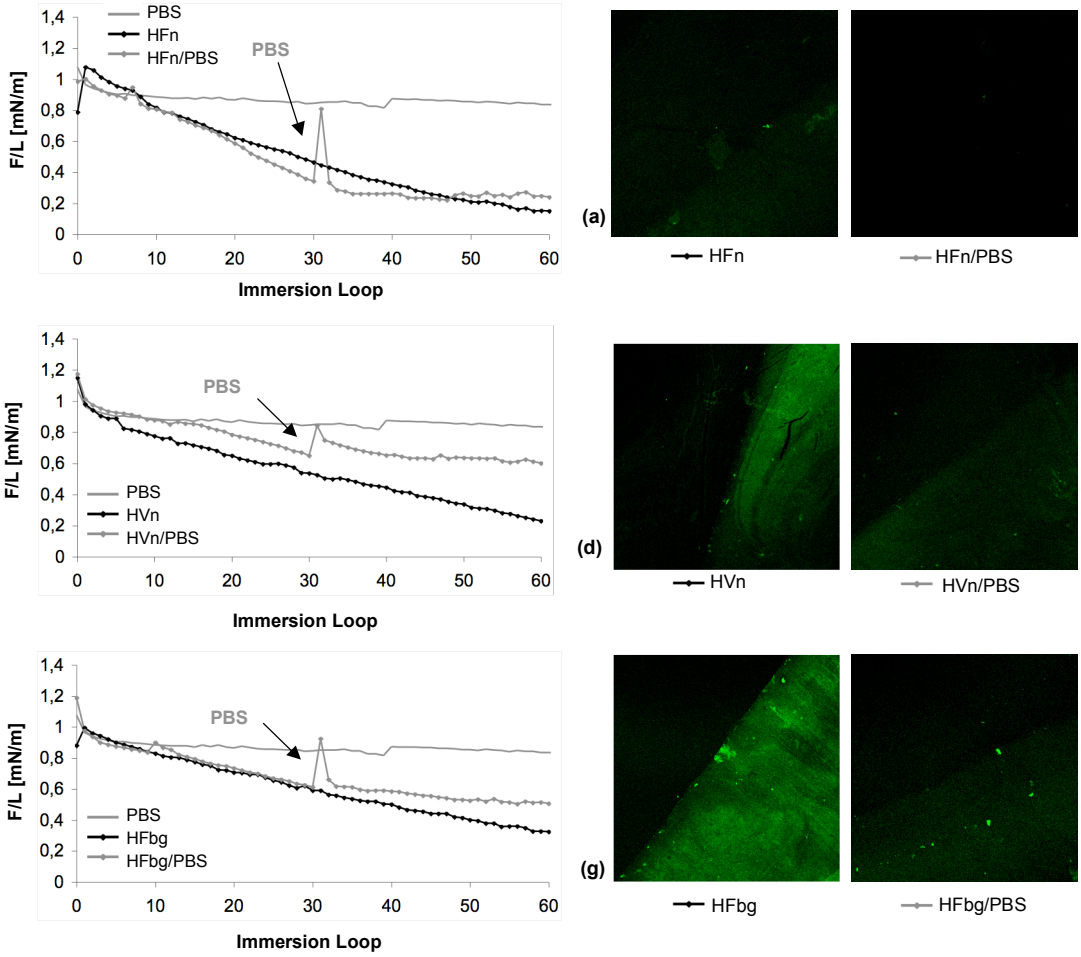

Figure 6. 60-loop DCA adsorption/desorption hysteresis of SEVA-C surfaces using 0.8 μg/mL HFn (a), 0.6 μg/mL HVn (b) and 0.4μg/mL HFbg (c) solutions; and protein visualization at loop 30 (b, e and h) and at loop 60 (c, f and i). Bar corresponds to 500 μm.

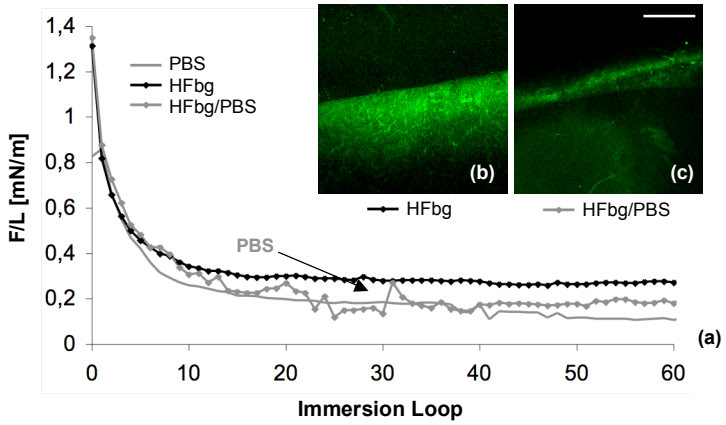

Figure 7. 60-loop DCA adsorption/desorption hysteresis of SPCL surfaces using PBS and 0.4 μg/mL HFbg solutions and protein visualization at loop 30 (b) and at loop 60 (c). Bar corresponds to 500 μm.

3.3.2. Competitive Protein Systems

Single protein solutions are generally emphasized over the complex protein mixtures when it comes to the experiments simplicity. In the present study complex protein solutions were analysed in order to closer evaluate the real biological environment.

To analyse the effect of protein competition on hysteresis, the data of 60 loop experiments was represented as a function of loop number (Figure 8).

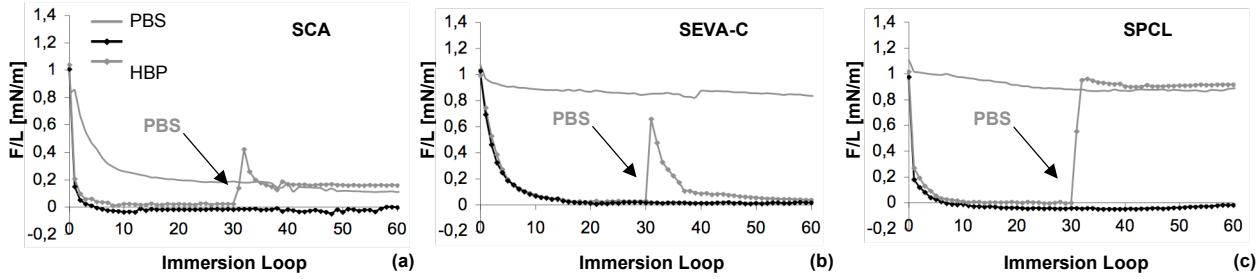

Figure 8. 60-loop DCA adsorption/desorption hysteresis of SCA (a), SEVA-C (b) and SPCL (c) surfaces using human blood plasma (HBP) solutions at 0.2% (V/V).

The adsorption and desorption experiments performed with human blood plasma strongly resembled those of HSA. The hysteresis curves and the hydrophobic shift obtained at loop 31 were very similar to the albumin single test solutions. In complex protein solutions, SCA and SPCL showed a hysteresis of zero before loop 10. In contrast only at loop 20, SEVA-C surfaces achieved complete wettability. Furthermore, SCA and SPCL desorption hysteresis did not recover to the original values obtained during the adsorption study (Figure 8a and 8c). In contrast, SEVA-C achieved a completely wettable surface at both the end of the adsorption and desorption phases, at loop 30 and loop 60 respectively (Figure 8b). This was not demonstrated for unitary HSA solutions (Figure 5d). The increase in hydrophilicity of all the surfaces was higher for the competitive system due to the protein concentration and species diversity in the human blood plasma solution.

Changes in DCA hysteresis profiles were demonstrated to be related to material, although changes in hysteresis with HFn, HVn and HFbg solutions were not detected on SCA and SPCL and adsorption and desorption profiles obtained for SEVA-C were similar for the different molecules. In addition, SEVA-C showed no reversibility of the advancing wetting tensions and consequently, no changes in hysteresis. The results suggested that SEVA-C presented irreversible adsorption.

It is known that the wettability of a polymer affects protein adsorption and that hydrophobic surfaces tend to be much more denaturing to adsorbed proteins than hydrophilic surfaces.[33] Surface hydrophobicity strongly affects the interactions of proteins with materials and thus, the irreversibility of the adsorption process. When hydrophilic proteins adsorb onto hydrophilic surfaces no net hydrophobic interactions occur. In addition, no significant conformational changes take place in the

tertiary and/or secondary structure of the protein.[34, 35] In previous studies, SEVA-C was determined to be the most hydrophobic surface and, in contrast, SCA showed the lowest values of static contact angles.[36] It was previously demonstrated that the adsorption of proteins to a hydrophobic surface is usually irreversible due to the increase of the free energy gain,[37, 38] whereas the adsorption onto hydrophilic surfaces is weaker, more sensitive to electrostatic interactions and more reversible.[39, 40] Hydrophobic, electrostatic and specific acceptor donor interactions are generally referred to as the most important forces involved in the adsorption process. The surface charge plays a key role in the early electrostatic attraction of the protein to the surface. Nevertheless, these are relatively long distance forces. In contrast, hydrophobic interactions involve the release of water from both hydrophobic protein residues and the solid surface, which results in strong binding of the nonpolar protein components with the surface. An irreversibly adsorbed protein layer is formed. Several other reversibly bound layers will then form on top of the irreversibly bound layer[41] to create the complex protein multilayers which mediate cell interaction.

The differences of adhesion strength observed in the desorption phase that can be considered an elution test indicated conformational changes of the proteins. The surface mediated structure that was adopted by the proteins could affect the biological activity of the proteins and modulate the cell biological response to the different SBB.

4. CONCLUSIONS

The biological environment was simulated to determine single and competitive adsorption profiles of proteins from the blood matrix onto the surface of materials. Insights into the dynamic equilibriums established between proteins and surfaces were obtained from DCA measurements. Hysteresis profiles obtained for the different protein solutions indicated that different interactions between these proteins and substrates were taking place during the adsorption process. The SCA surface was the most interactive in terms of albumin adsorption, revealing saturation at shorter time periods as observed by the higher hydrophilicity. Adsorption studies with SEVA-C and SPCL showed very similar results and were in contrast to SCA.

The most significant differences were observed in the study of protein desorption. The differences observed in the desorption phase indicated conformational changes of the proteins. SCA and SPCL were demonstrated to completely revert the hysteresis profile back to the original one obtained with protein-free buffer. In contrast, SEVA-C showed irreversible hysteresis independently of the protein used in the desorption study. The same results were obtained for the study of protein competition. The biological activity of the proteins on the SBB surfaces was affected by the material properties and could therefore modulate the cell biological response to the different starch-based materials.

Acknowledgements

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CHAPTER VIII

THE DYNAMICS OF IN VIVO ADSORPTION AND DIFFUSION OF PROTEINS ONTO BIODEGRADABLE MATERIALS

CHAPTER VIII

THE DYNAMICS OF IN VIVO ADSORPTION AND DIFFUSION OF PROTEINS ONTO BIODEGRADABLE MATERIALS*

Alves, CM[1,2], Marques, AP[1,2], Reis, RL[1,2], Hunt, JA[3]

[1]3B´s Research Group – Biomaterials, Biodegradables and Biomimetics, Dept. of Polymer Engineering, University of Minho, Campus Gualtar, 4710-057 Braga, Portugal

[2] IBB – Institute for Biotechnology and Bioengineering, PT Government Associated Laboratory, Braga, Portugal

[3]Clinical Engineering, UKCTE, University of Liverpool, P.O. Box 147, Liverpool L69 3QA, United Kingdom

Abstract

The physicochemical relationship between different biological molecules and organic materials was investigated *in vivo*. The objective of the research was to determine the dynamic and competitive interaction of albumin, fibronectin, vitronectin and fibrinogen on polymeric blends of corn starch with ethylene vinyl alcohol (SEVA-C). Samples were implanted subcutaneously and analyzed after 3, 7 and 14 days. Immunohistochemistry was performed to assess the spatial distribution, diffusion and displacement of the proteins albumin, fibronectin, vitronectin and fibrinogen. Results indicated that albumin and vitronectin were absent from the immediate tissue implant interface in contrast to fibrinogen and fibronectin that had adsorbed and formed layers on the surface of SEVA-C. For longer implantation periods, fibronectin adsorption presented a multilayer pattern that displaced fibrinogen from the immediate interface. The smaller molecular weight proteins diffused into the bulk of the materials, penetrating greater distances with increasing implantation time. The materials demonstrated molecule specific differences in affinity, enriching the surface with fibronectin and the bulk of the material with albumin and vitronectin that could be exposed or desorbed and subsequently delivered as part of the bulk material degradation.

Keywords

In vivo, protein adsorption, competition, biodegradable, albumin, vitronectin, fibronectin, fibrinogen.

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* **This chapter is based on the following publication:**

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1. INTRODUCTION

Research for several decades has emphasized the importance of protein adsorption to understand the interactions between cells and implanted material surfaces.[1, 2] Commonly used biomaterials are considered to be by design practically passive and non-toxic, but this is too a simplistic perspective; following blood-contact a wide variety of potentially adverse or at least interactive foreign body reactions (FBR) may be initiated.[3] The accumulation of large and small biological molecules from the surrounding fluid is one of the very first steps in the biological cascade of events that follow the implantation of a material,[4, 5] including the onset of FBR.[6] In either *in vivo* or *in vitro* environments different cell types[7, 8] including phagocytes[9] are known to spontaneously interact with biological molecules that have bound to surfaces rather than directly with the material itself. It is generally accepted that the type of molecule, it's conformation and the amount adsorbed, play critical roles in the cellular response[10, 11] and that the state of adsorption is strongly affected by surface properties, such as chemistry and wettability.[10]

The problems of biological molecule interaction with surfaces intended for biomedical applications are less commonly studied *in vivo* and the assessment of protein behaviour in living organisms is yet to be fully understood.[12] *In vitro* results provide valuable clues for the analysis of molecule interactions with surfaces in the *in vivo* environment, although studies using complex protein solutions can perform differently.[13, 14] It is accepted that a common problem in the study of protein adsorption phenomena is the apparent disparity between the *in vitro* and *in vivo* behaviour of biological molecules that continues to limit our understanding of the functionality of proteins in the *in vivo* interface and delays the applicability of materials in the biomedical field. Numerous proteins exist in the human body and the variety of molecules in physiological fluids affect important aspects of protein interaction with surfaces, such as protein competition, denaturation or blood clotting that directly modulate the overall biocompatibility and performance of implanted materials.[15-17]

Starch-based Biomaterials (SBB) have been investigated for a range of applications in the field of Tissue Engineering including scaffolding,[18, 19] bone[20] and drug delivery systems.[21] The *in vitro* biocompatibility[22] and *in vivo* host response[23] of starch-based biomaterials has been assessed and correlated with protein adsorption behaviour. The *in vitro* protein adsorption, competition and immunological cellular response has been determined previously[24] as well as the influence of SBB surfaces on protein adsorption and on the behaviour of osteoblast-like cells.[25] The use of a complex *in vivo* model to study protein adsorption onto and into materials intended for biomedical applications is of critical value to achieve implant success.

The hypothesis under investigation was that material bulk chemistry can directly affect protein adsorption in a manner that is protein specific and therefore, different for different proteins this effect can be determined *in vivo*. In this study proteins were selected for investigation based on their relevance in the biomedical field and their specific properties. The adsorption of albumin, fibronectin,

vitronectin and fibrinogen was studied. Albumin is a model molecule in protein adsorption studies due to its high concentration in physiological fluids like blood plasma and interstitial space[26] and to the ability to "passivate" biomaterial surfaces, reducing inflammatory and thrombogenic processes.[27] Members of the family of substrate adhesion molecules fibronectin[28, 29] and vitronectin,[30] were studied for their relevance in physiological processes such as the modulation of the immune system and tissue remodelling. These proteins are components of the extracellular matrix (ECM) and their integrin-binding sequences are responsible for modulating cell adhesion and migration. Finally, fibrinogen was selected considering its prominent role in multi-component processes such as the FBR in which fibrinogen is believed to adsorb immediately to the implanted materials.[6, 10]

Previous *in vivo* research that determined the analysis of protein adsorption is rare. This study investigated this critical phase of material interaction by analyzing the protein composition of the biolayer and tissue surrounding the *in vivo* implanted polymeric blends of corn starch and ethylene vinyl alcohol (SEVA-C). The results have been related to protein adsorption data from *in vitro* models that have utilised the same materials. In the *in vitro* studies, human serum was used as a dynamic multi-protein system and single and binary protein solutions of Human Serum Albumin (HSA), Human Fibronectin (HFN) and Human Vitronectin (HVN) were investigated.[24] One of the aims of the research was to determine the relationship between key biological molecules and the tissue reactions evoked by SEVA-C in the *in vivo* subcutaneous environment.[23] Protein adsorption patterns were analyzed for short and long implantation periods in order to relate *in vitro* protein adsorption with the *in vivo* inflammatory response.

2. MATERIALS AND METHODS

2.1. Starch-based Biomaterials (SBB)

Natural origin polymers were investigated consisting of biodegradable polymeric blends of corn starch with ethylene vinyl alcohol copolymer (SEVA-C). The amount of starch was 50% by weight (wt%) on SEVA-C. Conventional injection moulding technology was used to process samples under optimized conditions. Samples were cut into rectangular-shaped blocks 13 x 10 x 7 mm^3. Before implantation, the edges of the samples were trimmed and samples were rolled for 1 week in glass flasks to round machined edges and reduce the magnitude of edge effects.

Samples were sterilized by ethylene oxide in optimised conditions,[31] washed, and all subsequent experimental procedures were performed under sterile conditions.

2.2. Subcutaneous Implantation of SEVA-C Materials

The experiments were performed in Wistar rats, anaesthetized using Immobilon as previously described.[23] Briefly, four different materials were implanted subcutaneously in the back, two either side of the spine, for 3, 7 and 14 days, with three repeats for each material per time period. At the end

of the implantation period, rats were sacrificed by CO_2 and the tissue surrounding the implant was carefully dissected with the material *in situ* and snap frozen using isopentane in cardice. Explanted samples were stored at -80ºC until resin embedded.

2.3. Preparation of Implanted Materials

Samples of the SEVA-C material were embedded in Technovit 8100 New®-embedded (Heraeus Kulzer, Wehrheim, Germany) that is a low temperature glycolmethacrylate embedding system that facilitates the preservation of tissue antigenicity. Resin embedding of the implants was performed according to the manufacturer indications. Briefly, fixation was performed in a mixture of paraformaldehyde, lysine and periodate (PLP fixative) for 24h at 4ºC and washed in 50 mM ammonium chloride buffer. The implants were dehydrated in a cold acetone bath for 24h at 4 ºC. A mixture of 0.6 g of Technovit Hardener I in 100 ml base-liquid 100% Technovit was used as the infiltration mixture for 24 hours. An embedding mixture was then used (1 part of Technovit Hardener II and 30 parts of infiltration solution); the infiltration of the samples was performed at -55 ºC for 4 days followed by 2 days at -20 ºC for polymerisation. Sample blocks were then trimmed with a low speed circular saw (IsoMet® Low Speed Saw, Buehler LTD., USA). 7μm thick sections were cut using a Polycut Microtome (Leica, UK) and then mounted on 3-aminopropyl-triethoxysilane (APES)-coated slides, fixed with acetone for 5 min, air-dried and kept short term at 4 ºC until staining.

2.4. Immunohistochemistry of In Vivo Protein Adsorption

Antibody labelling of specific biological molecules was used to determine the adsorption of Albumin (Alb), Fibronectin (Fn), Vitronectin (Vn) and Fibrinogen (Fbg). For the detection of the biomolecules, tissue sections were stained using an avidin-biotin alkaline phosphatase technique, as described elsewhere.[32] In brief, materials were incubated with 0.1% trypsin solution to expose masked epitopes, exposed to horse serum for 20min and incubated with primary antibodies overnight at 4ºC, for the identification of the different proteins. After that time, materials were incubated with biotinylated rabbit anti-goat IgG antibody (Dako A/S, Denmark) for 1h at room temperature. The avidin and biotinylated horseradish peroxidase complex (Vector Laboratories Ltd., UK) was added to all materials for 30min and the substrate reaction was developed using the Alkaline Phosphatase Substrate Kit (Vector Laboratories Ltd., UK). Each incubation was followed by one wash with Phosphate Buffered Saline (PBS) for 5min. Materials were washed and mounted in permanent aqueous mounting medium (Serotec Ltd, UK). Each time period studied had one sample stained as a control replacing the primary antibody with buffer and parallel isotype reference staining was always conducted.

2.5. Image Analysis and Protein Diffusion

Immunostaining results were observed using transmitted light microscopy (Axioplan 2 Imaging, Zeiss, Germany) and image acquisition by digital camera equipment (AxioCam, Zeiss, Germany). Subsequently, measurements of protein diffusion and biolayer thickness were performed for the assayed molecules. For each staining, three sections per time period were analyzed and twenty repeats per section were used. To quantify protein diffusion KS400 3.0 image analysis software (Zeiss, Germany) was used.

2.6. Statistical Analysis

Data analysis of protein diffusion on and into the implanted materials was presented as arithmetic means/standard deviations of the mean (mean/SD). Statistical data evaluation was made by the ANOVA/Tukey multiple comparison tests to detect differences between groups (SPSS 13.0.1, Statistical Analysis Software, USA). In statistical evaluations, n>20 (by section) and $p<0.05$ was considered as statistically significant.

3. RESULTS AND DISCUSSION

3.1. Co-diffusion of Albumin and Vitronectin in the Bulk of the Implant

The results obtained by antibody staining showed that albumin (Figure 1) and vitronectin (Figure 2) adsorbed into the bulk of the implant and were distributed in the same area of the implanted material. Moreover, immunodetection of both molecules indicated an absence of layer formation on the surface of SEVA-C material.

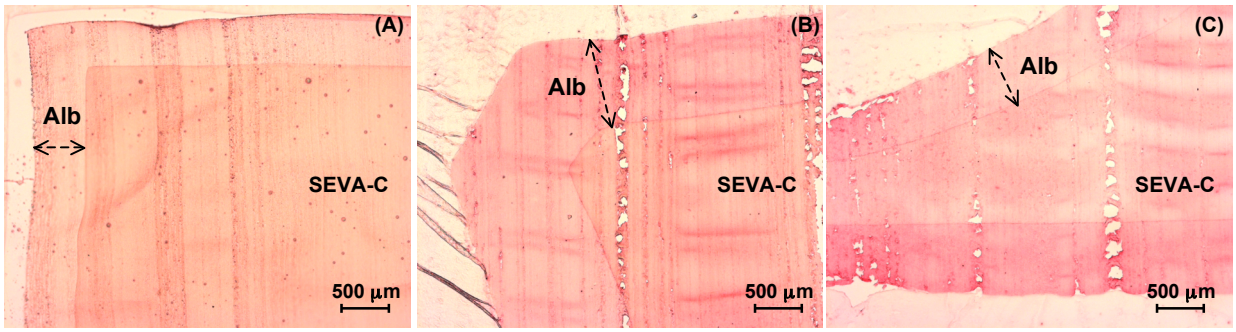

Figure 1. Immunohistochemical staining of SEVA-C sections for serum albumin (Alb) after subcutaneous implantation for 3 (A), 7 (B) and 14 (C) days.

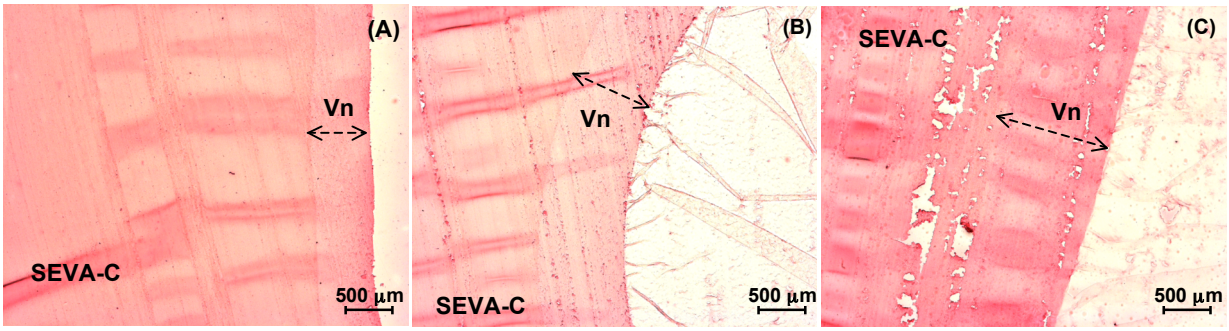

Figure 2. Immunohistochemical staining of SEVA-C sections for Vitronectin (Vn) after subcutaneous implantation for 3 (A), 7 (B) and 14 (C) days.

The analysis of Alb and Vn behaviour showed that the increase in the diffusion distance was proportional to the implantation period. Summarized in Figure 3 the analysis of Figures 1 and 2 indicated that for both proteins a staining increase was observed from day 3 up to day 14 of implantation. Overall, the analysis of the diffusion of both biomolecules indicated no differences in the distances measured, except for the shorter implantation period. At day 3 of implantation, albumin diffused 36.64 ± 2.14 µm in the bulk of the implant in contrast to vitronectin that showed increased diffusion of 45.98 ± 5.06 µm (p<0.05). From 3 and 7 days of implantation, a faster diffusion of albumin was observed when compared to vitronectin, thus resulting in similar measurements for both proteins. The data obtained for both proteins after 7 and 14 days of implantation was 115 µm and 120 µm, respectively (Figure 3).

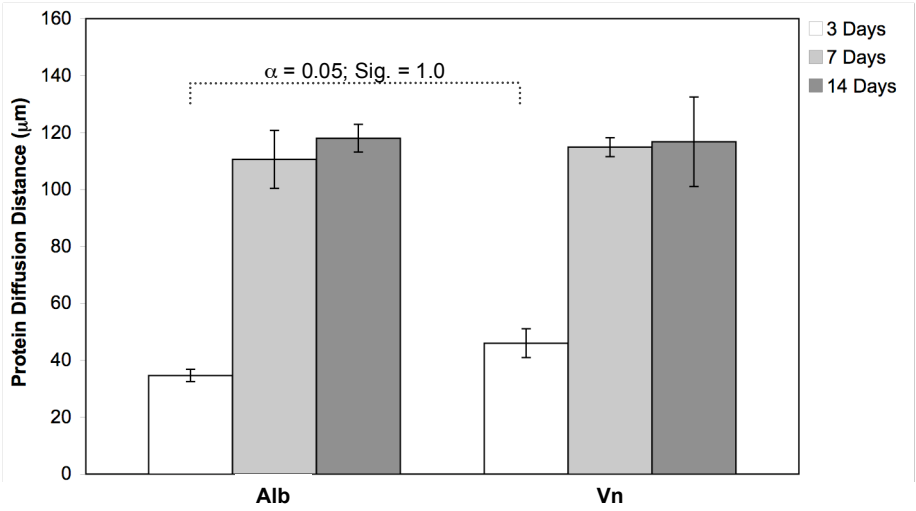

Figure 3. Diffusion of Albumin (Alb) and Vitronectin (Vn) detected by immunostaining of SEVA-C materials implanted for 3, 7 and 14 days.

Table 1. Diffusion of Albumin (Alb) and Vitronectin (Vn) determined by immunohistochemistry of SEVA-C materials implanted for 3, 7 and 14 days (A) and ANOVA analysis (B).

SEVA-C Materials	N	Subset for alpha = 0.05			
		1	2	3	4
Alb 3 days	26	**34,64**			
Vn 3 days	34		**45,98**		
Alb 7 days	36			110,55	
Vn 7 days	30			114,87	114,87
Vn 14 days	30				116,75
Alb 14 days	33				117,98
Sig.		*1.00*	*1.00*	*0.33*	*0.69*

The degradation and hydration effects that may have taken place in the *in vivo* environment possibly enabled the diffusion of small molecular weight biomolecules since Fn and Fbg were not found in the bulk of SEVA-C materials.

The glycoprotein Vn is known to circulate in plasma at concentrations of around 200 to 400 μg/mL. Vn is an asymmetrically shaped molecule found in fresh human plasma as a heterogeneous mixture of two structurally and functionally distinct forms: heparin-binding form (2%) and non-heparin-binding species (98%).[33] The monomer non-heparin-binding Vn presents a Stokes' radius of 3.9 nm consisting of a highly folded conformation and 75 kDa polypeptide.[33, 34] On the other hand, serum albumin, with a molecular weight and Stokes' radius lower than that of vitronectin, of 66 kDa[35] and 3.53 nm,[36] respectively, is the most abundant plasma protein in the interstitial space.[26] The physical properties of both proteins could suggest higher diffusion of Alb compared to Vn. Interestingly, the results indicate that for early implantation times the affinity of vitronectin was significantly higher than that observed for albumin (Table 1). In previous experiments[24] using diluted human blood plasma and SEVA-C polymeric blends, vitronectin showed considerable enrichment at the surface after 24 h incubation, when compared to other adhesive and non-adhesive proteins. The higher affinity of the glycoprotein to the starch based surface under competitive conditions could also provide an explanation for its increased diffusion into the bulk of the implant. Studies from Fabrizius-Homan *et al.*,[37, 38] found that the competitive potential of vitronectin could indicate a greater resistance to displacement when compared to other molecules such as fibrinogen, and that this protein could bind and activate platelets effectively forming thrombi even in sub monolayer surface concentrations. The results demonstrated the co-diffusion of molecules as the biomaterial equilibrated to the *in vivo* environment. As the material adsorbed water from the surrounding physiological fluids the porosity increased, proteins penetrated as a consequence of this increased permeability. The significant ability of a partially degradable material to self-select molecules could be utilised to provide material specific benefits; two advantages could be envisioned: 1) as the material degrades, molecules could become available for

cell tagging by exposing reactive epitopes; 2) as the desorption and exchange of proteins increases, SEVA-C could gradually deliver albumin and vitronectin at the immediate surface interface.

3.2. Fibrinogen Displacement by Fibronectin Multilayer Formation

The results of fibronectin immunostaining (Figure 4) contrasted albumin and vitronectin; fibronectin was clearly detected in the immediate implant-tissue interface (Figure 4A). Fn adsorbed to the surface in a multilayer pattern and was not found dispersed in the tissue surrounding the implant. Moreover, the deposition rate and final adsorbed amount were substantially higher when compared to fibrinogen (Figure 5). The increase in Fn layer thickness was proportional to the implantation period, reaching approximately 310 μm after day 7 (Figure 4B) and 1015 μm after day 14 of implantation (Figures 4C and 4D). This concurred with previous studies using hydroxyapatite or titanium[14]; in this study adsorption of higher molecular weight proteins increased with longer incubation periods. At the last implantation time point the area surrounding the implant was characterized by a multilayered fibronectin rich pattern.

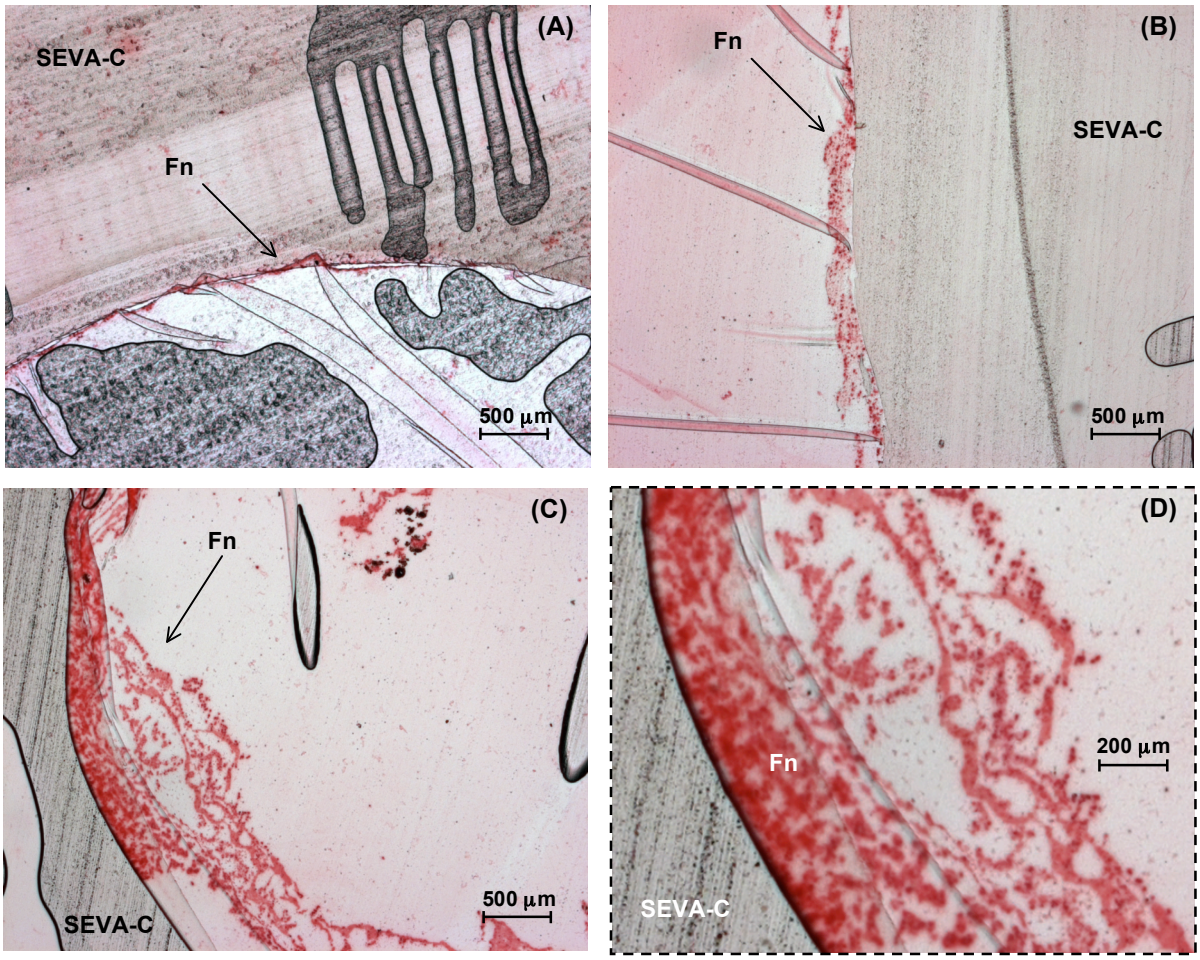

Figure 4. Immunohistochemical staining of SEVA-C sections for Fibronectin (Fn) after subcutaneous implantation for 3 (A), 7 (B) and 14 (C and D) days.

Several studies[39, 40] have demonstrated the influence of fibronectin in the development of the vascularisation. It is also known that following injury from the implantation process itself, *in situ* production of fibronectin occurs and thus, adsorbed fibronectin may not derive exclusively from circulating plasma and interstitial fluids. The high affinity and consequent adsorption of Fn onto the surface of SEVA-C materials can ultimately cause the remodelling of the surrounding tissue.

Antibody labelling of fibrinogen indicated the absence of recruited protein by SEVA-C at day 3 of implantation (Figure 5A). At day 7 a thin film of fibrinogen had accumulated on the implanted surface with thickness of 39.7 ± 8.8 μm (Figure 5B). Similar results were obtained for longer implantation, however fibrinogen was not detected in the implant tissue interface. In opposition, Fbg was displaced 1077.4 ± 192.5 μm from the surface (Figures 5C and 5D).

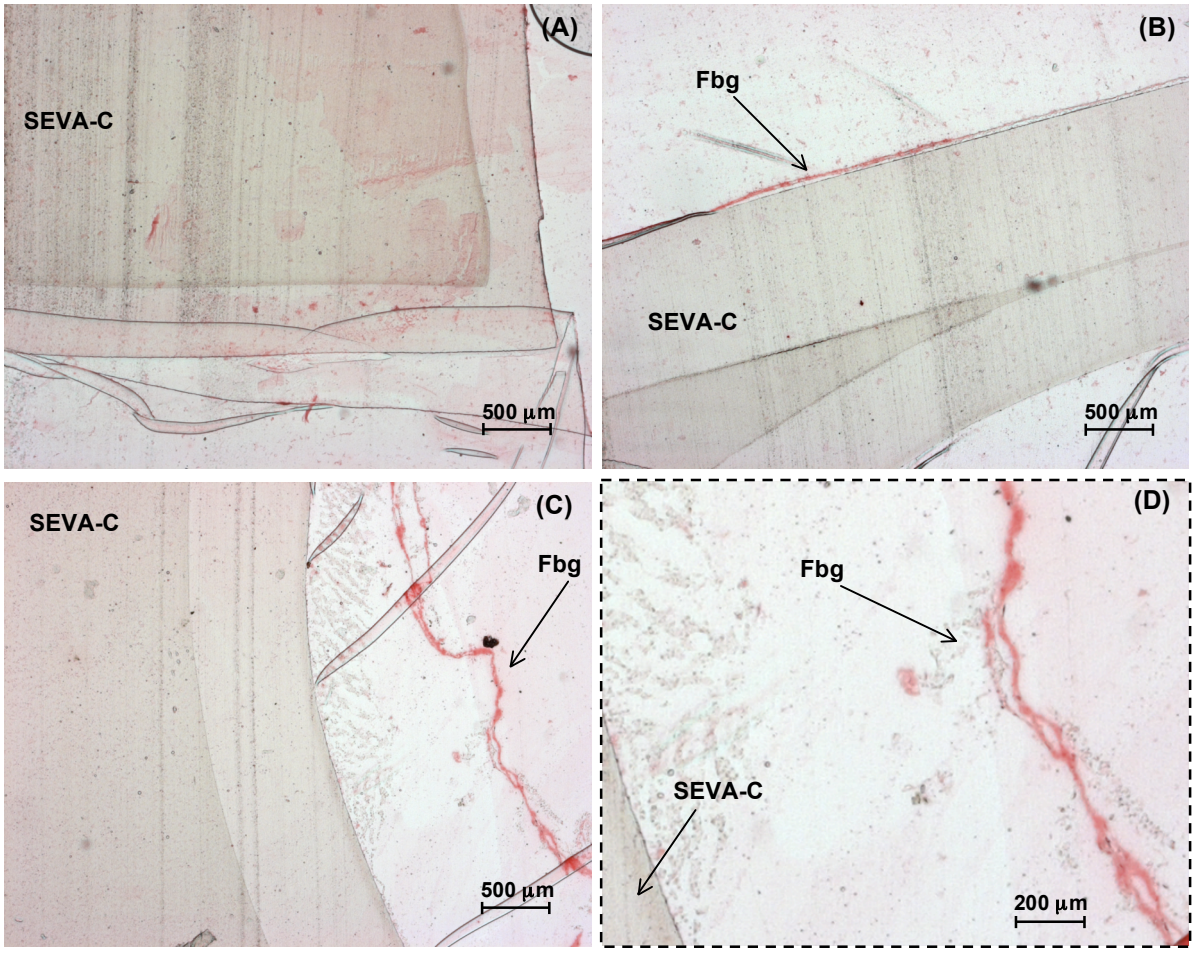

Figure 5. Immunohistochemical staining of SEVA-C sections for Fibrinogen (Fbg) after subcutaneous implantation for 3 (A), 7 (B) and 14 (C and D) days.

The relevant role of fibrinogen in a multi-component process such as the foreign body reaction is known.[6, 10] Previous studies have indicated immediate adsorption of fibrinogen to the implanted materials and the formation of multilayer patterns.[6, 41] Moreover, there is considerable data in the

literature showing that fibrinogen presents a high affinity for a large number of surfaces and is preferentially adsorbed from whole plasma, predominating in the deposited protein layer.[42, 43] According to Vroman *et al*.,[44, 45] fibrinogen was demonstrated to dominate the proteinaceous film during the early implantation time. In contrast, the results obtained in this study, indicated a low affinity of this protein to the surface of the starch-based material. Fibrinogen was absent at day 3 (Figure 5A) and was displaced from the interface tissue material after 14 days of implantation (Figure 5C). In opposition to fibronectin, the low intensity of the anti-fibrinogen staining and its subsequent displacement from the surface indicated a poor interaction between fibrinogen and the implanted material. From competition studies, it is known that exchanged proteins typically consist of the less tightly surface bound biomolecules.[46]

Previous *in vitro* studies[42] using protein-deficient plasmas indicated the possibility of fibrinogen displacement by other molecules such as high molecular weight kininogen and, to a lesser extent, by factor XII. An important result of the present study is the indication that fibrinogen displacement resulted from the adsorption of fibronectin. The dynamics of fibronectin adsorption onto the SEVA-C surface was highly selective for this protein as shown in Figure 4 and in *in vitro* studies,[24] this could be the reason for fibrinogen displacement from the interface instead of being a consequence of the activity of smaller size molecules as described by other authors. Effectively, the fibrinogen displacement distance (Figure 4C) suggests that the Fn multilayer (Figure 5C) was localized in between the implant surface and the thin Fbg layer.

Studies[10] already support the potential for the type of protein on the surface of a material to affect the host response in terms of inflammation following implantation, and specifically to influence fibrinogen in mediating a phagocyte response. Accordingly, previous studies using starch based materials[23] have verified that SEVA-C polymeric blends induced mild inflammatory reactions, as determined by the macrophage/monocyte and lymphocyte responses after 14 days of implantation. The previously observed immunogenic response can be related to these results that indicated a hypofibrinogeneic tissue surrounding the implant as well as the displacement of this protein from the surface after 14 days of implantation.

The role of fibrinogen in adverse body reactions has been supported by evidence that biomolecules tightly adsorb onto hydrophobic implanted surfaces as a consequence of conformational changes in their ternary structure.[47] The dynamics of fibrinogen adsorption was evaluated onto SEVA-C material characterised by its hydrophobic nature,[25] indicative that other properties such as surface chemistry could play a role in the overall fibrinogen behaviour. Moreover, the possibility of conversion of fibrinogen onto fibrin was not indicated as the layer determined after 14 days matched the thickness of that after 7 days. In this context, the presented results could indicate that due to its properties, SEVA-C could limit the complicated process of surface induced thrombosis.

4. CONCLUSIONS

In vivo modelling provided further insights into the dynamics and equilibriums established between different proteins and the implanted SEVA-C material. The *in vivo* behaviour of key proteins following SEVA-C implantation and their correlation to the previously characterised cellular host response has been demonstrated.

Albumin was localized in the same areas as vitronectin, diffusing into the bulk of the implant. The diffusion mechanism in place was dictated by the chemical characteristics of the proteins and indicated that for early implantation times the affinity of vitronectin was significantly higher than that for albumin. In contrast to albumin and vitronectin, fibronectin was observed mainly at the tissue implant interface and fibrinogen was displaced from the SEVA-C surface. The results suggest that the displacement of fibrinogen from the surface can be related to the high adsorption of fibronectin. Consistently, the distance from the surface at which fibrinogen was detected indicated the absence of interaction between these two proteins with fibrinogen, which is indicative of a lower affinity to the SBB surface and the fibrinogen distribution being dependent on the fibronectin layer. The tissue surrounding the implant was hypofibrinogeneic and rich in fibronectin possibly providing an explanation for the moderate inflammatory response of SEVA-C that has been determined by previous studies.

The SEVA-C high affinity for fibronectin effectively influenced the distribution of fibrinogen by displacement from the surface. Simultaneously, co-diffusion of vitronectin and albumin induced an enrichment of the bulk of the material with these proteins. The material could therefore self-select and incorporate clinically relevant molecules to enable SEVA-C implants to continuously expose cell adhesive epitopes as well as anti-thrombogenic molecules at a controlled rate that was related to bulk degradation.

Acknowledgements

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SECTION 4

CHAPTER IX

GENERAL CONCLUSIONS AND FINAL REMARKS

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1. IN VITRO ANALYSIS OF PROTEIN ADSORPTION

Analyzing the interaction of proteins with the surfaces of materials intended for biomedical applications is fundamental for understanding cellular events and the overall host response. By single protein adsorption studies the complexity of blood bio environment or the biocompatible potential of biomaterial surfaces can not be assessed. In the present work one of the major aims was to investigate the adsorption mechanisms of clinically relevant proteins onto the surface of starch based biomaterials (SBB). Single protein systems and the complex multi-protein environment were evaluated to assess different aspects of protein-surface dynamics.

1.1 Distribution of the proteins on the surfaces

The distribution and density of biomolecules onto surfaces for clinical use are of utmost importance. In general, proteins are the cell anchor points to surfaces aimed to be colonized and thus integrated in a certain biological environment.

The patterns of protein adsorption onto SBB materials were evaluated to determine whether it was a material or protein dependent phenomenon. The immunostaining technique provided important qualitative and semi-quantitative analysis of different starch-based blends studied in terms of protein adsorption pattern and intensity. For all proteins studied it was observed that SCA and, to some extent, SPCL presented a less homogeneous protein coating than SEVA-C. Considering the order SCA, SPCL and SEVA-C, we obtain an increase in miscibility and interaction between the starch and the synthetic polymeric phases, which also provides a decrease in surface chemical heterogeneity. It was demonstrated that independently of the biomolecule in study, proteins formed a smooth and homogeneous protein coating at SEVA-C surfaces when compared with SPCL and SCA. Thus, allowing concluding that the type of material defines the distribution of the studied biomolecules.

1.2. Protein adsorption, kinetics and competition

The adsorption amounts, the kinetic and the competitive behaviour of proteins onto SBB surfaces were investigated. Besides surface analysis provided by microscopy techniques, fluorimetry proved to be a useful technique. The type of adsorption obtained onto SBB surfaces was characteristic of a Freundlich type, in contrast to the control surfaces (TCPS) that fit the Langmuir model. The first model was more suitable for heterogeneous surfaces and consider further the dynamics of protein-protein as well as protein-surface interactions.

Onto SCA and SPCL the equilibrium of adsorption was achieved faster than that of SEVA-C. Albumin adsorption was affected by the material composition as well as by the concentration of the protein solution, preferentially adsorbing onto SCA and SPCL. Fibronectin adsorption reached higher values on SEVA-C and SPCL.

On the competitive behaviour of proteins for the different surfaces, fibronectin demonstrated a different adsorption activity for the different materials as assessed by single and competitive adsorption with albumin. There was no effect on the adsorption of HSA and HFN onto SPCL in competitive conditions. Fibronectin adsorption was reduced on SCA in the presence of albumin, for which adsorption simultaneously increased, while the opposite situation was observed for TCPS. Competitive conditions were favourable to the affinity of both molecules by improving the affinity of albumin and fibronectin onto SEVA-C surfaces.

When the complexity of the study was increased by the use of human blood serum as the protein source, differences in protein behaviour were observed: in opposition to vitronectin and to fibronectin, the competitive potential of albumin to adsorb onto starch-based surfaces was decreased. All studied surfaces showed, in general, good VN adsorption and SPCL showed the highest protein adsorption levels, regardless of the biomolecule studied. The adsorption isotherms and the competition of plasma proteins on starch-based biomaterials were elucidated.

1.3. Effect of surface modification on protein adsorption

The potential of surface properties in influencing protein adsorption, such as morphology, chemistry and hydrophobicity were evaluated. In this study, the capacity of oxygen-based plasma treatment to increase protein adsorption onto PDLLA, a gold standard biodegradable material, was assessed. The amounts of adsorbed proteins in single systems (albumin and fibronectin) were shown to increase after plasma treatment. The surface modification increased the wettability as well as surface energy, which polar component of surface energy was mainly affected. Moreover, treated DLLA showed increase in total O% of treated over non-treated samples. Higher wettability and the introduction of oxygen functionalities created sites for binding proteins by polar interactions or hydrogen bonding. Also, the simultaneous increase in surface micro-heterogeneity could have played a role in the increased protein adsorption. In general, oxygen plasma treatment resulted in PDLLA surfaces of higher adsorption affinities.

1.4. Protein adsorption/desorption studies

In the field of surface science the study of adsorption-desorption kinetics or adsorption reversibility related to the folding-unfolding events, have been investigated. The biological environment was simulated and insights into the dynamic equilibriums established between proteins and surfaces were obtained from DCA measurements.

Hysteresis profiles obtained for the different protein solutions indicated that different interactions between these proteins and substrates were taking place during the adsorption process. The SCA surface was the most interactive in terms of hysteresis changes due to immersion in PBS or to albumin adsorption, revealing saturation at shorter time periods as observed by the higher hydrophilicity. The most significant differences were observed in the study of protein desorption. The differences observed in the desorption phase indicated conformational changes of the proteins. SCA and SPCL were demonstrated to completely revert the hysteresis profile back to the original one obtained with protein-free buffer. In contrast, SEVA-C showed irreversible hysteresis independently of the protein used in the desorption study. The same results were obtained for the study of protein competition. On SEVA-C, stronger interactions such as hydrophobic forces were established and it is likely the rearrangement of protein conformation had occurred. The biological activity of the proteins on the SBB surfaces was affected by the material properties and could therefore modulate the cell biological response to the different starch-based materials.

2. IN VITRO CELL RESPONSE TO PROTEINS AND SURFACES

Cell response to a biomaterial surface is considered one of the major factors in determining the biocompatibility of a material because this step affects subsequent cell proliferation and differentiation pathways. It is known that interfacial reactions occurring when a material contacts a biological environment are modulated by both the surface and the biomolecules, such as proteins, that interact with it. The outcome of these interactions subsequently affect the cellular response.

2.1. Co-culture of monocytes/macrophages and lymphocytes

The effect of human albumin, fibronectin and vitronectin on modulating leukocyte adhesion gave rise to results that demonstrate leukocyte behaviour to be affected by the protein studied and by the culture time period: (*i*) short-term effects in cell adhesion were found to be developed in the presence of vitronectin, (*ii*) while at longer-term, albumin induced the increase in cell attachment.

In addition, it was shown that cell populations adhere to protein coated and non-coated surfaces, although monocytes and/or macrophages were found in higher numbers mainly for shorter incubation periods.

2.2. Bone related cells: FRCs and MG63 osteoblast like cells

The surface modification technique used in this study, oxygen-based plasma treatment successfully functionalize/activate the surface of SBB and PDLLA without affecting the bulk properties of both materials. Both, the effects of plasma modification and the presence of different protein systems on the viability and morphology of MG63 osteoblast-like cells were evaluated.

In the absence of pre-incubated proteins, the plasma treated SPCL surfaces were demonstrated to greatly improve osteoblast-like cells proliferation. Protein types and the presence of other proteins were shown to be the key for cell adhesion and proliferation numbers. In several cases, cell morphology was shown to be related to surface properties created by the plasma treatment. In contrast to SEVA-C surfaces, cell adhesion and proliferation on SCA were found to be enhanced for non-treated surfaces. Adhesion on treated and untreated SPCL was very similar, while plasma modification clearly promoted MG63 cells proliferation. The morphology of the cells was studied: on SEVA-C surfaces was primarily defined by the protein system used, while on SPCL it was mainly affected by the plasma treatment.

Onto PDLLA, MG63 osteoblast-like cells and primary cultures of FRC cells were used to assess both the effect of plasma treatment and protein adsorption on cell attachment and proliferation. In the absence of pre-adsorbed proteins, neither MG63 nor FRC cells could distinguish between treated and untreated surfaces. However, MG63 osteoblast-like cells showed higher proliferation rates for plasma treated PDLLA surfaces, indicating a direct effect of the oxygen based plasma technique. In turn, gas plasma treatment, by influencing protein adsorption on the surfaces, was shown to affect cell response to the surfaces. The effect on the treatment over the cultured cells was only observed by combining gas plasma modification of the surface with the protein adsorption, thus indicating a crucial role for adsorbed proteins in mediating the response of osteogenic cells to the plasma-treated PDLLA surface.

3. IN VIVO PROTEIN ADSORPTION: HOST RESPONSE TO SEVA-C POLYMERIC BLEND

To some extent, the *in vitro* results supported the idea that single solution studies were not good simulations of the real situation of the complex bioenviromnent. Consequently, *in vivo* modelling was performed, which provided further insights into the dynamics and equilibriums established between different proteins and the implanted SEVA-C material. The *in vivo* behaviour of key proteins following SEVA-C implantation and their correlation to the previously characterised cellular host response has been demonstrated.

Albumin was localized in the same areas as vitronectin, diffusing into the bulk of the implant. The diffusion mechanism in place was dictated by the chemical characteristics of the proteins and indicated that for early implantation times the affinity of vitronectin was significantly higher than that for albumin. In contrast, fibronectin was observed mainly at the tissue implant interface and fibrinogen was displaced from the SEVA-C surface. The results suggest that the displacement of fibrinogen from the surface can be related to the high adsorption of fibronectin. Consistently, the distance from the surface at which fibrinogen was detected indicated the absence of interaction between these two

proteins with fibrinogen, which is indicative of a lower affinity to the SBB surface and the fibrinogen distribution being dependent on the fibronectin layer.

The SEVA-C high affinity for fibronectin effectively influenced the distribution of fibrinogen by displacement from the surface. Simultaneously, co-diffusion of vitronectin and albumin induced an enrichment of the bulk of the material with these proteins. The material could therefore self-select and incorporate clinically relevant molecules to enable SEVA-C implants to continuously expose cell adhesive epitopes as well as anti-thrombogenic molecules at a controlled rate that was related to bulk degradation.