

Susana Maria Ribeiro e Sousa Mendes de Freitas

**Albumin and fibronectin adsorption
and osteoblast adhesion on titanium oxides**

Tese submetida à Faculdade de Engenharia da Universidade do Porto
para candidatura à obtenção do grau de Doutor em Engenharia Metalúrgica e de Materiais

Faculdade de Engenharia

Universidade do Porto

Fevereiro 2007

This thesis was supervised by:

Professor Mário Adolfo Barbosa (supervisor)

Faculdade de Engenharia, Universidade do Porto

INEB – Instituto de Engenharia Biomédica, Laboratório de Biomateriais, Universidade do Porto

Professor Pedro Moradas Ferreira (co-supervisor)

Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto

IBMC – Instituto de Biologia Molecular e Celular, Universidade do Porto

The research described in this thesis was conducted at:

INEB – Instituto de Engenharia Biomédica, Laboratório de Biomateriais

IBMC – Instituto de Biologia Molecular e Celular, Cellular and Applied Microbiology laboratory
and Advanced Light Microscopy facility

IST, Instituto Superior Técnico, Centro de Química Estrutural

IST, Instituto Superior Técnico, Departamento de Física

The research in this thesis was financially supported by:

Programa PRODEP acção 5.2

...to Miguel, Leonor and Francisca

Acknowledgements

I would like to express my sincere gratitude to the persons who helped me during this long-term project, and made this thesis possible.

I will start acknowledging my supervisors, Professor Mário Barbosa and Professor Pedro Moradas Ferreira for the opportunities they gave me, and for all of their help, support, patience, and friendship during the development of this work. It was for me a privilege to have the opportunity of working in renowned institutions, INEB and IBMC that have hosted me in a very friendly way, and always made available every necessary mean to carry out my research work. At these institutions I found exceptional researchers with whom I shared experiences and learned with, but I also made friends for life.

I would like to acknowledge Professor Fernando Jorge Monteiro for his friendship along these years.

I would also like to acknowledge the collaboration of several persons from the following institutions:

At INEB: I will probably face the most difficult of the tasks, which is to express my sincere gratitude to all my remarkable colleagues and friends at INEB, with whom I had the privilege of working with. As much as I could write here would never be enough to show how grateful I am to them all.

I would like to thank to a long time and dear friend Cristina Ribeiro, whose friendship goes far beyond laboratory relationship. Thank you for being there whenever I needed!

I also like to thank Isabel Amaral for being a special friend that also provided me with a considerably enrichment of knowledge of my work do to her excellent competence.

I am also very grateful to Manuela Brás and Meriem Lamghari for their friendship and support and for work collaboration in AFM and cell culture experiments.

I am thankful to Ana Paula Pêgo, Cristina Barrias, and Cristina Martins, for their encouragement, prompt advices, patience, and above all for their friendship. Ana Paula Filipe, Ana Queiroz, Judite Barbosa, Maria Ascensão Lopes, Maria Pia Ferraz, and Pedro Granja thank you for being always my friends and supported me in this work.

Although not influencing my work so directly, several other colleagues have also greatly contributed with their help and friendship and made my life at the lab much easier. I am very grateful to them all: Anabela Dias, Andreia Cabral, Alis Mateus, Ana Rosa Carvalho, Carla Monteiro, Carlos Fonseca, Cristina Matos, Daniel Duarte, Diamantino Falcão, Dulce Carqueijó, Eliana Vale, Fátima Pina, Hugo Oliveira, Inês Gonçalves, Lino Ferreira, Januário Lima, José Paulo Pereira, Marta Lima, Patrícia Cardoso, Pedro Sá, Rui Azevedo, Sandra Teixeira, Serafim Oliveira, Sofia Teixeira, Susana Carrilho, and Virgínia Fonseca.

At ISEP: I would like to express my sincere gratitude to my colleagues and friends, especially to Prof. Cristina Delerue Alvim Matos for all the support, and encouragement, but above all for being my friend.

At IBMC: To Dra. Amélia Amorim, to Victor Costa, Frederico Silva, for their availability whenever needed; to Paula Sampaio for her help in Confocal Laser Scanning Microscopy analyses; to Isabel Cardoso and M Rosário Almeida who guide me at the beginning of my radiolabelling work.

At CEMUP: To Doctor Carlos Sá for his assistance in XPS analyses.

At IST: To Professor Benilde Saramago, from Departamento de Engenharia Química, for scientific support in the characterization of surface energetics. To Professor Luís Viseu Melo, from Departamento de Física, for his assistance in AFM measurements.

At INESC: To Professor Paulo Freitas and Doctor Susana Freitas for the titanium oxide sputtered sample preparation.

Many thanks to all my other friends for their support, particularly in the most difficult times. No one is forgotten even if it is not mentioned! I would like to especially express my gratitude to Nina Maio who was always present and for being a true friend.

Eugénia Leitão and Neal for being exceptional friends all along these years, and for english assistance in the final phase of this thesis.

Finally, I would like to express my sincere gratitude to my parents, to whom I own what I am,

and who supported me during this long-term journey, in so many ways. To my parents-in-law, to my sister, to my sisters-in-law and brothers-in-law and to all of my nephews and nieces, I also want to acknowledge all the support and friendship.

At last, but above all, special thanks to Miguel for your support and patience, for being always unconditionally present when I needed you most, and for supporting my choices! Thank you dear Leonor and Francisca for your patience and comprehension for every weekends and holidays I had to work in my thesis instead of being available to be with you both. Your prayers were finally heard!

Several promising multifunctional biomaterials are currently being investigated and applied to new types of artificial organs and advanced medical devices. However, metals are still the more suitable biomaterials to replace hard tissue due to good bone matching mechanical properties. The physiological stability of titanium associated with its corrosion resistance as well as exceptional mechanical properties and favorable osteointegration, makes it one of the most used biomaterials for bone and cartilage replacement.

When biomaterials are implanted in the body, proteins immediately adsorb onto the implant surfaces. The tissue response to implanted materials arises mainly from differences in the adsorption of proteins that depend on surface parameters, such as chemical composition, surface roughness, and surface charge, among others.

The main objective of this thesis was to identify the main features that govern the protein adsorption on titanium oxide surfaces using different topographies, at the nanometric scale. The experimental approach involved two key proteins, albumin (HSA) and fibronectin (FN), adsorbed both from single protein, as well as from complex protein solutions. Albumin is the predominant plasma protein, and is generally considered to “passivate” surfaces preventing cell adhesion and greatly reducing the acute inflammatory response to the material. On the contrary, FN is an adhesive protein of the extracellular matrix (ECM) required for integrin-receptor-based cell adhesion, cytoskeleton reorganization, proliferation, and differentiation.

This work was based on the preparation and characterization of titanium oxide surfaces chemically similar to those used in clinical practice, with different roughnesses. Two surfaces, TiO₂ sputtered on Si (TiO₂ sp), and TiO₂ formed on commercially pure titanium after H₂O₂ immersion (TiO₂ cp), were developed. The two surfaces presented identical chemical composition but different topography, as shown by XPS, wettability studies, and AFM. Surface roughness was always significantly higher on the TiO₂ cp surfaces.

Subsequently, the above TiO₂ surfaces were evaluated regarding their ability to adsorb human serum albumin, HSA, or human plasma fibronectin, FN, both from single protein solutions, plasma, and diluted plasma. FN showed a contrasting affinity to the TiO₂ surfaces, as compared to HSA. A higher affinity of the FN solution to the TiO₂ cp surface was found, as compared to the TiO₂ sp. The radiolabelling data also shown more FN adsorbed onto TiO₂ cp than onto TiO₂

sp surfaces. However, FN molecules appeared to be more strongly attached to the former surface, as suggested by the work of adhesion and by the exchangeability studies, following the trend observed for albumin. AFM allowed direct visualization of the adsorbed FN in air and in liquid, on both surfaces tested. Upon adsorption, FN appeared to form globular aggregates or ellipsoids. The FN aggregates coalesced forming clusters as time of adsorption and concentration increased. AFM results provided novel evidence that FN adsorbs spontaneously as self-assembly at TiO_2 surfaces, as a function of time. The aggregate structure is an intermediate feature shared by some protein fibrillar assemblies at interfaces, which is believed to promote cell adhesion, and cytoskeleton organization.

Finally, the effect of competitive preadsorption of HSA and FN on both TiO_2 substrates from binary protein mixtures and from 10% plasma on osteoblast adhesion and morphology was investigated, using MC3T3-E1 osteoblasts. Our results showed a direct correlation between osteoblast adhesion and the amounts of FN and HSA adsorbed on TiO_2 surfaces. In addition, fewer osteoblasts adhered after 4 and 24h to both substrates after preadsorbed with HSA, HSA/FN=200 and 10% plasma, as compared to the surfaces preadsorbed with FN and HSA/FN=1. For the latter, FN was able to compensate the inhibitory effect of HSA on osteoblast adhesion. Therefore, the presence of lower amounts of co-adsorbed albumin may improve presentation of FN in a more integrin-recognized conformation. This fact suggests that some degree of molecular packing prevents loss of integrin-binding activity.

FN reversibility does not seem to be dependent on the HSA/FN adsorption mass ratio in solution, suggesting that FN competitively adsorbs to TiO_2 in a favorable conformation allowing exchange with other FN molecules in solution.

In conclusion, the HSA and FN adsorption pattern on TiO_2 and the subsequent response of osteoblasts (cell adhesion and morphology), depends on the type of TiO_2 surface. The presence of lower amounts of co-adsorbed albumin may improve the exposure of FN in a more integrin-recognized conformation, suggesting that some degree of molecular packing is likely to prevent loss of integrin-binding activity.

Plusieurs biomatériaux multifonctionnels sont actuellement étudiés et aussi utilisés dans différents types d'organes artificiels ainsi que dans des outils médicaux. Cependant, les métaux sont toujours considérés plus adéquats pour substituer le tissu osseux due à la proximité de leur propriétés mécaniques. La stabilité physiologique du titanium associée à sa résistance à la corrosion, ses propriétés mécaniques exceptionnelles et son potentiel d'ostéointégration ont permis qu'il soit le biomatériau le plus utilisé pour la substitution de l'os et du cartilage.

Quand les biomatériaux sont implantés dans l'organisme, les protéines adsorbent immédiatement à la surface de l'implant. La réponse du tissu hôte aux matériaux implantés résulte essentiellement des différences dans l'adsorption des protéines. Cette réponse dépend des paramètres de la surface de l'implant dont la composition chimique, la rugosité et la charge. Le principal objectif de ce travail a visé d'identifier les caractéristiques principales qui régulent l'adsorption des protéines aux surfaces du titanium oxydé en utilisant différentes topographies à l'échelle nanométrique. L'approche expérimentale inclu deux protéines clés, l'albumine (HSA) et la fibronectine (FN). L'adsorption de ces deux protéines a été réalisée à partir d'une solution de HSA ou de FN ou d'une solution protéique plus complexe. La HSA est la protéine prédominante du plasma et généralement considérée de type passivant des surfaces, évitant l'adhésion cellulaire et réduisant la réponse inflammatoire au matériau implanté. Contrairement à la HSA, la FN est une protéine d'adhésion de la matrice extracellulaire (MEC) nécessaire à l'adhésion cellulaire via les récepteurs d'intégrines, à la réorganisation du cytosquelette, à la prolifération et à la différenciation des cellules.

Ce travail a ciblé la préparation et la caractérisation des surfaces du titanium oxydé, chimiquement similaires à celles utilisées en clinique, avec différentes rugosités. Deux surfaces, TiO_2 'sputtered' en Si (TiO_2 sp) et TiO_2 obtenu à partir du titanium pur, commercialement disponible, après immersion dans H_2O_2 (TiO_2 cp), ont été développées. Les deux surfaces présentent une composition chimique identique mais une topographie différente comme a été démontré par le XPS, les études de mouillage et l'AFM. La surface du TiO_2 cp a montré une rugosité plus élevée.

Ultérieurement, l'étude de la capacité des deux surfaces de TiO_2 décrites ci-dessus d'adsorber l'albumine du sérum humain, la HSA, la fibronectine du plasma humain ou la FN à partir d'une

solution protéique, du plasma ou du plasma dilué a été réalisée. La FN a montré une affinité différente aux deux surfaces de TiO_2 en comparaison avec celle de l'HSA. La solution de FN a exhibé une plus grande affinité aux surface de TiO_2 cp en comparaison à celle observée en présence du TiO_2 sp. De plus, les études de radioactivité ont montré que la FN adsorbe préférentiellement aux surfaces TiO_2 cp qu'à celles du TiO_2 sp. Cependant, les molécules de FN semblent être plus fortement attachées à la surface du TiO_2 cp, comme a été suggéré par les études d'adhésion et de permutation, avec une tendance analogue à celle observé en présence d'albumin. La technique d'AFM a permis une visualisation directe de la FN adsorbée dans les conditions air ou liquide sur les deux surfaces testées. Après l'adsorption, la FN semble former des agrégats globulaires ou ellipsoïdes. Ces agrégats s'unissent et forment des "clusters" au fur et à mesure que le temps d'adsorption et la concentration augmentent. Les résultats d'AFM ont apporté une nouvelle évidence montrant que la FN adsorbe spontanément avec une autoorganisation aux surfaces de TiO_2 en fonction du temps. La structure des agrégats est une caractéristique intermédiaire partagée par certains rassemblement de protéines fibrillaires au niveau des interfaces considérées comme promotrices d'adhésion cellulaire et organisation du cytosquelette.

L'effet de la compétitivité de préadsorption de la HSA et de la FN sur les deux substrats de TiO_2 à partir de solution binaire de protéines ou de 10% de plasma sur l'adhésion et la morphologie des ostéoblastes a été étudié. Les ostéoblastes MC3T3-E1 ont été utilisés. Les résultats montrent une corrélation directe entre l'adhésion des ostéoblastes et les concentrations de la FN et de la HSA adsorbées au niveau des surfaces de TiO_2 . De plus, un faible nombre des ostéoblastes adhèrent après 4 et 24h aux deux surfaces préalablement préadsorbées avec la HSA, HSA/FN=200 et 10% de plasma en comparaison avec les surfaces préadsorbées avec la FN et HSA/FN=1. Cependant, la FN a pu compenser l'effet inhibiteur de l'HSA sur l'adhésion des ostéoblastes. Par conséquent, la présence de faible concentrations de l'HSA adsorbée peut améliorer la présentation de la FN sous forme d'une conformation permettant une reconnaissance de la protéine via les intégrines. Ceci suggère qu'un certain degré de compactage moléculaire évite la perte de l'activité résultante de la liaison des intégrines.

L'effet réversible de la FN ne semble pas être dépendant de l'adsorption de la masse du ratio HSA/FN en solution, suggérant que la FN adsorbe compétitivement au TiO_2 avec une conformation favorable permettant un échange avec d'autres molécules de FN en solution.

En conclusion, le profile d'adsorption de la HSA et la FN au TiO_2 et la réponse des ostéoblastes (adhésion cellulaire et morphologie) qui en résulte dépendent du type de surface de TiO_2 . La présence de faibles concentrations d'albumin co-adsorbée peut améliorer l'exposition de la FN sous une conformation permettant une reconnaissance de la protéine via les intégrines. Ceci suggère qu'un certain degré de compactage moléculaire paraît esquiver la perte de l'activité résultante de la liaison des intégrines.

Presentemente existe uma vasta gama de biomateriais promissores que estão a ser investigados ou aplicados no desenvolvimento de novos tipos de órgãos artificiais e dispositivos médicos. No entanto, os metais, devido às suas propriedades mecânicas, são ainda considerados os biomateriais mais apropriados para substituição de tecido duro. A estabilidade fisiológica do titânio associada à sua resistência à corrosão, assim como às propriedades mecânicas e osteointegração favorável, contribuem para que este seja um dos biomateriais mais utilizados para a substituição do osso e da cartilagem.

Quando um biomaterial é implantado, as proteínas adsorvem imediatamente à sua superfície. A resposta do tecido aos materiais implantados surge principalmente das diferenças na adsorção das proteínas que, por sua vez, dependem de parâmetros de superfície, composição química, rugosidade e carga de superfície, entre outras.

O objectivo principal desta tese foi o de identificar as principais características que medeiam a adsorção de proteínas em superfícies do óxido titânio com diferentes topografias à escala nanométrica. O trabalho envolveu a adsorção de duas proteínas chave na adesão celular, a albumina (HSA) e a fibronectina (FN), provenientes de soluções simples com uma só proteína, de soluções com duas proteínas, assim como de plasma. A albumina é a proteína predominante do plasma, e é conhecida como “passivante” das superfícies prevenindo a adesão celular e reduzindo a resposta inflamatória ao material implantado. Por outro lado, a FN é uma proteína de adesão da matriz extracelular (ECM) conhecida como fundamental na adesão, na reorganização do citoesqueleto, na proliferação, e na diferenciação celular.

A primeira parte desta tese visou a preparação e caracterização de superfícies de óxido de titânio quimicamente idênticas às usadas na prática clínica, com duas rugosidades distintas. Para isso preparam-se duas superfícies de óxido de titânio, TiO_2 “sputtered” em Si (TiO_2 sp) e TiO_2 formado por imersão de titânio comercialmente puro em H_2O_2 (TiO_2 cp). Através das técnicas de espectroscopia de fotoelectrões de raios-X (XPS), dos estudos de molhabilidade, e por microscopia de força atómica (AFM), foi possível confirmar uma composição química idêntica e topografia diferente. A rugosidade de superfície foi sempre significativamente mais elevada nas superfícies de TiO_2 cp.

Subsequentemente, avaliou-se a adsorção de HSA e FN nas referidas superfícies de TiO_2 , partindo de soluções simples de proteína, de plasma e de plasma diluído. A FN mostrou diferente afinidade para as superfícies de TiO_2 , quando comparada com a HSA. Através da técnica de marcação radioactiva observou-se que a FN adsorve mais à superfície de TiO_2 cp do que à superfície de TiO_2 sp. No entanto, as moléculas de FN pareceram estar mais fortemente aderentes à primeira superfície, como sugerido pelo trabalho de adesão e pelos estudos de desorção, seguindo a mesma tendência observada para a HSA. Os estudos de AFM permitiram a visualização directa ao ar e em água da FN adsorvida, para as duas superfícies testadas. Após adsorção, foi possível observar agregados globulares de FN, que coalesceram com o tempo de adsorção assim como com o aumento de concentração. Os agregados são estruturas intermediárias partilhadas por algumas proteínas fibrilares em interfaces, que se crê promoverem a adesão celular e a organização do citoesqueleto.

Por último, avaliou-se o efeito da competição na preadsorção de HSA e FN aos substratos de TiO_2 , a partir de soluções binárias das duas proteínas e do plasma a 10%, na adesão e morfologia de osteoblastos MC3T3-E1. Os resultados mostraram que existe uma correlação entre a adesão dos osteoblastos e as quantidades de FN e HSA adsorvidas às superfícies de TiO_2 . Existem menos osteoblastos aderentes aos dois substratos às 4 e 24h após preadsorção com HSA, HSA/FN=200 e 10% plasma, quando comparados com a preadsorção com FN e HSA/FN=1. Para esta última condição, a FN conseguiu compensar o efeito inibidor da HSA na adesão de osteoblastos. A presença de pequenas quantidades de albumina a adsorverem simultaneamente com a FN, parece contribuir para que esta última assuma uma conformação adequada ao reconhecimento pelas integrinas, sugerindo que um certo grau de empacotamento molecular parece prevenir a perda de actividade por ligação às integrinas. A reversibilidade da FN não parece ser dependente da razão de HSA/FN em solução, facto que sugere que a FN adsorve competitivamente ao TiO_2 numa conformação favorável à permuta com outras moléculas de FN em solução.

Em resumo, a adsorção de HSA e FN ao TiO_2 e subsequente resposta dos osteoblastos (adesão celular e morfologia), dependem do tipo de superfície de TiO_2 . A presença de pequenas quantidades de HSA a coadsorverem com a FN parecem contribuir para que esta proteína adsorva numa conformação favorável ao reconhecimento por integrinas, sugerindo

que um certo grau de empacotamento molecular parece prevenir a perda de actividade por ligação às integrinas.

	Acknowledgements	i
	Abstract	v
	Résumé	vii
	Resumo	xi
	Contents	xv
	List of Tables and Figures	xvii
	List of abbreviations	xxiii
Chapter I	Introduction	1
	1. Titanium as a biomaterial for bone replacement	1
	2. Titanium oxide	3
	2.1 Physico-chemical properties of titanium oxide	3
	2.2 Composition, thickness and structure of oxide films on pure titanium	5
	2.3 Modification and physico-chemical properties of titanium oxide films in contact with electrolytes and body fluids	10
	2.4 Surface treatments of titanium	14
	3. Protein adsorption at solid/liquid interfaces	15
	3.1 Protein structure	15
	3.2 Protein adsorption	16
	3.2.1 Type of interactions	18
	3.2.1.1 Electrostatic interactions	18
	3.2.1.2 Hydrophobic interactions	19
	3.2.2 Conformational stability of proteins	19
	3.2.3 Structure of the adsorbed protein layer	20
	3.2.4 Competitive protein adsorption	21
	3.2.5 Reversibility of protein adsorption	21
	4. Cell/material interactions	22
	4.1. The role of adsorbed proteins in tissue response to biomaterials	22
	4.1.1 Adhesion proteins	23

4.1.2 Non-adhesion proteins	26
5. Techniques for protein adsorption study at biomaterials surfaces	26
6. Aim and structure of the thesis	29
7. References	34
Chapter II Human Serum Albumin Adsorption on TiO₂ from Single Protein Solutions and from Plasma	47
Chapter III TiO₂ type influences fibronectin adsorption	83
Chapter IV Dynamics of fibronectin adsorption on TiO₂ surfaces	101
Chapter V Osteoblast adhesion and morphology on TiO₂ depends on the competitive preadsorption of HSA and FN	131
Chapter VI Concluding Remarks and Future work	155

List of Tables and Figures

Chapter I

Table 1 - Purity grades of CP Titanium according to ISO 5832-2:1993. The C and H wt % are, for all grades, 0.1 and 0.015, respectively. [adapted from ref ³]	2
Figure 1 – Crystallographic unit cell of the two most common forms of titanium oxide, TiO ₂ , a) rutile and b) anatase. Grey small balls represent titanium cations, Ti ⁴⁺ , black balls represent oxygen anions, O ²⁻ . [adapted from ref. ⁸]	4
Table 2 - Selected physico-chemical properties of the most stable titanium oxide, TiO ₂ . ^{1,4,8,9}	4
Figure 2 – Typical XPS survey spectrum of Ti CP mechanically polished with a native, room temperature grown oxide film. ¹²	6
Table 3 – Typical XPS oxide film composition (at %) and oxide layer thickness of TiCP after different surface treatments. [adapted from ref. ¹²]	6
Figure 3 – XPS Ti2p emission peak recorded on an evaporated high-purity titanium film. The oxide film composition shows contributions from Ti in oxidation states +IV (TiO ₂), +III (Ti ₂ O ₃), +II (TiO) and 0 (Ti metal). ⁸	7
Table 4 – XPS Binding energies for the titanium/titanium oxide film ^{5-7,10,13-17}	8
Figure 4 – Schematic representation of the early interfacial processes taking place at the interface between the titanium oxide and the biological fluid in contact with the implant. The dimensions of features are not to scale. The oxide films are shown as ordered structures for convenience, but are often amorphous or only partially crystalline. ⁸	13, 14
Figure 5 – The primary, secondary, tertiary, and quaternary structure of the hemoglobin molecule. ³¹	16
Table 5 - Properties that determine protein adsorption at titanium surface [adapted from references ^{37,38}]	17
Figure 6 - Interactions between cells and the extracellular matrix. The association between cells and the proteoglycan of the ECM is mediated by a membrane protein (integrin) and by FN an ECM protein with binding sites for both integrin and the proteoglycan. [adapted from ref ³²]	22

Figure 7 – Schematic representation of fibronectin dimer showing its modular structure and functionality of various domains. Two subunits are shown, with amino termini to the left and carboxyl termini to the right. Regions implicated in adhesion and assembly are shown. [adapted from ref. ¹⁰¹].	24
Figure 8 – Schematic diagram showing the synergy site and the RGD-loop that are located on the 9th and 10th type III modules, FN-III ₉ and FN-III ₁₀ , respectively. ¹¹²	25
Table 6 - Selected techniques for protein adsorption study at biomaterials surfaces ^{35-37,84,119,120}	27, 28

Chapter II

Figure 1 - 3D AFM images of a (a) TiO ₂ sputtered surface, (b) Ti cp surface (before H ₂ O ₂ treatment), and (c) TiO ₂ cp surface (after H ₂ O ₂ treatment).	56
Table 1 - Ra, Rq, and Rmax Values for all the Ti substrates studied.	57
Table 2 – Contact angles and their standard deviations of water and diiodomethane (n=10) on TiO ₂ surfaces, at t=0 s.	59
Figure 2 - Representative deconvolution of the XPS envelope for (a) C1s, (b), N1s and (c) O1s peaks (after 60 min of 0.20 mg/mL HSA adsorption at a TiO ₂ cp surface) at 0°.	61
Figure 3 - Evolution of the XPS element envelope with increased time of albumin immersion ([HSA]=0.20 mg/mL).	62
Figure 4 - Changes in atomic % with [HSA] in solution after 60 min of adsorption at a TiO ₂ cp surface: (a) C%; (b) N%; (c) Ti% and O%.	63
Figure 5 - Time dependence contact angle of several solutions at a TiO ₂ cp surface.	65
Table 3 - Contact angles and their standard deviation of PBS and different protein solutions on TiO ₂ surfaces at t=600 s.	66
Figure 6 - Work of adhesion between TiO ₂ substrates and (a) water, (b) [HSA]=4.0 mg/mL, and (c) plasma.	67
Figure 7 - Work of adhesion between TiO ₂ cp and HSA at different concentrations and between TiO ₂ cp and plasma solutions.	68
Figure 8 - Work of adhesion between HSA molecules and the TiO ₂ substrates as a function of HSA concentration in solution (t=600 s).	69

Figure 9 - HSA adsorption isotherms for TiO ₂ substrates after 1 h of incubation.....	70
Figure 10 - Elution of HSA by other HSA molecules from TiO ₂ cp and TiO ₂ sp as a function of HSA concentration in solution. The elution time was 24 h or 72 h for the TiO ₂ cp and 24 h for the TiO ₂ sp.....	70
Figure 11 - Comparison of the kinetics of HSA adsorption (0.20 and 4.0 mg/mL) and desorption (with HSA) from a TiO ₂ cp surface.....	71
Figure 12 - Comparison of the kinetics of HSA adsorption (0.20 and 4.0 mg/mL) on TiO ₂ sp and desorption (with HSA).....	72
Figure 13 - Comparison between adsorption of albumin onto a TiO ₂ cp surface by ¹²⁵ I-HSA radiolabeling and XPS measurements of N%.....	75

Chapter III

Figure 1 - Representative deconvolution of XPS envelope for (a) C1s, (b) N1s and (c) O1s peak (after 60 min of FN 200 µg/mL adsorption at TiO ₂ sp surface) at 0°.....	90
Figure 2 - Atomic % as a function of [FN] in solution after 60 min of adsorption on a TiO ₂ cp surface.....	91
Table 1 - Contact angle values of different solutions at TiO ₂ surface (n = 10).....	92
Figure 3 - Work of adhesion between TiO ₂ substrates and FN ([FN] = 200µg/mL).....	93
Figure 4 - Work of adhesion between TiO ₂ cp and protein solutions.....	93
Figure 5 - Adsorption isotherms of FN on TiO ₂	94
Figure 6 - Elution of FN molecules by other FN molecules from TiO ₂ sp and from TiO ₂ cp as a function of FN concentration in solution. The elution time was 24 h.....	95

Chapter IV

Figure 1 – AFM images and cross sections of a clean TiO ₂ cp substrate surface, a) topography and b) phase image. The image was performed using MAC mode in water.....	109
Figure 2 – AFM height and phase images of TiO ₂ cp surfaces, before and after protein adsorption recorded in air and in water (lateral scan size: 2000x2000 nm ²). The	

height (a, b, c and d) and phase images (e, f, g, and h) respectively, show that contrast from the surface is high enough to observe protein.....	110
Figure 3 - Surface roughness of TiO ₂ cp and TiO ₂ sp determined in air and water, before and after protein adsorption (scan area: 1000x1000 nm ²), a) R _q and b) R _{max}	111
Figure 4 - AFM height (a) and phase (b) images of FN adsorbed on a TiO ₂ sp surface after 10 min of incubation with 50 µg/mL of FN and correspondent cross sectional profiles [1000x1000 nm ²]. The images were performed using MAC mode in water.....	112
Figure 5 – Topographic AFM images recorded in air (a and c) and in water (b and d) of TiO ₂ sp surfaces, before and after 30 min. with 75 µg/mL of FN adsorption (lateral scan size: 2000x2000 nm ² and 1000x1000 nm ² for the higher magnification images).....	113
Table 1 – Particle dimensions of FN adsorbed on TiO ₂ sp, under different adsorption conditions.....	114
Figure 6 – Evolution of XPS element envelope with increased time of FN immersion (FN =20 µg/mL): a) Ti% and O%; b) N% and C%.....	115
Figure 7 – Kinetics of FN adsorption (20 µg/mL) on TiO ₂ surfaces.....	116
Figure 8 – Elution of FN molecules after adsorption of FN at 20 µg/mL (60 min.) from TiO ₂ sp and from TiO ₂ cp. a) Elution of FN by other FN molecules or by HSA, and b) Elution of FN by other FN in solution, as a function of time of adsorption. The elution time was 24 h.....	117
Figure 9 – Comparison between adsorption of FN as a function of time onto a TiO ₂ cp surface by ¹²⁵ I-FN radiolabelling and XPS measurements of N%.....	120

Chapter V

Figure 1 – Effect of protein preadsorption on MC3T3-E1 osteoblast cell adhesion on TiO ₂ after 4 and 24 h of incubation, expressed as OD/cm ² . TCPS was used as a control (data not shown). Values reported are the average ± SD of four to six cultures. Asterisks (*) indicate a significant difference from a substrate to those pre-adsorbed with FN (p≤0.05). The hash symbol (#) indicates a significant difference between a substrate and those samples without pre-adsorbed protein (p≤0.05).....	139
---	-----

Figure 2 - MC3T3-E1 cell morphology and cytoskeletal organization on TiO ₂ sp imaged with confocal microscopy after 4 h, with the preadsorption conditions: a) HSA, b) FN, c) HSA/FN=200. F-actin is indicated in green while cell nuclei were counterstained in blue with DAPI dye. Scale bar = 20 µm.....	141
Figure 3 - MC3T3-E1 cell morphology and cytoskeletal organization on TiO ₂ cp imaged with confocal microscopy after 24 h, with the preadsorption conditions: a) HSA, b) FN, c) 10 % plasma. F-actin is indicated in green while cell nuclei were counterstained in blue with DAPI dye. Scale bar = 20 µm.....	142
Figure 4 - Competitive adsorption of HSA and FN to TiO ₂ sp substrates as a function of HSA/FN mass ratio in solution (1, 10, 50 and 200). Calculations were performed considering as 100 % adsorption the concentration of HSA or FN adsorbed from a pure albumin solution or from a pure fibronectin solution, respectively.....	144
Figure 5 - Relative a) HSA and b) FN amount competitively adsorbed from 10 % plasma solutions to TiO ₂ sp substrates. Calculations were performed considering as 100 % adsorption the concentration of HSA or FN adsorbed from a pure albumin solution or from a pure fibronectin solution, respectively.....	145
Figure 6 - Exchangeability of HSA and FN competitively adsorbed to TiO ₂ sp surfaces, following 24 h exposure to HSA and FN solutions, respectively.....	146

List of abbreviations

¹²⁵ I	Iodine-125
¹²⁵ I-FN	Radiolabelled fibronectin with iodine-125
¹²⁵ I-HSA	Radiolabelled albumin with iodine-125
AES	Auger electron spectroscopy
AFM	Atomic force microscopy
ATR	Attenuated total reflectance technique
ATR-FTIR	Attenuated total reflectance - Fourier transform infrared technique
BCA	Bicinchoninic acid
BE	Binding energy
BSA	Bovine serum albumin
CCD	Charge-coupled device
cp	Chemical polishing
CP	Commercially pure
ECM	Extracellular matrix
FN	Human plasma fibronectin
HSA	Human serum albumin
IR	Infrared
IRAS	Infrared reflection-absorption spectroscopy
MAC	Magnetic alternated current
NR	Neutral red
OD	Optical density
OWRK	Owens-Wendt-Rabel and Kaelbe method
PBS	Phosphate buffered saline
RGD	Arg-Gly-Asp sequence
SA	Surface area
SEM	Scanning electron microscopy
sp	Sputtering
SPR	Surface plasmon resonance technique
STM	Scanning tunneling microscopy
TCA	Trichloroacetic acid
TEM	Transmission electron microscopy
Tof-SIMS	Time-of-flight secondary ion mass spectrometry
XPS	X-ray photoelectron spectroscopy

Introduction

1. Titanium as a biomaterial for bone replacement

At present the metallic implants have a significant contribution in treatment of health problems. Therefore this utilization generates a high economical impact. The worldwide market for all types of biomaterials was estimated at over 4 billion euros in the late 1980's and is likely to exceed 18 billion euros by 2005. Approximately 3.6 million orthopedic operations per year are made in the United States, and four of the ten most frequent involve metallic implants.¹ The class of implantable metallic biomaterials can be divided into four subgroups: stainless steels, the cobalt-based alloys, titanium metals, and miscellaneous others, including tantalum, gold, dental amalgams as well as other specific metals. The main considerations in selecting metallic implants for biomedical applications are biocompatibility, appropriate mechanical properties, corrosion resistance and reasonable cost.²

In the last 30 years titanium has become the best material for implants in bone. This is in part because Ti is among the most biocompatible biomaterials and partly because of its high strength and low weight. Commercially pure (CP) titanium is mainly used as dental implant material and in osteosynthesis, because the strength of CP titanium is too low for load-bearing orthopedic applications. For orthopedic load-bearing implants alloying elements are added to titanium to increase the strength of the material. CP titanium is available in four different purity grades (according to ISO 5832-2:1993), which are listed in Table 1.³ The four basic grades of pure titanium differ primarily in oxygen and iron content and represent about 24% of the titanium production. Grade 1 has the highest purity and the lowest strength.⁴

Table 1 – Purity grades of CP Titanium according to ISO 5832-2:1993. The C and H wt % are, for all grades, 0.1 and 0.015, respectively. [adapted from ref ³]

Grade	Ti (wt %)	O (wt %)	N (wt %)	Fe (wt %)
1	>99.5	max. 0.18	max. 0.03	max. 0.15
2	>99.4	max. 0.25	max. 0.03	max. 0.20
3	>99.2	max. 0.35	max. 0.05	max. 0.25
4	>99.0	max. 0.45	max. 0.05	max. 0.30

The elastic moduli of Ti and Ti-6Al-4V are much lower than that of stainless steels or cobalt-chromium molybdenum alloys, but still at least an order of magnitude higher than that of natural bone. As a consequence bone resorption (stress-shielding effect) in the vicinity of metallic implants may occur. This clinical complication arises because preferential distribution of mechanical loading through the metallic prosthesis withdraws bone of the mechanical stimulation needed to maintain homeostasis.²

The earliest applications of titanium as a material for medical, surgical and dental devices started after the progress in titanium manufacturing processes for aviation industry and military requirements on the post-World War II. The four CP titanium grades (ASTM F 67), the extra low interstitial Ti6Al4V ELI (ASTM F 136) and standard Ti6Al4V (ASTM F 1472) were the first titanium biomaterials used in implantable components and devices. The corrosion resistance and biocompatibility for those “non-aerospace” titanium grades was studied and documented and increasing interest was generated since they were more suitable for medical, surgical and dental devices than other metals used until then. Titanium manufacturers, taking advantage of titanium properties, created new devices and improved versions of old ones. This stimulated the need for a better understanding the titanium formulations available and initiated research into improved titanium biomaterials more suited for the specific needs of dental applications, total joint replacement and fracture fixation devices. The widespread and successful clinical use of titanium as an implant material, indicated that titanium showed improvements in materials performance, device design and physician technique, relative to the other metallic implants

used.³ The observation of corrosion, wear problems, bone resorption and inflammation in stainless steels, especially in long term implantation, accelerated the progressive use of titanium.

2. Titanium oxide

Titanium biomaterials (pure titanium as well as several alloys), rely upon the formation of an extremely thin (3-7 nm thick),⁵⁻⁷ hard, adherent, protective oxide film.

This oxide forms naturally and spontaneously and is responsible for the passive properties of titanium surfaces. The excellent chemical inertness, corrosion resistance and repassivation ability of titanium and its alloys are a direct consequence of the chemical stability of the titanium oxide film.⁸

2.1 Physico-chemical properties of titanium oxide

A variety of stoichiometries of titanium oxide are known, being the most common TiO, Ti₂O₃, and TiO₂. The wide range of oxygen to titanium ratios is a consequence of two issues: the existence of various and relatively stable titanium oxidation states and the fact that oxygen presents a reasonable solubility in titanium. The titanium oxides, especially TiO₂, are thermodynamically very stable. The Gibbs free energy for the formation of the oxide is highly negative ($\Delta G_0^{298} = -888.8$ KJ/mol of oxide formed) for oxidation media such as oxygen, water or oxygen containing organic molecules. However, the most stable oxide of titanium is TiO₂, where titanium is in the preferred oxidation state, +IV.⁸

The TiO₂ film formed at room temperature on pure titanium is amorphous or nanocrystalline. TiO₂ exists in three different crystallographic forms: rutile and anatase with tetragonal and brookite with orthorhombic structures. In the two more common forms of TiO₂, anatase and rutile, the titanium ion is coordinated to six oxygen anions (Figure 1).⁸

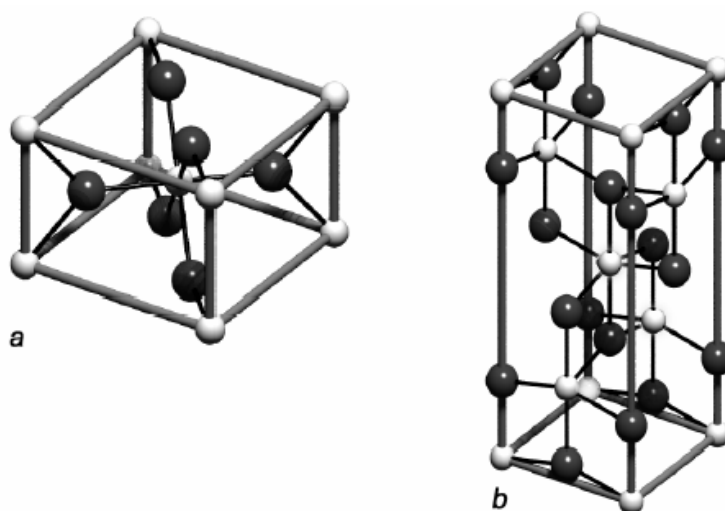


Figure 1 – Crystallographic unit cell of the two most common forms of titanium oxide, TiO_2 , a) rutile and b) anatase. Grey small balls represent titanium cations, Ti^{4+} , black balls represent oxygen anions, O^{2-} . [adapted from ref.⁸]

There are some physico-chemical properties of the oxide that are believed to confer the outstanding stability and biological performance to TiO_2 (Table 2).^{1,4,8,9} The titanium oxide presents a low overall solubility in water with a slightly negative charge at physiological pH, and with no significant tissue response. The mean dielectric constant is similar to the one of water ($\epsilon_w=78$) with the consequence that the Coulombic interaction of charged species is similar to that of water molecules.^{1,4,8,9}

Table 2 - Selected physico-chemical properties of the most stable titanium oxide, TiO_2 .^{1,4,8,9}

	TiO_2
Isoelectric point (ISP)	ca. 6.2
Charge at pH=7.4	negative
Dielectric constant of oxide	48 (anatase) 110 (rutile) 78 (brookite)
Solubility at pH=7 (mol/L)	$3 \cdot 10^{-6}$
Typical tissue response	inertness
Refractive index, 550 nm	2.54-2.75

Hydroxide and chemisorbed water are strongly bound to the titanium cations at the outermost surface with properties depending on the surface fabrication conditions and history of the sample; additionally, a film of weakly bound, physisorbed water is present.⁸

2.2 Composition, thickness and structure of oxide films on pure titanium

The composition, thickness and structure of native room-temperature grown films are the properties that are believed to be the most important in what concerns the performance of titanium as biomaterial. For that reason there are an extensive number of papers on the subject in the literature and only a fraction of them is being cited here.^{5,6,8-11}

Quantitative surface composition

The determination of surface composition of titanium oxide can be obtained from X-ray photoelectron spectroscopy (XPS) data. This method gives information from depths in the range of 2-10 nm, provides semi-quantitative to quantitative surface concentrations and senses different chemical environments of the atoms present at the surface ('chemical shifts'). Survey spectrum can be created and the overall surface composition determined. Normally, peaks that can be attributed to titanium, oxygen and carbon dominate the spectrum. The carbon peak is mainly adventitious hydrocarbon contamination that cannot be avoided for samples that have been exposed to the ambient atmosphere. An example of a XPS survey spectrum of Ti cp mechanically polished with a native, room-temperature grown oxide film is shown in Figure 2.¹²

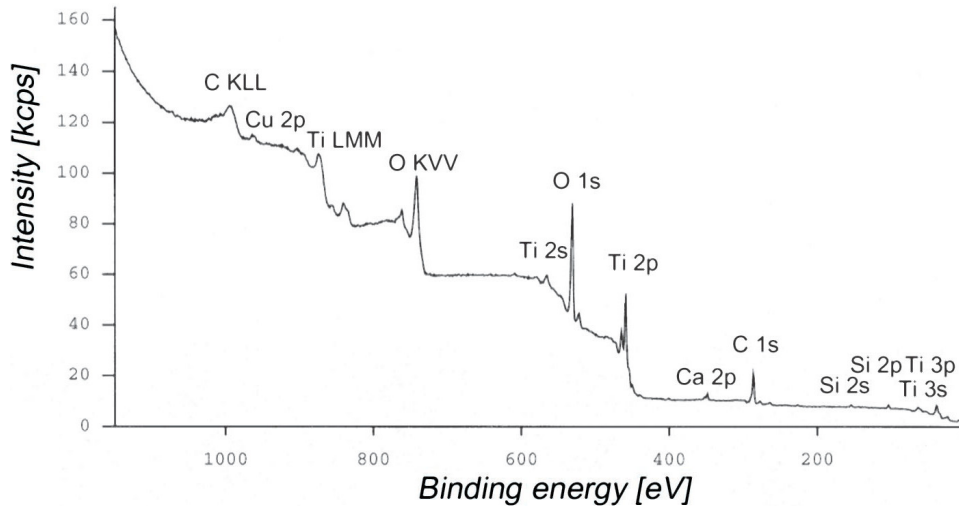


Figure 2 – Typical XPS survey spectrum of Ti CP mechanically polished with a native, room-temperature grown oxide film.¹²

The overall quantification of the referred surface is shown in Table 3. Carbon concentrations of 20-30 at% determined with XPS seem high, but are typical for even well cleaned surfaces that have been exposed to air for a period of time.

Table 3 – Typical XPS oxide film composition (at %) and oxide layer thickness of Ti CP after different surface treatments. [adapted from ref.¹²]

Element	Ti CP	
	Mechanically polished (at %)	Plus HNO ₃ passivation (at %)
Ti	14.8±1.6	26.1±0.9
O	46.8±1.9	54.4±2.0
C	30.9±2.1	19.0±2.9
N	0.6±0.2	0.5±0.1
Additional detected elements	Si, Ca, Zn, Cu, Pb	-
Relative oxide thickness, nm	4.3±0.2	4.3±0.2

An additional cleaning step may be applied immediately before the surface analysis, such as oxygen plasma treatment or UV/ozone treatment, showing carbon concentrations as low as 5-15 at%. However, if surfaces are subsequently exposed to air, the carbon concentration increases again, attaining a steady state value that depends on the environmental conditions.

Oxide film stoichiometry

A typical high-resolution spectrum for Ti2p is shown in Figure 3.⁸ The Ti2p signal can provide detailed information on the chemical composition of titanium oxide, namely oxidation state and stoichiometry, after peak deconvolution. The Ti2p peak is a doublet peak, Ti2p_{3/2} and Ti2p_{1/2}, revealing a Ti⁴⁺ oxide state for CP Ti surfaces exposed to air (Binding energies, BE, in Table 4)⁵⁻

7,10,13-17

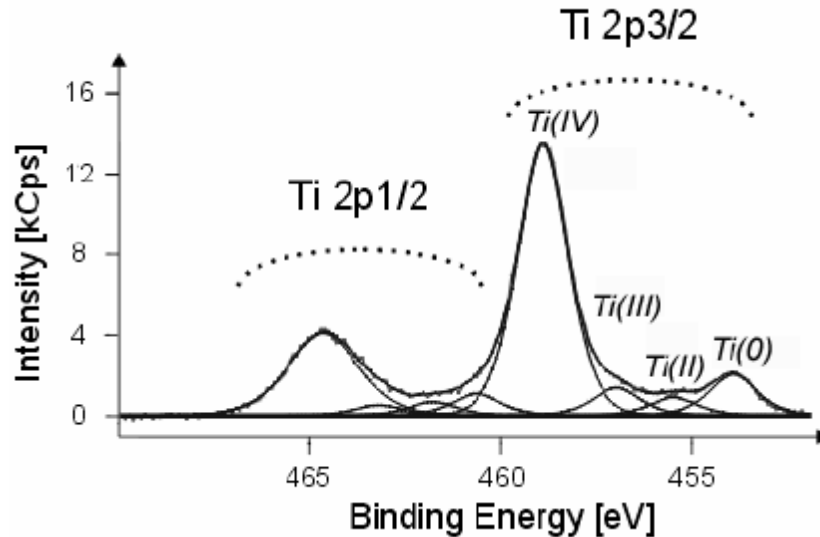


Figure 3 – XPS Ti2p emission peak recorded on an evaporated high-purity titanium film. The oxide film composition shows contributions from Ti in oxidation states +IV (TiO₂), +III (Ti₂O₃), +II (TiO) and 0 (Ti metal).⁸

However, this spectrum gives clear evidence that although at lower concentration, TiO_2 can coexist with other sub-oxides, like TiO and Ti_2O_3 at the Ti surface. If the film is not thick enough, photoelectrons from the bulk metal can reach the surface and Ti (0) can also be detected. The peak positions can be attributed to Ti in the following oxidation states: +IV (TiO_2), +III (Ti_2O_3), +II (TiO) and 0 (Ti metal).⁸

Table 4 – XPS Binding energies for the titanium/titanium oxide film^{5-7,10,13-17}

	Ti 2p _{3/2}	Ti 2p _{1/2}
TiO ₂	459.0	465.0
	459.4	465.2
	459.1	-
	458.9	464.6
	459.1	465.0
Ti ₂ O ₃	457.5	462.5
	457.9	463.6
	457.2	-
	457.2	462.9
	457.6	463.4
TiO	455.5	461.0
	460.0	461.7
	455.4	-
	455.2	461.0
	455.7	461.4
Ti	453.9	460.0
	454.2	460.3
	454.2	-
	453.6	460.0

All values in eV relative to C1s=285.0 eV

The high resolution O1s spectra obtained from Ti CP surface often reveals a shoulder on the higher binding energy side, justifying, the separation of O1s spectrum into two or more components. The BE assigned to titanium oxide (O_M) (530.4 eV)^{18,19} appears in the low-energy range followed by other components at higher BE, namely hydroxide/hydroxyl groups plus chemisorbed water (O_H), (532.2 eV).^{10,19}

Armstrong et al.²⁰ and Leitão et al.²¹ found that binding energy differences between two major transitions may indicate the chemical composition of a metal oxide. The BE difference between the O1s and the Ti2p3, is $\Delta BE = O(1s) - Ti(2p/3) = 71.5$ eV, which, according to the data obtained by the same authors, corresponds to TiO₂.

Thickness

The oxide thickness on machined titanium surfaces can be estimated either by using the relative intensities of the oxide and metallic contributions in the Ti2p spectra, or by XPS or Auger electron spectroscopy, AES, sputter depth profiling.⁵

As it was previously referred the TiO₂ film thickness is between 3 and 7 nm. However, the exact value for the oxide thickness clearly depends on the history of the sample, as it was mentioned before. Factors known or believed to influence oxide layer growth during exposure to the atmosphere include temperature, time and humidity. At room temperature the native oxide film forms within a very short time on fresh titanium metal surfaces, but afterwards grows only very slowly (only ca 1 nm after 20 days of exposure to ambient room temperature atmosphere).⁶ More noticeable changes in oxide film thickness occur at elevated temperature including thermal sterilization and in particular during anodic oxidation.

Oxides, hydroxides and water

Despite surface composition and oxide film thickness, the nature of oxygen species present at the outermost oxide surface is believed to be important to the behavior of materials in contact with biological environments. Hydroxide is frequently associated with the outermost oxide layer and the oxide/hydroxide surface accounts with water strongly adsorbed to titanium ions (chemisorbed) and water molecules weakly adsorbed (physisorbed) as mono or multilayer. The combined effect of hydroxide formation and water adsorption is often called as “hydration”. The

XPS O1s spectrum can demonstrate the presence of titanium oxide at 530.3 eV, hydroxide at ca. 531.3 eV and water at ca. 532.5 eV, as it was previously referred.^{7,16,17,19}

The oxide layer presents in many cases oxygen-containing organic compounds arising from contamination and as a result from reaction of organic molecules with titanium oxide, forming alcoholates (Ti-O-R) or carboxylates (Ti-OOC-R).^{5,10,22} These compounds easily adsorb or reactively form at titanium oxide surface, as a consequence of the high oxide adsorptivity and high reactivity of oxide towards organic species.

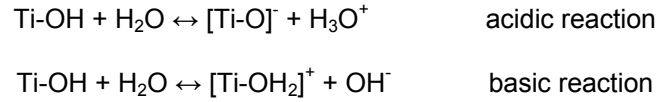
It is also possible to found oxygen-containing inorganic species such as nitrate, phosphate, silicate or sulfate as traces.⁸

2.3 Modification and physico-chemical properties of titanium oxide films in contact with electrolytes and body fluids

Titanium oxide layer is very stable in air but, there is clear evidence that in contact with aqueous body fluids more significant changes, such as oxide thickness growth, and inorganic and organic (protein) molecule adsorption, takes place. It is obvious that is the interface titanium oxide/biological fluids the most important to the use of titanium in biomedical field. However, there are much more information on the interface titanium oxide/air than the interface titanium oxide/biological fluids. This mainly reflects the fact that solid/liquid interfaces are much more difficult to study and that there are still lack of suitable and reliable techniques to investigate such interfaces.⁸ In general, the processes that are expected to occur at the titanium oxide solid/liquid interface are the hydration and the dissolution and growth of oxide films, formation of electric charges at the oxide/fluid interface following acid/base reactions, interaction of inorganic ions, such as calcium and phosphate and organic molecules like proteins, peptides and polysaccharides, with the titanium oxide.

The titanium oxide in contact with water or aqueous solutions is believed to be highly hydroxylated and to carry a slight negative charge near the physiological pH. The hydroxides of

multivalent cations like Ti^{4+} exhibit both acid and alkaline properties. The reactions showing the formation of such hydroxides may be represented as,



The acidic reaction leads to the formation of negative charges at the surface, and the basic reaction to the positive charges. The overall charge of the surface will depend on the equilibrium constant (K_a) of the reactions and on the pH of the aqueous solution. In water at neutral pH titanium oxide will be moderately negative due to the formation of a fraction of acidic hydroxides being deprotonated, while almost all of the basic and a large part of acidic groups are still present in neutral form.

Regarding the mechanism of the oxide growth in contact with aqueous fluids, both oxygen diffusion through the oxide film to the titanium/metal interface,²³ electric-field assisted transport of titanium ions into the oxide lattice⁷ and chemical reactions of the titanium surface with enzymatically-produced peroxides (H_2O_2) and superoxides^{24,25} have been proposed for *in vitro*. The generally observed increase of titanium oxide thickness *in vivo* has been partly attributed to metabolic activity and to the formation of reactive oxygen metabolites. Oxide film growth and changes in its chemical composition have been reported for *in vitro* and for *in vivo* investigations.^{26,27}

In what concerns the microstructure of titanium oxide, changes have been observed upon immersion in electrolytes, such as growth of the oxide crystals. These crystals are believed to be polycrystalline or single crystals of rutile and anatase, assessed by transmission electron microscopy (TEM). The growth is believed to be due to newly-formed oxide during immersion and not due to crystallization of the originally-present native oxide film.⁸

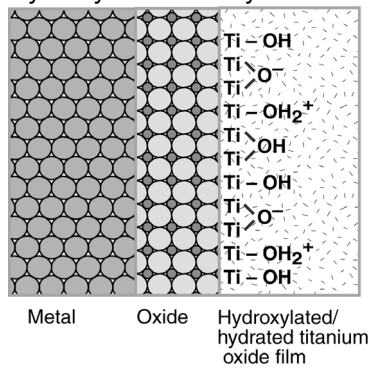
When the solution contacting titanium oxide presents ions the charge will be modified. For example, in simulated body fluid containing calcium ions the surface charge of titanium oxide will rise as a consequence of calcium ions being adsorbed. An electric double layer will be built up that depends on the oxide surface and the type and concentration of cations and anions in

solution.^{8,9} Ca^{2+} is believed to electrostatically bind to the negatively charged surface.²⁸ On the contrary PO_4^{3-} , HPO_4^{2-} and H_2PO_4^- ions seems to replace hydroxides at the basic hydroxides sites, forming a strong complexation bond.

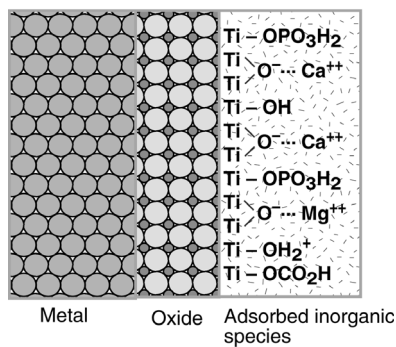
The surface processes that lead to the adsorption of organic species such as for example proteins, glycoproteins, glycolipids, lipoproteins are also very important in what concerns the interaction of titanium oxide film with biological fluids. The two main arguments why an improved knowledge of the interfacial biomolecule/surface processes may be important are: cells are unlikely to sense directly the titanium oxide surface and proteins are believed to be the mediating layer and to the complexity of the interfacial zone between the bone and titanium implants, that has been found to be constituted by titanium oxide with calcium phosphate deposits, followed by a layer of proteoglycans and proteins and then a layer of collagen. The complexity of this interface has motivated investigation towards simpler biomolecules, namely amino acids and proteins.

Textor et al.⁸ summarise the interfacial processes that might occur at the titanium electrolyte interface in a simplistic diagram (Figure 4) although very illustrative of what might happen.

Hydroxylation and hydration of the titanium oxide film in contact with aqueous fluid.



Adsorption of cations and anions from the electrolyte constituents of the body fluid, in particular calcium and phosphate



Adsorption of biomolecules (proteins, glycoproteins, glycolipids, lipoproteins, proteoglycans, polysaccharides), attachment of platelets, formation of fibrin network in contact with blood.

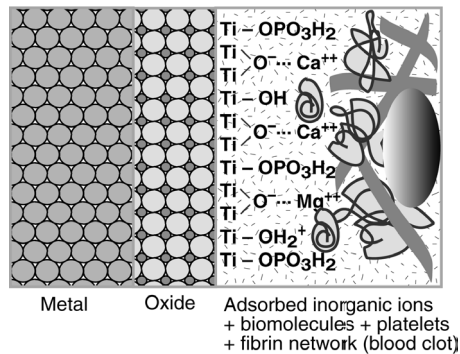
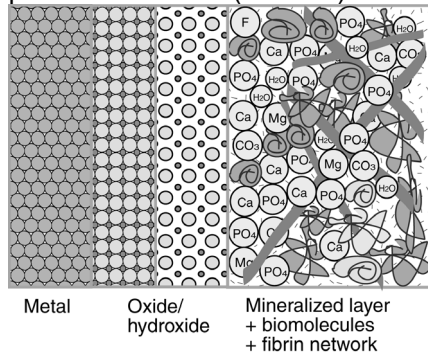


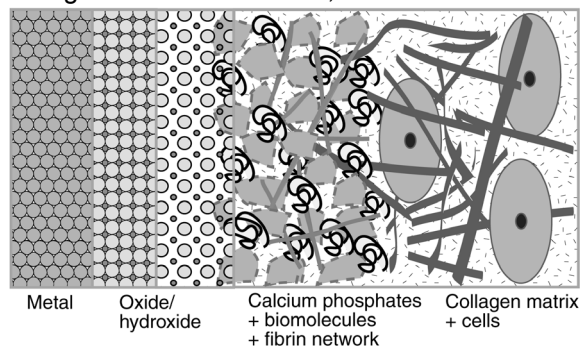
Figure 4 – Schematic representation of the early interfacial processes taking place at the interface between the titanium oxide and the biological fluid in contact with the implant. The dimensions of features are not to scale. The oxide films are shown as ordered structures for convenience, but are often amorphous or only partially crystalline.⁸

Figure 4, cont.

Further modifications of the interface: growth of the oxide/hydroxide layer (porous, gel-like outer layer) and precipitation of calcium phosphates containing also other species (magnesium, carbonate, fluoride, etc.) present in the fluid. Exchange reactions and reorganization within the proteinaceous film ("biofilm").



Formation of an interface architecture in the course of cellular activity: matrix deposition, collagen network formation, mineralization.



2.4. Surface treatments of titanium

There is clear evidence that the biological performance of a biomaterial is a function of its surface properties. In fact, it is generally accepted that the outermost atomic/molecular layers of the material can play a key role. This is the primordial reason why surface treatments such as mechanical, thermal, chemical, and electrochemical methods, are performed. The main effects obtained with each type of method can be broadly divided in three categories: cleaning or removal of native surface layers, modification of surface structure and topography and finally

modification of composition and structure of the oxide layer or controlled formation of a new surface layer.

Mechanical methods are useful mainly for modifying surface topography and roughness. Standard chemical treatments are used essentially for etching or chemical passivation, and generally lead to thin (<10 nm) oxides composed mainly of TiO₂ for pure Ti. Alternative chemical or electrochemical treatments namely heat treatments, immersion in H₂O₂, alkaline etching, apatite precipitation, electropolishing, anodic oxidation, vacuum techniques, can be used for preparing in a controlled way a wide range of surface films with varying morphology, thickness, microstructure and/or chemical composition.²⁹

3. Protein adsorption at solid/liquid interfaces

3.1 Protein structure

Proteins consist of amino acid copolymer chains that interact with each other to give the molecule a three-dimensional structure.³⁰ They play a key role in nearly all biological processes. Enzymes, the catalysts of biochemical reactions, are mostly proteins. Proteins also facilitate a wide range of other functions, such as transport and storage of vital substances, coordinated motion, mechanical support, and protection against diseases. The human body contains an estimate of 10⁵ different kinds of proteins each of which has a specific physiological function. The chemical composition and structure of these complex natural polymers are the basis of their specificity.³¹

Protein structure has been described at four different scales^{31,32} (Figure 5).³¹ The primary structure refers to the order and number of amino acids in a copolymer chain. From the 20 amino acids building blocks (residues), 8 have nonpolar (hydrophobic) side chains, 7 have neutral polar side chains and 5 have charged polar side chains,² rendering the protein amphoteric and amphiphilic.³³ It is therefore not surprising that most proteins are highly surface active.³⁴ Proteins do not exist simply as long, extended chains of amino acids. Instead, interactions between amino acids cause folding, bending, and coiling of the chain to give a

specific three dimensional structure (conformation). Secondary structure results from hydrogen bonding associated with the amide linkages in the backbone of the proteins to form structures as α -helix and β -pleated sheet. Tertiary structure refers to the three-dimensional structure of the whole protein subunit resulting from intramolecular associations, including ionic interactions, salt bridges, hydrophobic, hydrophilic interactions, hydrogen bonding and covalent disulphide bonds. Quaternary structure refers to interactions between subunits, or individual polypeptide chains, in multichain proteins.²

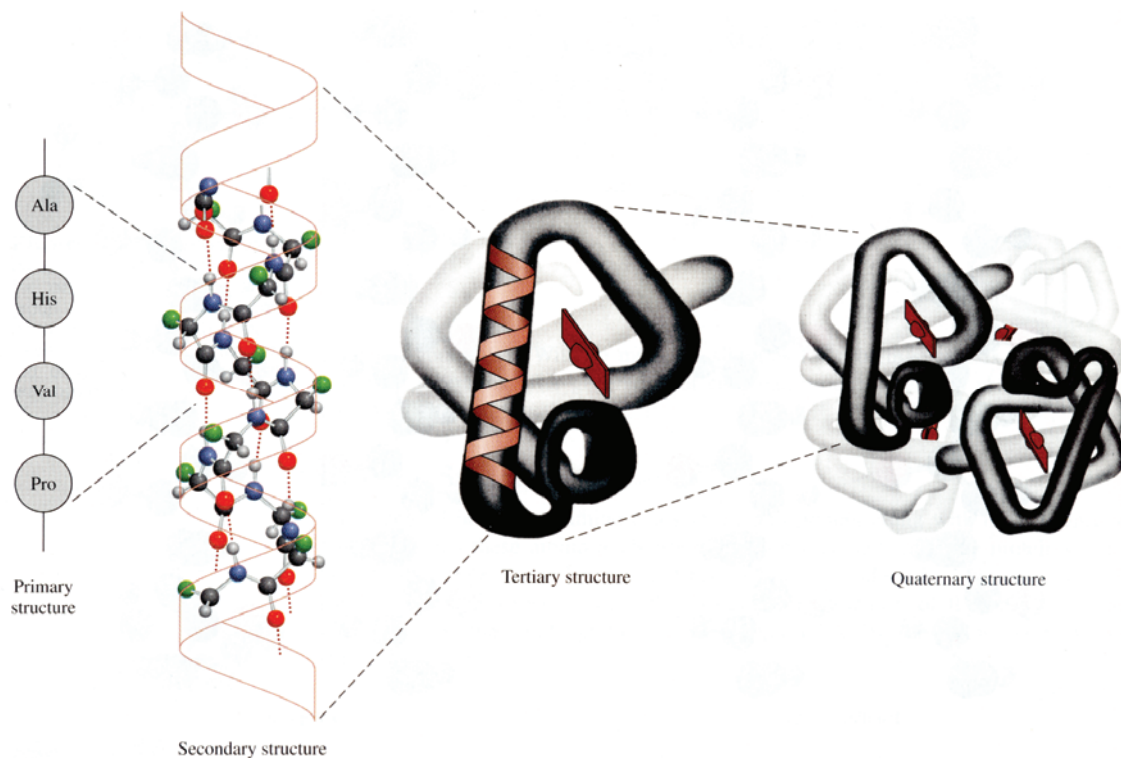


Figure 5 – The primary, secondary, tertiary, and quaternary structure of the hemoglobin molecule.³¹

3.2 Protein adsorption

Most reactions in biology occur, not in solution, but at interfaces. Typical interfaces of biological importance include the cell surface/synthetic biomaterial, extracellular matrix(ECM)/biomolecule, ECM/cell, hydrated tissue/air (lung) and mineral/protein (bone). Some of the advantages that

surfaces offer are high accessibility for reaction, a low energy barrier to mobility in the plane of the surface facilitates complex reactions (clustering and conformational changes), high surface area geometries enhance reaction turnover rates, surface energy minimization can orient specific structures to interfaces and molecular recognition can be readily implemented.³⁵

Biomaterials readily adsorb proteins from biological fluids when inserted in the human body. Indeed, the protein adsorption event occurs well before cells arrive at the surface. Therefore, cells see primarily a protein layer, rather than the actual surface of the biomaterial. Since cells respond specifically to proteins, this interfacial protein film is believed to be the event that controls subsequent reaction to implants. Protein adsorption is also concern for biosensors, immunoassays, array diagnostics, haemodialysis, food processing, marine fouling and a large number of other phenomena.³⁶ It is evident that the scope of the investigations in the different areas above must be very different. A common interest is, however to measure and understand protein adsorption, protein layer structure, and exchange reactions on the solid surfaces. Some important properties for protein-metal surface interactions are presented in Table 5.^{37,38}

Table 5 - Properties that determine protein adsorption at titanium surface [adapted from references^{37,38}]

Titanium surface properties	Protein properties
Free energy	Overall hydrophobicity
Charge	Charge
Ion binding	Ion binding
Redox/corrosion potential	Prosthetic groups
Hydrogen bonding capacity	Hydrogen bonding residues
Surface water structure/ binding strength	Water binding
Acid/basic groups ("surface pH")	Isoelectric point (pI)
Surface impurities	Specific interacting residues
Surface topography	Quaternary, tertiary and secondary structure
Oxide (thickness, growth, dielectric constant, water and ion sorption)	Number of disulphide bridges
Interactions with small (organic) molecules	Size

The properties are lined up although in practice it is not such a simple relation between the adsorption of a given protein molecule on a given metal surface. In fact protein adsorption is very complex, being electrostatic and hydrophobic interactions and the intrinsic structural stability of the protein the most relevant phenomena.³³

In addition to the protein and surface properties described above, adsorption also depends on the availability of molecules for interaction with substrate. Molecules can be brought to the surface by one or more of four major transport mechanisms: diffusion, thermal convection, flow, and coupled transport, and as the combination of convection and diffusion. Variables such as concentration, velocity, and molecular size are important in determining the arrival of protein molecules at surface.²

Several papers have been published on protein adsorption on titanium dealing with specific proteins such as albumin,^{28,39-41} fibronectin,^{23,42,43} vitronectin,^{44,45} fibrinogen,³⁹ glycosaminoglycans,⁴⁶ laminin.⁴⁷⁻⁴⁹ However there are no general accepted conclusions as to the importance of the protein film, or specific proteins, on biological reaction to Ti.⁵⁰

3.2.1 Type of interactions

3.2.1.1 Electrostatic interactions

Protein adsorption to hydrophilic surfaces is usually determined by electrostatic interactions.^{51,52} Although interactions may occur at the atomic scale, the global charges on proteins and surfaces appears to dominate electrostatically driven adsorption.^{33,53-55} This is observed especially with adsorption to hydrophilic surfaces of proteins that are less susceptible to structural changes.^{56,57}

It is intuitive that opposite charges will be mutually attracted and this is indeed observed with respect to protein adsorption. However, the situation becomes complicated in aqueous solutions, in which surface charges are shielded by hydrating water, modulated by pH and counter-balance by small ions. When liked charges at physiological pH are present, maximum

adsorption generally occurs at the isoelectric point of the protein-surface system (pH at which the net charge is minimized).⁵⁶

Coadsorption of counter-ions may contribute to this process and, indeed, the presence of such ions is implied by adsorption of proteins to like-charged surfaces.⁵⁷ This may account for the observed involvement of calcium ions in some adsorption processes.^{28,58,59}

3.2.1.2 Hydrophobic interactions

Hydrophobic interactions play an important role in protein adsorption. Protein molecules tend to minimise exposition of their hydrophobic groups to the aqueous environment. However, Haynes and Norde³³ estimate that 40-50 % of the accessible surface of proteins is often occupied by non-polar groups. For a protein to adsorb, both adsorbate and surface must (at least partially) dehydrate.^{54,60} Dehydration of hydrophobic parts of the protein and the sorbent surfaces is driven by entropy gain and, therefore, promotes adsorption to occur spontaneously.^{57,61,62} The adsorption to hydrophobic substrates may induce conformation change of the protein by hydrophobic-hydrophobic interactions with the substrate contributing to irreversible adsorption.^{61,62}

Hydrophobic dehydration is relatively unimportant for hydrophilic surfaces and/or rigid hydrophilic proteins.^{61,63}

Apparently due to a greater number of possible adsorption-promoting interactions, hydrophobic surfaces are usually reported to adsorb more protein than hydrophilic surfaces.⁶⁴⁻⁶⁷

3.2.2 Conformational stability of proteins

Protein molecules must not be thought of as rigid structures, as they are flexible chains that have been coiled, folded, and bent to assume a particular conformation. Changes in the microenvironment of the proteins, such as pH, temperature, and ionic strength, can alter the conformation of the molecule.² Structural stability of proteins is of particular importance in the

adsorption process and reversibility of adsorption. This is dependent on intramolecular forces within the protein molecules that may lead to an alteration of protein conformation.^{63,68}

Protein molecules with low conformational stability (soft protein), such as albumin, are found to adsorb on a great variety of surfaces (hydrophilic and hydrophobic) even under adverse electrostatic condition.⁶⁷ In contrast, proteins with high conformational stability (hard protein), such as lysozyme, adsorb mainly on hydrophobic surfaces, adsorbing on hydrophilic surfaces only under favourable electrostatic interactions.^{55,63,67,69-73}

Upon adsorption on hydrophobic surfaces, soft proteins change their conformation to a greater extent than hard proteins, due to a loss of secondary structure.⁷⁴⁻⁷⁷ Although conformational alterations may result in protein denaturation and/or loss of functional activity in some situations they can be beneficial rather than detrimental.⁷⁸⁻⁸⁰ As an example enzymes are often more stable in immobilized formulations than in solution, and enzymatic activity can be enhanced in the presence of an interface.^{78,80,81}

3.2.3 Structure of the adsorbed protein layer

Maximum plateau adsorption is usually assumed as a more or less well-packed layer of proteins. The surface concentration corresponding to a monolayer falls in the range expected for a close-packed monolayer of protein (ca. 1 and 5 mg/m²). This value is dependent on the protein, its molecular orientation, conformational state, and the type of substrate.^{55,71,82}

In a simple way it is generally considered that non-spherical proteins may adsorb with two different orientations, i.e., one with the long axis (end-on type) and the other with the short axis (side-on type) perpendicular to the surface.^{57,83,84}

Though proteins often adsorb as monolayers, multilayer adsorption can also occur, especially when the solution concentration is high.⁸²

3.2.4 Competitive protein adsorption

Body fluids, including blood, tears, and saliva, contain numerous types of biomolecules. For example, blood contains more than 150 proteins, apart from lipids, carbohydrates, and hormones.² Adsorption from these types of fluids is very complex since there are many proteins competing for the surface. However adsorption from mixtures of proteins is selective, leading to enrichment of the surface phase in proteins that have higher surface affinity. Studies of plasma interaction with solid surfaces results in a hierarchical adsorption process known as the “Vroman effect”.⁸⁵⁻⁸⁷ The high concentration proteins dominate the surface at short times due to the higher collision rates. As time passes, various exchange processes occur and proteins with higher surface affinities dominate the surface. Finally, at very long times only the highest affinity proteins are present on the surface, even if their bulk solution concentration is very low.^{55,85,88}

3.2.5 Reversibility of protein adsorption

Protein adsorption to hydrophobic surfaces is usually reported as irreversible.^{57,64,89} The irreversibility of adsorption may occur from a range of causes. One explanation could be that hydrophobic peptide moiety in the protein molecule changes its orientation in order to interact with the hydrophobic region on the surface, resulting in a multipoint attachment.^{2,71,90} Also by increasing the adsorption period, the protein undergoes structural deformations.^{74,89} The extent of conformation alterations after adsorption increases as protein solution concentration decreases and therefore with the decrease of the surface coverage.⁹⁰ At low solution concentration, the protein has no neighbours on the surface and thus can optimally adapt to the surface, maximising the number of binding interactions. At high solution concentration, any adsorbed protein is surrounded by neighbours, minimising the probability that it can conformationally adapt to the surface. Consequently more protein may be present on the surface, but with each molecule having fewer surface contacts.^{2,55}

4. Cell/material interactions

4.1. The role of adsorbed proteins in tissue response to biomaterials

Cell adhesion to adsorbed proteins is primarily mediated by integrin receptors.^{91,92} Integrins represent a widely expressed family of heterodimeric (α and β subunits) transmembrane protein receptors that bind to adhesive motifs present in various extracellular matrix proteins, including fibronectin (FN), vitronectin (VN), laminin, and collagen.³² Following ligand binding, integrins cluster and associate with cytoskeletal elements to form focal adhesions, supramolecular assemblies of structural and signaling proteins that provide anchorage forces and activate signaling cascades regulating cell cycle progression and differentiation in developing tissue (Figure 6).³²

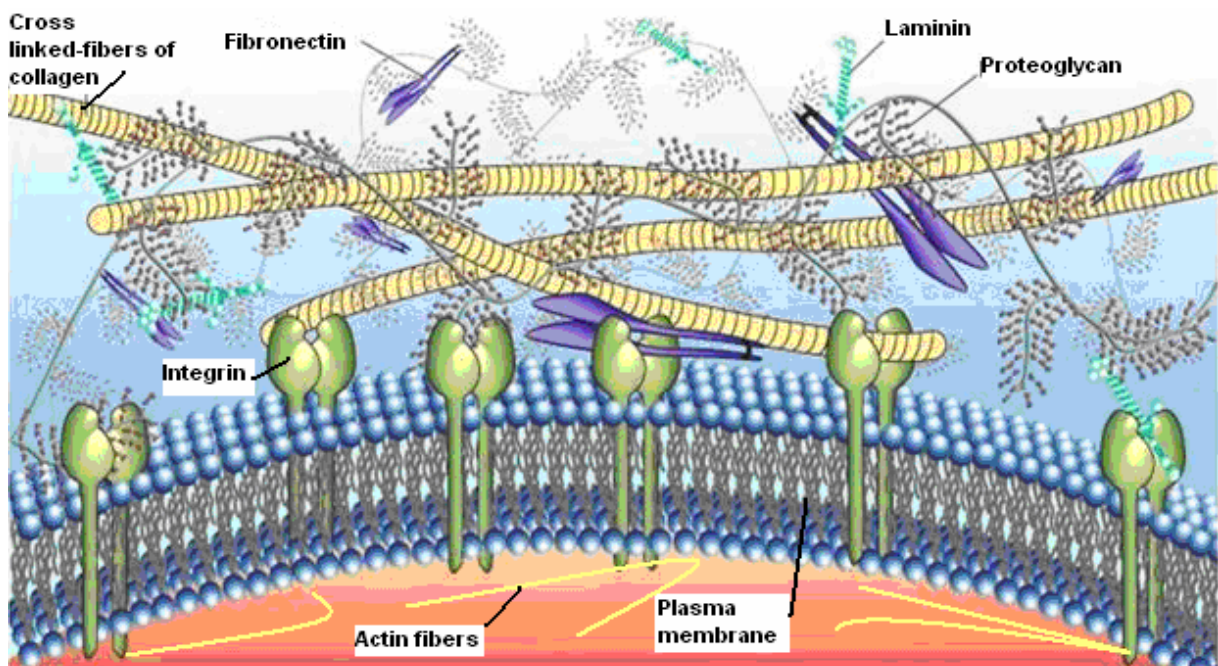


Figure 6 - Interactions between cells and the extracellular matrix. The association between cells and the proteoglycan of the ECM is mediated by a membrane protein (integrin) and by FN an ECM protein with binding sites for both integrin and the proteoglycan. [adapted from ref ³²]

Serum or plasma contains proteins such as albumin and IgG that decrease or inhibit cell attachment while FN and VN can facilitate the adhesion of cells.^{2,55,93-96} It is believed that the efficiency of cell adhesion and growth is dependent on the balance between of adhesion-promoting versus adhesion-inhibiting proteins which competitively adsorb to a surface.

The protein conformation upon adsorption plays a key role in controlling cell behavior including cell adhesion, spreading, migration, and differentiation. For example, fibronectin adsorption to different surfaces alters the structure of the adsorbed protein influencing cell adhesion, spreading, migration, and differentiation.^{97,98}

4.1.1 Adhesion proteins

Fibronectin

Fibronectin is one of the most abundant extracellular matrix glycoproteins that adsorbs to biomaterials, mediating cell adhesion. Apart from FN, there are other glycoproteins containing cell-binding domains such as vitronectin, laminin, and collagen. However, FN is an important substratum for cell adhesion, migration, and wound healing, inflammation, and metastasis.⁹⁹ FN exists in a soluble dimeric form in micromolar concentration in blood plasma and in an insoluble multimeric form in ECM. In soluble form, van der Waals and electrostatic interactions between modules stabilize FN in a compact conformation, unreactive to other extracellular matrix proteins and to self-assembly.⁹⁹ FN partially opens its arms upon surface adsorption, exposing adhesion sites that are buried in the soluble form.¹⁰⁰ Fibronectin is composed of two homologous subunits, each with a molecular weight of ca. 220 kDa, connected near their carboxy termini by a pair of disulfide bonds. Each dimer arm is approximately 70 nm long and consists of a series of globular modules of three types of structural homology (classified as fibronectin repeats FN-I, FN-II, and FN-III). The modules are grouped together into functional domains that bind to cell surfaces (integrins), fibrin, heparin, and collagen (Figure 7).¹⁰¹

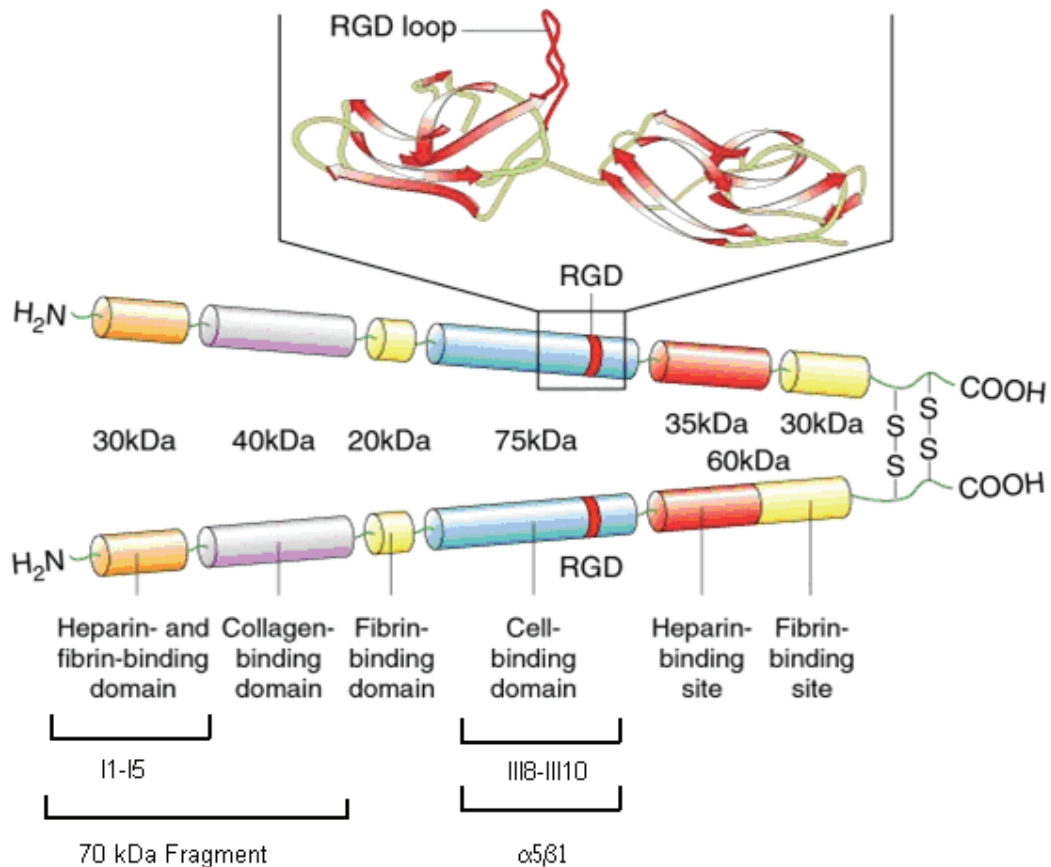


Figure 7 – Schematic representation of fibronectin dimer showing its modular structure and functionality of various domains. Two subunits are shown, with amino termini to the left and carboxyl termini to the right. Regions implicated in adhesion and assembly are shown. [adapted from ref.¹⁰¹]

These modules, though all part of the same amino acid chain, can be envisioned as "beads on a string," each one joined to its neighbors by short linkers. FN binds to integrins ($\alpha_5\beta_1$) through a cell adhesive site comprising modules III8-III10. The most critical site for cell adhesion is the Arg-Gly-Asp (RGD) sequence in III10 module, which is in the carboxy terminal domain.¹⁰² FN-III-9 contains a synergy site which enhances the integrin binding of RGD loop on FN-III-10 (Figure 8).¹¹²

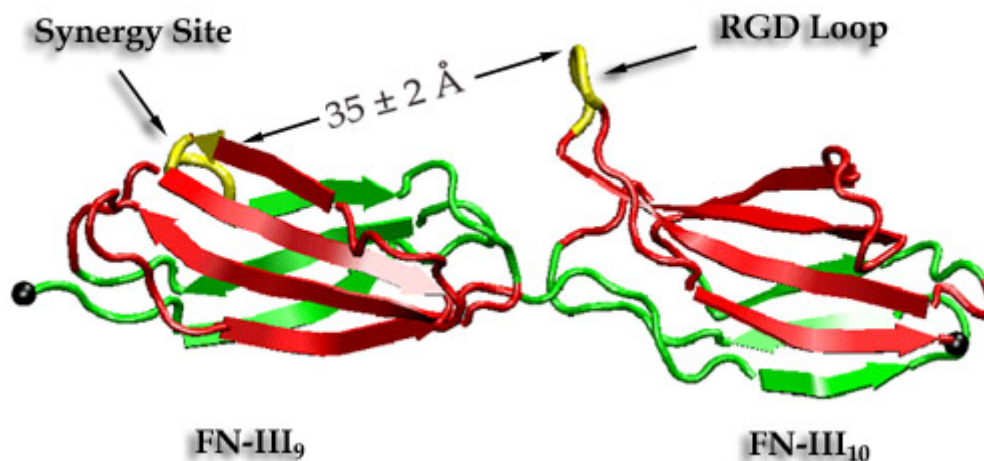


Figure 8 – Schematic diagram showing the synergy site and the RGD-loop that are located on the 9th and 10th type III modules, FN-III₉ and FN-III₁₀, respectively.¹¹²

After binding cell integrins via RGD sequence, FN assembles into fibrils that constitute the primitive extracellular matrix before collagen deposition in wound healing and development.^{91,103}

The mechanism controlling polymerization is likely to involve cryptic sites required for self-assembly exposed by integrin-mediated stretching of FN.^{100,103} FN self-association is known to rely on interactions between specific domains of the protein among which the N-terminal domain (70 kDa fragment) is the most prominent in particular within modules I1-I5.¹⁰⁴

The isoelectric point of FN ranges from 5.6 to 6.1,¹⁰⁵ indicating that at physiological pH FN has a slightly negative charge.

FN dimensions present a higher variability that is account to the extreme flexibility of the protein.^{43,106} FN has been reported to have globular and filamentous forms depending on substrate and solution conditions.^{107,108} Factors like temperature, pH, and ionic strength of the local environment also play a role in the final shape of the FN molecule. Electrostatic interactions between regions of opposite net charge within or between chains could hold the molecule in a relatively compact conformation which becomes more open in high salt or when pH is shifted more than a few units from neutrality. In solution under normal physiological conditions, FN has been reported to assume a globular or compact form.¹⁰⁹⁻¹¹¹

4.1.2 Non-adhesion proteins

Albumin

Human serum albumin (HSA) is the predominant plasma protein, making up 60-70% of plasma in a concentration of ca. 40 mg/mL (human adults). HSA is a globular protein of 585 amino acids^{113,114} with a molecular weight of 66 KDa.¹¹³ HSA has 17 disulfide bridges, which contributes to the stability of the protein in the blood circulation.¹¹³

HSA is composed of three structurally similar globular domains, each of which contains two subdomains, denominated subdomain IA, IB, IIA, IIB, IIIA and IIIB.¹¹⁵ The net charge of HSA at physiological pH for domains I, II and III is -9, -8, and +2, respectively. The isoelectric point of HSA is 4.7, indicating that in physiological conditions this protein has a negative charge.¹¹³ The protein was described to have an ellipsoid form on silica with dimensions of 4 x 4 x 14 nm.¹¹⁶

HSA is a relatively flexible protein, and is a key protein in biomaterials applications. The immobilized protein can be used to minimize surface-blood interactions greatly reducing the acute inflammatory response to the material.^{38,117,118}

5. Techniques for protein adsorption study at biomaterials surfaces

There are several techniques that can be used to study the adsorption of a protein to a surface, since the thickness of adsorbed protein monolayer (1-10 nm) are in the range of most surface analysis instrumentation. A non exhaustive list of some important and commonly used techniques to characterize biomaterials surfaces with adsorbed proteins are presented in Table 6.^{35-37,84,119,120} The referred techniques are essentially those that give direct information about adsorption isotherms, adsorption/desorption kinetics, structure/conformation of the protein layer on the surface, interaction of protein molecules on surfaces and visualization of proteins.³⁵⁻³⁷ Information can be enhanced by combination of techniques.

Table 6 – Selected techniques for protein adsorption study at biomaterials surfaces, ^{35-37,84,119,120}

Techniques	Principle	Information depth	Analytical sensitivity	Spatial resolution	Information
Radiolabelling techniques	Use of radioisotopes on certain residues of protein	Uppermost molecular level	High	Poor; droplet size ca. 2 µm	Adsorbed amount from single and complex protein solutions, adsorption kinetics and isotherms, exchange of molecules on surfaces.
Immunofluorescence	Use of an unlabelled primary antibody of a particular protein and a fluorochrome labelled secondary antibody	Uppermost molecular level	Very high	Poor	Detection of the location and relative abundance of a particular protein for which an antibody exists; conformation of an adsorbed protein
Ellipsometry	Detection of polarization change of light reflected on a sample.	100-300 nm	0.1-1 nm	Lateral resolution ca. 2 µm	Adsorbed amount from single and complex protein solutions (when associated to antibodies), adsorption kinetics, structural information of the adsorbed layer.
SPR	Influence of adsorbed molecules on electron charge density waves in the surface of a thin metal film (Au or Ag)	50-300 nm	0.1-1 ng/cm ²	Lateral resolution <10 µm for ex situ, 10-50 µm for in situ measurements in the imaging mode	Adsorbed amount from single and complex protein solutions (when associated to antibodies), adsorption kinetics, structural information of the adsorbed layer, specificity of adsorption when associated to antibodies.
ATR-FTIR	IR spectroscopy using evanescent field above the surface of a specially cut crystal in which the light propagates between the crystal surfaces	1-5 µm	1-10 mol %	10 µm, ca. 5 µm in the microscope mode	Identification of chemical functional groups and chemical bonds, characterization of thin films of proteins, conformation of adsorbed proteins
IRAS	Absorption of IR light hitting a surface under grazing incidence	0.05-1 µm	<10 ¹⁵ atoms/cm ²	ca. 5 µm diameter may be achieved, in the microscope mode	Identification of chemical functional groups and chemical bonds, examination of the orientation and average conformation of adsorbed proteins on reflective surfaces.

Table 6, cont.

Techniques	Principle	Information depth	Analytical sensitivity	Spatial resolution	Information
AFM	Atomic interaction between surface and tip are used to generate an atom-scale, electron density image of a surface.	Uppermost atomic or molecular level	Single atoms/molecules	0.1 nm (z), 2 nm (x) (depending on the shape and diameter of the tip)	Topography, protein visualization, dimensions of proteins, molecular conformation, adhesion, molecular recognition, friction forces, and force spectroscopy studies.
STM	Quantum tunnelling current is used to generate an atom-scale, electron density image of a surface.	0.5 nm	Single atoms	0.1 nm (z), 0.1 nm (x)	Topography, protein visualization, atomic and small molecules organization, surface dynamics.
SEM	Secondary electron emission induced by a focused electron beam is measured and spatially imaged	0.5 nm	High, but not quantitative	4 nm	Coverage and structure of protein films and individual molecules; correlation with surface structure
Contact angle measurement	Measurement of solid/liquid/vapour contact angles	0.1-2 nm (uppermost atomic or molecular level)	Low or high depending on the chemistry	Poor, droplet size ca. 1 mm	Surface energy and its components, hydrophobicity/hydrophobicity and affinity of a protein to a surface.
XPS	Analysis of the energy distribution of photoemitted electrons from a surface	2-10 nm	10^{12} - 10^{13} atoms/cm ²	10-150 μ m	Qualitative and semi-quantitative information of elemental surface composition, information about the molecular environment (oxidation state, bonding atoms, etc.).
ToF-SIMS	Ion bombardment sputters secondary ions from the surface. The ions are characterized according to their mass-to-charge ratio using a time-to flight mass spectrometer	1 nm in "static mode", 1 μ m in the "dynamic mode"	Very high	Lateral resolution >100 nm	Qualitative information of chemical elements, charge molecular fragments, chemical structure of the compound; map of protein distribution, identification of different proteins; protein conformation, degree of denaturation of an adsorbed protein film, surface coverage.

6. Aim and structure of the thesis

In this thesis, the adsorption of two proteins, albumin and fibronectin, from single protein solutions to TiO_2 substrates was analysed. The importance of albumin arises essentially due to the inhibitory effect of albumin on cell adhesion, and to the high albumin concentration presented in blood and plasma. The fibronectin significance occurs from the adhesive properties required for integrin-receptor-based cell adhesion and proliferation on surfaces. The latter protein is one of the most important components of extracellular matrix (ECM). However, the extracellular fluids are protein rich and competition between them occurs. Therefore, the final part of the work is related with the influence of competitive preadsorption of HSA and FN from binary protein mixtures and from 10 % plasma on MC3T3-E1 osteoblasts adhesion and morphology on TiO_2 substrates.

In **Chapter I** a general introduction is presented on the subject of titanium as a biomaterial for bone replacement. Protein adsorption at solid/liquid interfaces and cell/material interactions were also addressed.

The experimental work is presented along four chapters (**Chapter II to V**):

Chapter II describes albumin adsorption from single protein solutions and from plasma at two titanium oxides surfaces. The oxides were developed in order to obtain stable and homogeneous surfaces similar to those applied in clinical use but with different roughnesses. One was a titanium oxide layer sputtered on Si (TiO_2 sp) and the other a TiO_2 formed in H_2O_2 solution (TiO_2 cp). Some surface treatments of Ti cp were tried, in which H_2O_2 concentration and time of immersion was varied. The two titanium oxides developed in this chapter were used along all the experimental work.

The surfaces were characterized by atomic force microscopy (AFM), imaging and roughness analysis, using Tapping® mode AFM in air, by X-ray photoelectron spectroscopy (XPS) and by wettability studies leading to the surface free energy.

The adsorption kinetics and isotherms were evaluated by two different techniques radiolabelling of albumin (^{125}I -HSA) and XPS. With the latter technique it was possible to follow albumin adsorption in TiO_2 through the increase of nitrogen and carbon groups and the decrease of titanium and oxygen intensity. The work of adhesion between protein solutions and TiO_2 substrates was evaluated through wettability studies. HSA exchangeability was also estimated by ^{125}I -HSA.

The two TiO_2 surfaces are identical chemically and have distinct roughnesses; the TiO_2 sputtered has a mean R_a value 14 times smaller than the one for TiO_2 cp.

Differences in HSA affinity to the TiO_2 substrates were observed when the protein was assayed from a single protein solution and plasma. TiO_2 cp adsorb less albumin than the TiO_2 sp surfaces as shown by the radiolabelling data but the adherent molecules are strongly attached to the former surface as indicated by the work of adhesion and by the desorption studies. The data obtained by ^{125}I -HSA also suggests that albumin adsorbs as a multilayer.

In **Chapter III** the adsorption of human fibronectin (FN) on the two titanium oxide layer referred in Chapter II is presented. Adsorption isotherms were assessed by radiolabelling of FN with ^{125}I (^{125}I -FN) and by X-ray photoelectron spectroscopy (XPS). Exchangeability of bound FN by free FN, was also evaluated by the radiolabelling technique. The work of adhesion was evaluated through wettability studies.

Differences in FN affinity to the TiO_2 substrates were observed when the protein was assayed from a single protein solution and plasma. TiO_2 cp adsorbs more FN from a single protein solution than the TiO_2 sp surfaces as shown by the radiolabelling data. In contrast, when a plasma solution was assayed a higher affinity of the solution to the TiO_2 sp surface occurred. Besides FN molecules are more strongly attached to the former surface as indicated by the work of adhesion and by the exchangeability studies. Comparing the theoretical amount for monolayer formation and the data obtained by ^{125}I -FN, it can be suggested that FN may only adsorb as a multilayer in the side-on position, for concentrations higher than 100 $\mu\text{g/mL}$.

In **Chapter IV** fibronectin (FN) adsorption on the two titanium oxides used before was further characterized. Imaging and roughness analysis before and after FN adsorption used Atomic Force Microscopy (AFM) in Tapping® mode, in air, and in Magnetic Alternating Current (MAC) mode, in liquid (water). This is the first report on the use of MAC mode to address the adsorption of FN on Ti. FN adsorption as a function of time was followed by X-ray photoelectron spectroscopy (XPS), by radiolabelling of FN with ^{125}I (^{125}I -FN) and by ellipsometry. Exchangeability studies were performed using FN and HSA.

Direct visualization of FN adsorbed on TiO_2 sp using Tapping® mode AFM in air and MAC mode AFM in water, was possible. Phase imaging AFM detects successfully adsorbed FN on the rougher TiO_2 surfaces, TiO_2 cp, and the roughness parameters, R_q and R_{max} , also confirm the presence of FN on both substrates.

It was revealed that FN forms globular aggregates in an ellipsoidal conformation, when adsorbed to the TiO_2 sp surface. As the time of incubation is prolonged, the FN tends to extend on the surface with incoming molecules adsorbing on top on existing molecules. Increasing FN concentration, the aggregates coalesce forming clusters.

TiO_2 cp adsorbs more FN than the TiO_2 sp surfaces, after 60 min. of adsorption, as shown by the radiolabelling data. TiO_2 cp retains more than the necessary amount for cell adhesion as opposed to the TiO_2 sp that retains only the minimum, according to the amount of FN adsorbed needed for cell adhesion, referred by other authors.¹²¹ FN molecules are also more strongly attached to the former surface as indicated by the exchangeability studies.

Regarding the amount of FN adsorbed at TiO_2 surface, the data obtained by ellipsometry correlate well with those obtained by the radiolabelling technique, showing also that are below, to the theoretical value for a side-on FN monolayer. AFM data agree with these results as showed by the fact that FN does not adsorbed as a monolayer but instead as islands or clusters.

In **Chapter V** the influence of competitive preadsorption of HSA and FN on MC3T3-E1 osteoblasts adhesion and morphology on TiO_2 substrates was investigated. The strategy applied in the present investigation was to compare osteoblasts adhesion to surfaces

preadsorbed with HSA, FN, HSA/FN=1, HSA/FN=200 and 10% plasma. The adsorption of the referred proteins was also evaluated measuring the amount and the effectiveness of binding with radiolabelled proteins, ^{125}I -FN and ^{125}I -HSA.

MC3T3-E1 osteoblasts cell adhesion correlates well with the amounts of FN and HSA adsorbed on TiO_2 surfaces. The results suggest that HSA competes with FN for the TiO_2 surface sites.

Fewer osteoblasts adhered to both substrates preadsorbed with HSA, HSA/FN=200 and 10% plasma, than to the surfaces preadsorbed with FN and HSA/FN=1. For the latter protein preadsorption, FN was able to compensate the inhibitory effect of HSA on osteoblasts adhesion, with exception of TiO_2 sp after 4 h of MC3T3-E1 incubation. Therefore, the presence of lower amounts of co-adsorbed albumin may improve presentation of FN in a more integrin-recognized conformation, suggesting that some degree of molecular packing prevents loss of integrin-binding activity.

FN reversibility does not seem to be dependent on the HSA/FN adsorption mass ratio in solution, suggesting that FN competitively adsorbs to TiO_2 in a favorable conformation and does not suffer subsequent conformational changes allowing exchange with other FN molecules in solution.

In the last part of the thesis (**Chapter VI**), a short general discussion is presented, since a detailed discussion is already provided in each of the preceding chapters. Possible directions for future research are also given.

The work described in this thesis (**Chapter II to V**) has been published, accepted or for publication in international journals.

Papers resulting from this thesis:

1. Sousa SR, Moradas-Ferreira P, Melo LV, Saramago B, Barbosa MA. Human Serum Albumin Adsorption on TiO_2 from Single Protein Solutions and from Plasma. *Langmuir* 2004;20(22):9745-9754.
2. Sousa S, Moradas-Ferreira P, Barbosa M. TiO_2 type influences fibronectin adsorption. *Journal of Materials Science: Materials in Medicine* 2005;16(12):1173-1178.
3. Sousa SR, Brás M, Moradas-Ferreira P, Barbosa MA. Dynamics of fibronectin adsorption on TiO_2 , accepted for publication in *Langmuir*.
4. Sousa SR, Lamghari M, Moradas-Ferreira P, Barbosa MA. Osteoblast adhesion and morphology on TiO_2 depends on the competitive preadsorption of HSA and FN, *Journal of Biomedical Materials Research – Part A*, *in press*.

7. References

1. Brunski JB. Metals. In: Ratner BR, Hoffman AS, Schoen FJ, Lemons JE, editors. Biomaterials science: an introduction to materials in medicine. New York: Elsevier Academic Press, Inc.; 2004. p 37-50.
2. Bizios R, Dee KC, Puleo DA. An introduction to tissue-biomaterial interactions. New Jersey: John Wiley and sons, Inc.; 2003.
3. Freese HL, Volas MG, Wood JR. Metallurgy and technological properties of titanium and titanium alloys. In: Brunette DM, Tengvall P, Textor M, Thomsen P, editors. Titanium in Medicine: Material Science, Surface Science, Engineering, Biological Responses and Medical Applications: Springer; 2001.
4. Kauffman GB. Kirk-Othmer Encyclopedia of Chemical Technology: Sigma Xi, The Scientific Research Society; 1999.
5. Lausmaa J. Surface spectroscopic characterization of titanium implant materials. Journal of Electron Spectroscopy and Related Phenomena 1996;81(3):343-361.
6. Sittig C, Textor M, Spencer ND, Wieland M, Vallotton PH. Surface characterization of implant materials cp Ti, Ti-6Al-7Nb and Ti-6Al-4V with different pretreatments. Journal of Materials Science-Materials in Medicine 1999;10(1):35-46.
7. McCafferty E, Wightman JP. An X-ray photoelectron spectroscopy sputter profile study of the native air-formed oxide film on titanium. Applied Surface Science 1999;143(1-4):92-100.
8. Textor M, Sittig C, Frauchiger V, Tosatti S, Brunette DM. Properties and biological significance of natural oxide films on Titanium and its alloys. In: Brunette DM, Tengvall P, Textor M, Thomsen P, editors. Titanium in Medicine: Material Science, Surface Science, Engineering, Biological Responses and Medical Applications. Berlin: Springer-Verlag; 2001. p 172-230.
9. Tengvall P, Lundstrom I. Physico-chemical considerations of titanium as a biomaterial. Clinical Materials 1992;9(2):115-134.

-
10. Lausmaa J, Kasemo B, Mattsson H. Surface spectroscopic characterization of titanium implant materials. *Applied Surface Science* 1990;44(2):133-146.
 11. Kasemo B, Lausmaa J. Biomaterial and Implant Surfaces - on the Role of Cleanliness, Contamination, and Preparation Procedures. *Journal of Biomedical Materials Research-Applied Biomaterials* 1988;22(A2):145-158.
 12. Wieland M. Experimental determination and quantitative evaluation of the surface composition and topography of medical implant surfaces and their influence on osteoblastic cell-surface interactions [PhD thesis]. Zurich; 1999. 247 p.
 13. Carley AF, Chalker PR, Riviere JC, Roberts MW. The Identification and Characterization of Mixed Oxidation-States at Oxidized Titanium Surfaces by Analysis of X-Ray Photoelectron-Spectra. *Journal of the Chemical Society-Faraday Transactions I* 1987;83:351-370.
 14. Hanawa T, Ota M. Characterization of Surface-Film Formed on Titanium in Electrolyte Using XPS. *Applied Surface Science* 1992;55(4):269-276.
 15. Ramqvist L, Hamrin K, Johansson G, Fahlman A, Nordling C. Charge Transfer in Transition Metal Carbides and Related Compounds Studied by ESCA. *Journal of Physics and Chemistry of Solids* 1969;30(7):1835-&.
 16. Sousa SR, Moradas-Ferreira P, Melo LV, Saramago B, Barbosa MA. Human Serum Albumin Adsorption on TiO₂ from Single Protein Solutions and from Plasma. *Langmuir* 2004;20(22):9745-9754.
 17. Kuznetsov MV, Zhuravlev JF, Gubanov VA. XPS Analysis of Adsorption of Oxygen Molecules on the Surface of Ti and Thin Films in Vacuum. *Journal of Electron Spectroscopy and Related Phenomena* 1992;58(3):169-176.
 18. Shirkhanzadeh M. XRD and XPS Characterization of superplastic TiO₂ coatings prepared on Ti6Al4V surgical alloy by an electrochemical method. *Journal of Materials Science-Materials in Medicine* 1995;6(4):206-210.
 19. Kilpadi DV, Raikar GN, Liu J, Lemons JE, Vohra Y, Gregory JC. Effect of surface treatment on unalloyed titanium implants: Spectroscopic analyses. *Journal of Biomedical Materials Research* 1998;40(4):646-659.

20. Armstrong NR, Quinn RK. Auger and X-Ray Photoelectron Spectroscopic and Electrochemical Characterization of Titanium Thin-Film Electrodes. *Surface Science* 1977;67(2):451-468.
21. Leitao E, Barbosa MA, deGroot K. XPS characterization of surface films formed on surface-modified implant materials after cell culture. *Journal of Materials Science-Materials in Medicine* 1997;8(7):423-426.
22. Sittig C. Charakterisierung der Oxidschichten auf Titan und Titanlegierungen sowie deren Reaktionen in Kontakt mit biologisch relevanten Modellösungen [PhD]. Zurich; 1998.
23. Steinemann SG. Titanium - the material of choice? *Periodontology* 2000 1998;17:7-21.
24. Moreno-Herrero F, Colchero J, Gomez-Herrero J, Baro AM. Atomic force microscopy contact, tapping, and jumping modes for imaging biological samples in liquids. *Physical Review E* 2004;69(3).
25. Tengvall P, Lundstrom I, Sjoqvist L, Elwing H, Bjursten LM. Titanium-Hydrogen Peroxide Interaction - Model Studies of the Influence of the Inflammatory Response on Titanium Implants. *Biomaterials* 1989;10(3):166-175.
26. Sundgren JE, Bodo P, Lundstrom I. Auger-Electron Spectroscopic Studies of the Interface between Human-Tissue and Implants of Titanium and Stainless-Steel. *Journal of Colloid and Interface Science* 1986;110(1):9-20.
27. Albrektsson T, Hansson HA, Ivarsson B. Interface Analysis of Titanium and Zirconium Bone Implants. *Biomaterials* 1985;6(2):97-101.
28. Ellingsen JE. A Study on the Mechanism of Protein Adsorption to TiO₂. *Biomaterials* 1991;12(6):593-596.
29. Lausmaa J. Mechanical, Thermal, Chemical and Electrochemical Surface Treatment of Titanium. In: Brunette DM, Tengvall P, Textor M, Thomsen P, editors. *Titanium in Medicine: Material Science, Surface Science, Engineering, Biological Responses and Medical Applications*: Springer Verlag; 2001. p 231-266.
30. Chinn JA. Biomaterials: Protein-Surface Interactions. In: Bronzino JD, editor. *The Biomedical Engineering Handbook*. USA: CRC Press; 1995. p 1597-1608.

-
31. Chang R. Chemistry: The McGraw-Hill Companies, Inc.; 2005.
 32. Nelson DL, Cox MM. Lehninger Principles of Biochemistry: W. H. Freeman; ; 2004. 1100 p.
 33. Haynes CA, Norde W. Globular proteins at solid/liquid interfaces. Colloids and Surfaces B: Biointerfaces 1994;2(6):517-566.
 34. Gitlin I, Carbeck JD, Whitesides GM. Why are proteins charged? Networks of charge-charge interactions in proteins measured by charge ladders and capillary electrophoresis. Angewandte Chemie-International Edition 2006;45(19):3022-3060.
 35. Castner DG, Ratner BD. Biomedical surface science: Foundations to frontiers. Surface Science 2002;500(1-3):28-60.
 36. Ratner BD. Surface properties and surface characterization of materials. In: Ratner BD, Hoffman AS, Schoen FJ, Lemons JE, editors. Biomaterials Science: An Introduction to Materials in Medicine. San Diego, CA: Elsevier Academic Press; 2004. p 40-59.
 37. Ivarsson B, Lundstrom I. Physical Characterization of Protein Adsorption on Metal and Metaloxide Surfaces. CRC Critical Reviews in Biocompatibility 1986;2(1):1-96.
 38. Tengvall P. Proteins at titanium interfaces. In: Brunette DM, Tengvall P, Textor M, Thomsen P, editors. Titanium in Medicine: Material Science, Surface Science, Engineering, Biological Responses and Medical Applications. Berlin: Springer-Verlag; 2001.
 39. Wälivaara B, Aronsson BO, Rodahl M, Lausmaa J, Tengvall P. Titanium with Different Oxides - in-Vitro Studies of Protein Adsorption and Contact Activation. Biomaterials 1994;15(10):827-834.
 40. Serro APV, Fernandes AC, Saramago BDV. Dynamic interfacial behaviour of bovine serum albumin solutions on titanium surfaces. Colloids and Surfaces 1997;125(2-3):209-219.
 41. Hook F, Vörös J, Rodahl M, Kurrat R, Boni P, Ramsden JJ, Textor M, Spencer ND, Tengvall P, Gold J and others. A comparative study of protein adsorption on titanium oxide surfaces using in situ ellipsometry, optical waveguide lightmode spectroscopy,

- and quartz crystal microbalance/dissipation. *Colloids and Surfaces B-Biointerfaces* 2002;24(2):155-170.
42. Francois P, Vaudaux P, Taborelli M, Tonetti M, Lew DP, Descouts P. Influence of surface treatments developed for oral implants on the physical and biological properties of titanium .2. Adsorption isotherms and biological activity of immobilized fibronectin. *Clinical Oral Implants Research* 1997;8(3):217-225.
43. MacDonald DE, Markovic B, Allen M, Somasundaran P, Boskey AL. Surface analysis of human plasma fibronectin adsorbed to commercially pure titanium materials. *Journal of Biomedical Materials Research* 1998;41(1):120-130.
44. Zhang H, Bremmell K, Kumar S, Smart RSC. Vitronectin adsorption on surfaces visualized by tapping mode atomic force microscopy. *Journal of Biomedical Materials Research Part A* 2004;68A(3):479-488.
45. Howlett CR, Evans MDM, Walsh WR, Johnson G, Steele JG. Mechanism of Initial Attachment of Cells Derived from Human Bone to Commonly Used Prosthetic Materials During Cell-Culture. *Biomaterials* 1994;15(3):213-222.
46. Collis JJ, Embery G. Adsorption of Glycosaminoglycans to Commercially Pure Titanium. *Biomaterials* 1992;13(8):548-552.
47. Goto T, Yoshinari M, Kobayashi S, Tanaka T. The initial attachment and subsequent behavior of osteoblastic cells and oral epithelial cells on titanium. *Bio-Medical Materials and Engineering* 2004;14(4):537-544.
48. Uchida M, Oyane A, Kim HM, Kokubo T, Ito A. Biomimetic coating of laminin-apatite composite on titanium metal and its excellent cell-adhesive properties. *Advanced Materials* 2004;16(13):1071-+.
49. El-Ghannam A, Starr L, Jones J. Laminin-5 coating enhances epithelial cell attachment, spreading, and hemidesmosome assembly on Ti-6Al-4V implant material in vitro. *Journal of Biomedical Materials Research* 1998;41(1):30-40.
50. Ratner BD. A perspective on titanium biocompatibility. In: Brunette DM, Tengvall P, Textor M, Thomsen P, editors. *Titanium in Medicine: Material Science, Surface*

-
- Science, Engineering, Biological Responses and Medical Applications. Berlin: Springer-Verlag; 2001. p 1-12.
51. Andrade JD, Hlady V, Jeon SI. Polyethylene Oxide and Protein Resistance - Principles, Problems, and Possibilities. Abstracts of Papers of the American Chemical Society 1993;206:31-PMSE.
 52. Shirahama H, Lyklema J, Norde W. Comparative Protein Adsorption in Model Systems. Journal of Colloid and Interface Science 1990;139(1):177-187.
 53. Wilson CJ, Clegg RE, Leavesley DI, Pearcy MJ. Mediation of biomaterial-cell interactions by adsorbed proteins: a review. Tissue Eng 2005;11(1-2):1-18.
 54. Horbett TA. Proteins: structure, properties and adsorption to surfaces. In: Ratner BD, Hoffman AS, Schoen FJ, Lemons JL, editors. Biomaterials Science - An Introduction to Materials in Medicine. San Diego, CA, USA: Academic Press Inc.; 1996. p 133-141.
 55. Horbett TA. The role of adsorbed proteins in tissue response to biomaterials. In: Ratner BD, Hoffman AS, Schoen FJ, Lemons JE, editors. Biomaterials Science: An Introduction to Materials in Medicine. San Diego, CA, USA: Elsevier Academic Press; 2004. p 237-247.
 56. Haynes CA, Sliwinsky E, Norde W. Structural and Electrostatic Properties of Globular-Proteins at a Polystyrene Water Interface. Journal of Colloid and Interface Science 1994;164(2):394-409.
 57. Norde W. Adsorption of Proteins from Solution at the Solid-Liquid Interface. Advances in Colloid and Interface Science 1986;25(4):267-340.
 58. Serro APVA, Fernandes AC, Saramago BDV, Norde W. Bovine serum albumin adsorption on titania surfaces and its relation to wettability aspects. Journal of Biomedical Materials Research 1999;46(3):376-381.
 59. Underwood PA, Steele JG, Dalton BA. Effects of Polystyrene Surface-Chemistry on the Biological-Activity of Solid-Phase Fibronectin and Vitronectin, Analyzed with Monoclonal-Antibodies. Journal of Cell Science 1993;104:793-803.

60. Norde W, Giacomelli CE. BSA structural changes during homomolecular exchange between the adsorbed and the dissolved states. *Journal of Biotechnology* 2000;79(3):259-268.
61. Andrade JD, Hlady V. Protein Adsorption and Materials Biocompatibility - a Tutorial Review and Suggested Hypotheses. *Advances in Polymer Science* 1986;79:1-63.
62. van Oss CJ. Hydrophobicity and hydrophilicity of biosurfaces. *Current Opinion in Colloid and Interface Science* 1997;2:503-512.
63. Martins MCL. Interaction between Blood Proteins and Surfaces: Self-Assembled Monolayers (SAMs) and Biomedical Polymers [PhD]. Porto: Porto; 2003.
64. Jonsson U, Ivarsson B, Lundstrom I, Berghem L. Adsorption Behavior of Fibronectin on Well-Characterized Silica Surfaces. *Journal of Colloid and Interface Science* 1982;90(1):148-163.
65. MacDonald DE, Deo N, Markovic B, Stranick M, Somasundaran P. Adsorption and dissolution behavior of human plasma fibronectin on thermally and chemically modified titanium dioxide particles. *Biomaterials* 2002;23(4):1269-1279.
66. Absolom DR, Zingg W, Neumann AW. Protein Adsorption to Polymer Particles - Role of Surface-Properties. *J Biomed Mater Res* 1987;21(2):161-171.
67. Malmsten M. Formation of adsorbed protein layers. *Journal of Colloid and Interface Science* 1998;207(2):186-199.
68. Hlady V, Buijs J. Protein adsorption on solid surfaces. *Current Opinion in Biotechnology* 1996;7(1):72-77.
69. Giacomelli CE, Esplandiu MJ, Ortiz PI, Avena MJ, De Pauli CP. Ellipsometric study of bovine serum albumin adsorbed onto Ti/TiO₂ electrodes. *Journal of Colloid and Interface Science* 1999;218(2):404-411.
70. Giacomelli CE, Avena MJ, DePauli CP. Adsorption of bovine serum albumin onto TiO₂ particles. *Journal of Colloid and Interface Science* 1997;188(2):387-395.
71. Norde W, Anusiem ACI. Adsorption, Desorption and Readsorption of Proteins on Solid-Surfaces. *Colloids and Surfaces* 1992;66(1):73-80.

-
72. Renner L, Pompe T, Salchert K, Werner C. Fibronectin displacement at polymer surfaces. *Langmuir* 2005;21(10):4571-4577.
 73. Renner L, Jorgensen B, Markowski M, Salchert K, Werner C, Pompe T. Control of fibronectin displacement on polymer substrates to influence endothelial cell behaviour. *Journal of Materials Science-Materials in Medicine* 2004;15(4):387-390.
 74. Malmsten M, Lassen B. Competitive Adsorption at Hydrophobic Surfaces from Binary Protein Systems. *Journal of Colloid and Interface Science* 1994;166(2):490-498.
 75. Giacomelli CE, Bremer M, Norde W. ATR-FTIR study of IgG adsorbed on different silica surfaces. *Journal of Colloid and Interface Science* 1999;220(1):13-23.
 76. Zeng HT, Chittur KK, Lacefield WR. Analysis of bovine serum albumin adsorption on calcium phosphate and titanium surfaces. *Biomaterials* 1999;20(4):377-384.
 77. Cheng SS, Chittur KK, Sukenik CN, Culp LA, Lewandowska K. The Conformation of Fibronectin on Self-Assembled Monolayers with Different Surface-Composition - an FTIR/ATR Study. *Journal of Colloid and Interface Science* 1994;162(1):135-143.
 78. Zoungrana T, Findenegg GH, Norde W. Structure, stability, and activity of adsorbed enzymes. *Journal of Colloid and Interface Science* 1997;190(2):437-448.
 79. Maste MCL, Norde W, Visser A. Adsorption-induced conformational changes in the serine proteinase savinase: A tryptophan fluorescence and circular dichroism study. *Journal of Colloid and Interface Science* 1997;196(2):224-230.
 80. Barrias CC, Martins CL, Miranda CS, Barbosa MA. Adsorption of a therapeutic enzyme to self-assembled monolayers: effect of surface chemistry and solution pH on the amount and activity of adsorbed enzyme. *Biomaterials* 2005;26(15):2695-2704.
 81. Kobayashi A, Sato Y, Mizutani F. Adsorption properties and activities of lipase on a gold substrate modified by self-assembled monolayers. *Bioscience Biotechnology and Biochemistry* 2001;65(11):2392-2396.
 82. Feng L, Andrade JD. Protein adsorption on low temperature isotropic carbon. III. Isotherms, competitiveness, desorption and exchange of human albumin and fibrinogen. *Biomaterials* 1994;5(15):323-333.

83. Watanabe N, Shirakawa T, Iwahashi M, Ohbu K, Seimiya T. Effect of Surface-Charge on Adsorption of Bovine Serum-Albumin as Studied by Ellipsometry .1. Adsorption on Cationic Monolayer. *Colloid and Polymer Science* 1986;264(10):903-908.
84. Nakanishi K, Sakiyama T, Imamura K. On the adsorption of proteins on solid surfaces, a common but very complicated phenomenon. *Journal of Bioscience and Bioengineering* 2001;91(3):233-244.
85. Vroman L, Adams AL, Fischer GC, Munoz PC. Interaction of High Molecular-Weight Kininogen, Factor-Xii, and Fibrinogen in Plasma at Interfaces. *Blood* 1980;55(1):156-159.
86. Horbett TA. Principles Underlying the Role of Adsorbed Plasma-Proteins in Blood Interactions with Foreign Materials. *Cardiovascular Pathology* 1993;2(3):S137-S148.
87. Horbett TA. Mass-Action Effects on Competitive Adsorption of Fibrinogen from Hemoglobin-Solutions and from Plasma. *Thrombosis and Haemostasis* 1984;51(2):174-181.
88. Brash JL, Tenhove P. Effect of Plasma Dilution on Adsorption of Fibrinogen to Solid-Surfaces. *Thrombosis and Haemostasis* 1984;51(3):326-330.
89. Bohnert JL, Horbett TA. Changes in Adsorbed Fibrinogen and Albumin Interactions with Polymers Indicated by Decreases in Detergent Elutability. *Journal of Colloid and Interface Science* 1986;111(2):363-378.
90. Norde W, Favier JP. Structure of Adsorbed and Desorbed Proteins. *Colloids and Surfaces* 1992;64(1):87-93.
91. Hynes RO. Integrins - Versatility, Modulation, and Signaling in Cell-Adhesion. *Cell* 1992;69(1):11-25.
92. Horbett TA. The role of adsorbed proteins in animal cell adhesion. *Colloids and Surfaces B: Biointerfaces* 1994;2(1-3):225-240.
93. McFarland CD, Mayer S, Scotchford C, Dalton BA, Steele JG, Downes S. Attachment of cultured human bone cells to novel polymers. *Journal of Biomedical Materials Research* 1999;44(1):1-11.

-
94. McFarland CD, Thomas CH, DeFilippis C, Steele JG, Healy KE. Protein adsorption and cell attachment to patterned surfaces. *Journal of Biomedical Materials Research* 2000;49(2):200-210.
 95. Yang YZ, Cavin R, Ong JL. Protein adsorption on titanium surfaces and their effect on osteoblast attachment. *Journal of Biomedical Materials Research Part A* 2003;67A(1):344-349.
 96. Koenig AL, Gambillara V, Grainger DW. Correlating fibronectin adsorption with endothelial cell adhesion and signaling on polymer substrates. *Journal of Biomedical Materials Research Part A* 2003;64A(1):20-37.
 97. Keselowsky BG, Collard DM, Garcia AJ. Surface chemistry modulates fibronectin conformation and directs integrin binding and specificity to control cell adhesion. *Journal of Biomedical Materials Research Part A* 2003;66A(2):247-259.
 98. Michael KE, Vernekar VN, Keselowsky BG, Meredith JC, Latour RA, Garcia AJ. Adsorption-induced conformational changes in fibronectin due to interactions with well-defined surface chemistries. *Langmuir* 2003;19(19):8033-8040.
 99. Magnusson MK, Mosher DF. Fibronectin - Structure, assembly, and cardiovascular implications. *Arteriosclerosis Thrombosis and Vascular Biology* 1998;18(9):1363-1370.
 100. Vogel V, Thomas WE, Craig DW, Krammer A, Baneyx G. Structural insights into the mechanical regulation of molecular recognition sites. *Trends in Biotechnology* 2001;19(10):416-+.
 101. Cella. *Cell biology*. Shaoxing College of Life Sciences College of arts and sciences; 2006.
 102. Johansson S, Svineng G, Wennerberg K, Armulik A, Lohikangas L. Fibronectin-integrin interactions. *Frontiers in Bioscience* 1997;2:126-146.
 103. Baneyx G, Baugh L, Vogel V. Coexisting conformations of fibronectin in cell culture imaged using fluorescence resonance energy transfer. *Proceedings of the National Academy of Sciences of the United States of America* 2001;98(25):14464-14468.

104. Aguirre KM, McCormick RJ, Schwarzbauer JE. Fibronectin Self-Association Is Mediated by Complementary Sites within the Amino-Terminal One-Third of the Molecule. *Journal of Biology Chemistry* 1994;269(45):27863-27868.
105. Boughton BJ, Simpson AW. The biochemical and functional heterogeneity of circulating human plasma fibronectin. *Biochemical and Biophysical Research Communications* 1984;119(3):1174-1180.
106. Bough L, Vogel V. Structural changes of fibronectin adsorbed to model surfaces probed by fluorescence resonance energy transfer. *Journal of Biomedical Materials Research Part A* 2004;69A(3):525-534.
107. Ugarova TP, Zamarron C, Veklich Y, Bowditch RD, Ginsberg MH, Weisel JW, Plow EF. Conformational Transitions in the Cell-Binding Domain of Fibronectin. *Biochemistry* 1995;34(13):4457-4466.
108. Johnson KJ, Sage H, Briscoe G, Erickson HP. The compact conformation of fibronectin is determined by intramolecular ionic interactions. *Journal of Biological Chemistry* 1999;274(22):15473-15479.
109. Rocco M, Aresu O, Zardi L. Conformational State of Circulating Human-Plasma Fibronectin. *Febs Letters* 1984;178(2):327-330.
110. Rocco M, Carson M, Hantgan R, McDonagh J, Hermans J. Dependence of the Shape of the Plasma Fibronectin Molecule on Solvent Composition - Ionic-Strength and Glycerol Content. *Journal of Biological Chemistry* 1983;258(23):4545-4549.
111. Rocco M, Infusini E, Daga MG, Gogioso L, Cuniberti C. Models of Fibronectin. *Embo Journal* 1987;6(8):2343-2349.
112. Krammer A, Craig D, Thomas WE, Schulten K, Vogel V. A structural model for force regulated integrin binding to fibronectin's RGD-synergy site. *Matrix Biology* 2002;21(2):139-147.
113. Peters T. All about albumin: biochemistry, genetics and medical applications. San Diego, CA: Academic Publishers; 1996.
114. Carter DC, He XM, Munson SH, Twigg PD, Gerner KM, Broom MB, Miller TY. Three-dimensional structure of human serum albumin. *Science* 1989;244:1195-1198.

-
115. Dockal M, Carter DC, Ruker F. The three recombinant domains of human serum albumin - Structural characterization and ligand binding properties. *Journal of Biological Chemistry* 1999;274(41):29303-29310.
 116. Malmsten M. Ellipsometry studies of the effects of surface hydrophobicity on protein adsorption. *Colloids and Surfaces B: Biointerfaces* 1995;3(5):297-308.
 117. Tang LP, Eaton JW. Inflammatory Responses to Biomaterials. *American Journal of Clinical Pathology* 1995;103(4):466-471.
 118. Tang LP, Eaton JW. Natural responses to unnatural materials: A molecular mechanism for foreign body reactions. *Molecular Medicine* 1999;5(6):351-358.
 119. Vörös J, Wieland M, Taylor-Ruiz L, Textor M, Brunette DM. Characterization of titanium surfaces. In: Brunette DM, Tengvall P, Textor M, Thomsen P, editors. *Titanium in Medicine: Material Science, Surface Science, Engineering, Biological Responses and Medical Applications*: Springer-Verlag; 2001. p 87-144.
 120. Yu XJ, Brash JL. Measurement of protein adsorption to solid surfaces in relation to blood compatibility using radiolabelling methods. In: Dawids S, editor. *Test procedures for the blood compatibility of biomaterials*. Dordrecht, The Netherlands: Kluwer Academic Publishers; 1993. p 287-330.
 121. Tamada Y, Ikada Y. Effect of Preadsorbed Proteins on Cell-Adhesion to Polymer Surfaces. *Journal of Colloid and Interface Science* 1993;155(2):334-339.

Human Serum Albumin adsorption on TiO₂ from single protein solutions and from plasma

S. R. Sousa^{1,2,3}, P. Moradas Ferreira⁴, B. Saramago⁵, L. Viseu Melo⁶ and M. A. Barbosa^{1,2}

¹ INEB, Instituto de Engenharia Biomédica, Laboratório de Biomateriais, R. do Campo Alegre, 823, 4150-180 Porto, Portugal

² Universidade do Porto, Faculdade de Engenharia, Departamento de Engenharia Metalúrgica e de Materiais, R. Dr. Roberto Frias, s/n, 4200 - 465 Porto, Portugal

³ ISEP, Instituto Superior de Engenharia do Porto, Departamento de Engenharia Química, R. Dr. Bernardino de Almeida, 431, 4200 - 072 Porto, Portugal

⁴ IBMC, Instituto de Biologia Molecular e Celular, Grupo de Microbiologia Celular Aplicada, R. do Campo Alegre, 823, 4150-180 Porto, Portugal

⁵ IST, Instituto Superior Técnico, Centro de Química Estrutural, Av. Rovisco Pais, 1049-001 Lisboa, Portugal

⁶ IST, Instituto Superior Técnico, Departamento de Física, Av. Rovisco Pais, 1200 Lisboa, Portugal

ABSTRACT

In the present work, the adsorption of human serum albumin (HSA) on commercially pure titanium with a titanium oxide layer formed in a H₂O₂ solution (TiO₂ cp) and on TiO₂ sputtered on Si (TiO₂ sp) was analysed. Adsorption isotherms, kinetic studies and work of adhesion determinations were carried out. HSA exchangeability was also evaluated. Surface

characterization was performed by atomic force microscopy (AFM), X-ray photoelectron spectroscopy (XPS), and by wettability studies.

The two TiO₂ surfaces have very distinct roughnesses, the TiO₂ sp having a mean Ra value 14 times smaller than the one of TiO₂ cp.

XPS analysis revealed consistent peaks representative of TiO₂ on sputtered samples as well as on Ti cp substrate after 48 h of H₂O₂ immersion. Nitrogen was observed as soon as protein was present, while sulphur, present in disulphide bonds in HSA, was observed for concentrations of protein higher than 0.30 mg/ml.

The work of adhesion was determined from contact angle measurements. As expected from the surface free energy values, the work of adhesion of HSA solution is higher for the TiO₂ cp substrate, the more hydrophilic one, and lower for the TiO₂ sp substrate, the more hydrophobic one. The work of adhesion between plasma and the substrates assumed even higher values for the TiO₂ cp surface, indicating a greater interaction between the surface and the complex protein solutions.

Adsorption studies by radiolabelling of albumin, ¹²⁵I-HSA, suggest that a rapid HSA adsorption takes place on both surfaces, reaching a maximum value after ca. 60 min. of incubation. For the higher HSA concentrations in solution a multilayer coverage was observed on both substrates.

After the adsorption step from single HSA solutions, the exchangeability of adsorbed HSA molecules by HSA in solution was evaluated. The HSA molecules adsorbed on TiO₂ sp seem to be more easily exchanged by HSA itself than those adsorbed on TiO₂ cp after 24 h. In contrast, after 72 h, nearly all the adsorbed albumin effectively exchange with other albumin molecules.

INTRODUCTION

At present several promising multifunctional biomaterials are available that are being applied to new types of artificial organs and advanced medical devices.¹ Nevertheless, metals are still the more suitable biomaterials for replacing hard tissue due to good bone matching mechanical properties.²

The physiological inertness of titanium associated with its exceptional mechanical properties and favourable osteointegration makes it available as a replacement for bones and cartilage in a variety of surgeries, namely orthopaedic, dental, spinal, as well as in reconstructive and plastic surgery.³ However, the insertion of a foreign material into the human body activates defence mechanisms and triggers the coagulation system when the material is brought into contact with blood. White cells may be recruited to the wound site, with subsequent release of reactive oxygen species such as H₂O₂ and O₂⁻. It has been reported by Tengvall et al. that Ti forms stable Ti-peroxy complexes in the presence of large amounts of H₂O₂.⁴ The differential response of tissue to implanted materials may arise mainly from differences in the adsorption of proteins.⁵ The organisation of the adsorbed proteins depends on the type of the surface, since proteins display different relative affinities for different surfaces. Consequently, since each cell type has a different set of specific receptors for proteins, the efficiency of cell attachment, proliferation, and viability is dependent on surface parameters, namely, oxide composition, surface roughness, surface charge, and contaminants, among other characteristics.

This work aimed at assessing albumin adsorption on two different surfaces, TiO₂ sputtered on Si (TiO₂ sp) and TiO₂ formed on commercially pure titanium after H₂O₂ immersion (TiO₂ cp). The objective was to have titanium oxide chemically similar to those used in clinical practice, stable and with different roughnesses. The protein solutions were human serum albumin and plasma. Surfaces were characterized using atomic force microscopy (AFM), imaging and roughness analysis, X-ray photoelectron spectroscopy (XPS), and wettability studies leading to the surface free energy. The adsorption kinetics and isotherms were evaluated by two different techniques radiolabelling of albumin (¹²⁵I-HSA) and XPS. With the latter technique it was possible to follow albumin adsorption in TiO₂ through the increase of nitrogen and carbon groups and the decrease of titanium and oxygen intensity. The work of adhesion between protein solutions and TiO₂ substrates was evaluated through wettability studies. HSA exchangeability was also estimated by ¹²⁵I-HSA.

MATERIALS AND METHODS

TiO₂ substrates

Commercially pure titanium sheets, 1 mm thick, were used. The nominal composition (wt %, max.) was the following: 0.20 Fe, 0.05 N, 0.08 C and 0.015 H.

The surface was roughened by chemical polishing (cp) with an etching acid solution (2:3 parts by volume, 40 % of HF + 60 % of HNO₃ (v/v)), for 30 s. The substrates were pre-incubated in H₂O₂ 10 mM for 48 h to obtain a regular titanium oxide film.

TiO₂ sputtered substrates were prepared using an automated, load locked ion beam deposition system (Nordiko N3000), which is described elsewhere.⁶

The TiO₂ films (50 nm) were deposited on Si wafers (polished/etched, crystal orientation <100>, from AUREL GmbH) by ion beam sputtering from a Ti cp target, in the presence of a mixed Ar-O₂ beam coming from the assist gun (15.02% O₂ in Ar). The deposition rate was 0.038 nm/s.

An acceleration voltage of +1450 V (33 mA Xe beam, 1 sccm Xe, 110W RF plasma) and extraction voltage of -300 V was used at the deposition gun. The Ar-O₂ beam was created by accelerating the plasma inside the assist gun with +200V (15 mA beam, 10 sccm Ar-O₂, 50W RF plasma) and using an extraction voltage of -100V. The pressure during the assisted deposition was 1.3x10⁻⁴ Torr. A filamentless neutraliser (40 mA, 3 sccm Ar-O₂) next to ion source avoided surface charging during the dielectric deposition.

The wafers were diced into pieces (5X5 μm²) using a DISCO DAD321 automated saw. Before dicing, all wafers were coated with 1.5 μm of photoresist (reference PFR7790EG, from JSR Electronics), to protect the film surface.

Just before being used, TiO₂ sputtered films were cleaned twice in acetone and immersed in a "piranha" solution (7:3 parts by volume, 98 % H₂SO₄ + 30 % H₂O₂ (v/v)) for 90 s (caution: *this solution reacts violently with many organic materials and should be handled with great care*). Substrates were washed in ethanol, water (distilled and deionized) and again in ethanol for 2 min. in an ultrasonic bath.

Protein Solutions

Human serum albumin (Sigma, ref. A1653) solutions were prepared in phosphate buffered saline, PBS (Sigma, pH=7.4, ref. P-3813), at concentrations of 0.2, 0.4, 1.0, 4.0 and 10.0 mg/mL.

Plasma was obtained from healthy blood donors from Portuguese Blood Institute (IPS). The concentrations used were 100% and 10 % (v/v). The water used was distilled and deionised with a resistivity > 16 MΩcm.

Atomic Force Microscopy (AFM)

Surfaces morphology and roughness were determined using a commercial atomic force microscope (Digital Instruments Nanoscope). The instrument was operated in tapping mode and the sizes of images were 1x1 μm². Three samples of each type were analysed at a randomly chosen location. The surface roughness was obtained from scanned areas of 1000x1000 nm². To characterize the roughness, the average roughness (R_a), was obtained as well as the standard deviation of the amplitude (R_q) and the maximum peak (R_{max}).

X-ray Photoelectron Spectroscopy (XPS)

XPS measurements were carried out on a VG Scientific ESCALAB 200A (U.K.) spectrometer using MgKα (1253.6 eV) as a radiation source. The photoelectrons were analysed at two takeoff angles: 0 and 60°.

Survey XPS spectra were acquired from 0 to 1100 eV before and after hydrogen peroxide treatment, as well as after protein adsorption. High-resolution spectra for Ti2p, O1s, C1s, N1s and S1s were also obtained. Elemental atomic percentages of Ti, O, C, N and S were determined after deconvolution of high-resolution spectra, using an XPS peak-fitting program (XPS PEAK, Version 4.1). To take into account shifts caused by charging of the sample surface,

all spectra were adjusted taking the C1s peak at 285.0 eV as reference for the adventitious carbon contamination.

The elemental atomic percentages of Ti, O, C, N and S were determined for the adsorption times of 5, 10, 30, 60 and 360 min at HSA concentrations of 0.20 and 4.0 mg/mL (kinetics) and, after 60 min of HSA adsorption, as a function of HSA concentration in the range of 0.20 – 4.0 mg/mL (adsorption isotherms).

Wettability Studies

Contact angle measurements were performed with a contact angle measuring system from Data Physics, model OCA 15, equipped with a video charge-coupled device (CCD) camera and SCA 20 software. The equipment incorporates an electronic syringe unit with a gas tight 500 μ l dosing syringe (Hamilton).

Contact angles were obtained by the sessile drop method, at 25 °C in a chamber saturated with the liquid sample. The drop was recorded as soon as stabilisation was achieved, and images were recorded every 2 s up to 600 s. Digital images of the drop were acquired by the CCD-camera and used for the calculation of the contact angle. For the contact angle determination an ellipse function was fitted to the measured contour line, obtaining the best accuracy. The contact angle value was extrapolated to $t=0$ s for water and to $t=600$ s for protein solutions, using a curve fitting program. In each experiment at least 10 drops of ~ 4 μ L were used.

Surface tension of the protein solution in equilibrium with its own vapour (γ_{LV}) was obtained through the analysis of the shape of a pendant drop kept at 25 °C in a chamber saturated with the liquid sample. As drop stabilization was achieved, images were taken every 2 s during 600 s. In each experiment, at least 10 drops of ~ 20 μ L were analysed. The liquid densities at 25 °C were determined using an Anton Paar Density meter.

Surface free energy of all types of titanium was assessed by the Owens-Wendt-Rabel and Kaelble,⁷ (OWRK), method using as testing liquids distilled and deionised water and diiodomethane (>99%, from Sigma-Aldrich). The polar and dispersive components of the surface tension of the liquids at 25 °C were estimated from the correspondent values at 20 °C,

taken from the literature ⁸ [γ_{LV} of water and diiodomethane: 71.52 ± 0.13 mN/m ($\gamma_d = 21.42$ mN/m; $\gamma_p = 50.11$ mN/m) and 50.46 ± 0.07 mN/m ($\gamma_d = 50.46$ mN/m; $\gamma_p = 0$ mN/m)]. The estimation was based on the assumption that the ratio between the dispersive and the polar components of the liquid surface tension did not vary with the temperature.

This determination was based on Young's equation which relates the surface tension of a liquid in equilibrium with its vapour, γ_{LV} , with the contact angle (θ) of the same liquid drop on the surface of an ideally smooth flat solid. The difference $\gamma_{SV} - \gamma_{SL}$ can be obtained from Young's equation and experimentally measured values of γ_{LV} and θ .

The work of adhesion (the negative part of the Gibbs energy of adhesion) for a liquid in contact with a solid (W) may be obtained by the Young-Dupré equation:

$$W = \gamma_{LV} + \gamma_{SV} - \gamma_{SL} = \gamma_{LV} (1 + \cos \theta) \quad (1)$$

The interfacial free energy between the solid and the liquid can be calculated, according to Owens and Wendt ⁷, considering that the total surface free energy is expressed as a sum of two components arising from dispersive and polar contributions, γ^d and γ^p , respectively. Assuming further the geometric mean approach, for the polar and dispersive components of the work of adhesion, the solid-liquid interfacial free energy may be written as:

$$\gamma_{SL} = \gamma_{SV} + \gamma_{LV} - 2\sqrt{\gamma_{SV}^d \gamma_{LV}^d} - 2\sqrt{\gamma_{SV}^p \gamma_{LV}^p} \quad (2)$$

γ_{SV} as well as the dispersive (γ_{SV}^d) and the polar (γ_{SV}^p) components can be calculated from Young's equation and equation 2 using experimental values of contact angles measured with a pair of testing liquids with known polar and dispersive components.

Protein quantification by radioactivity

Quantification of albumin adsorbed on the TiO₂ surface was performed using ¹²⁵I labelled albumin.

Human plasma albumin (Sigma) was labelled with ¹²⁵I using the Iodogen method.^{9,10} ¹²⁵I-HSA was passed through a Sephadex G-25 M column (PD-10 column from Amersham Pharmacia Biotech) to remove unbound ¹²⁵I. The yield of iodination was always higher than 98 %, as determined by precipitating the ¹²⁵I-labelled albumin with 20% trichloroacetic acid (the TCA method).

Several authors reported¹¹⁻¹³ that some measured amounts of adsorbed protein were erroneously high due to interactions of residual free ¹²⁵I ion, present in the protein solution, with metal surfaces. The contribution of free ¹²⁵I to the total radioactivity found on the metal surfaces was estimated using unlabelled albumin solutions at a concentration of 1.0 mg/mL, and an amount of free ¹²⁵I ion equivalent to that present as free ¹²⁵I ion in the labelled protein solutions.

In all studies in which radiolabelling methods are used, it must be kept in mind that the behaviour of the labelled protein may or may not reflect the behaviour of unlabelled protein.¹⁴ To assess if preferential adsorption of ¹²⁵I labelled albumin occurred on TiO₂ surfaces, a series of control experiments were performed varying the ratio of labelled to unlabelled albumin (10 - 50 %). Final concentration of the albumin solutions (1.0 mg/mL) was confirmed by the bicinchoninic acid (BCA) assay using BCA assay kit from Pierce and BSA as standard.¹⁵

Protein solutions for adsorption experiments were prepared adding ¹²⁵I-HSA to unlabelled albumin solution to obtain a final activity of ~ 10⁸ cpm/mg.

HSA adsorption tests were performed placing the Ti substrates in a 24-well tissue plate (Sarsted). A drop of 10 µl ¹²⁵I-HSA solution was added onto each surface. To avoid evaporation, PBS was added to the wells in the periphery. Adsorption tests were carried out at 25 °C over a period of 60 min. After this period, the drop was absorbed from the surface with absorbent paper and the surface was washed three times with PBS. The samples were transferred to radioimmunoassay tubes and surface activity was measured using a gamma counter. All experiments were done in triplicate. The counts from each sample were averaged, and the surface concentration was calculated by the following equation:

$$\text{HSA (mg/m}^2\text{)} = \frac{\text{counts(cpm)} * |\text{HSA}|_{\text{solution}} \text{ (mg / ml)}}{A_{\text{solution}} \text{ (cpm / ml)} * SA \text{ (m}^2\text{)}} \quad (3)$$

where the counts measure the radioactivity of the samples, $|\text{HSA}|_{\text{solution}}$ is the albumin concentration solution, A_{solution} is the specific activity of the albumin solution, and SA is the surface area.

To understand the albumin adsorption on titanium oxide substrates, kinetics of adsorption of HSA for concentrations of 0.20 and 4.0 mg/mL (during 5, 30 and 60 min) and adsorption isotherms were investigated in a concentration range of 0.20 – 10.0 mg/mL.

Displacement tests were carried out by immersing the labelled Ti surface over 24 and 72h in an unlabelled HSA pure solution (exchange). The substrates were washed three times with PBS, and the residual activity was counted.

Statistical analysis of the experimental results was performed using the Kurskall-Wallis, non-parametric tests.

RESULTS

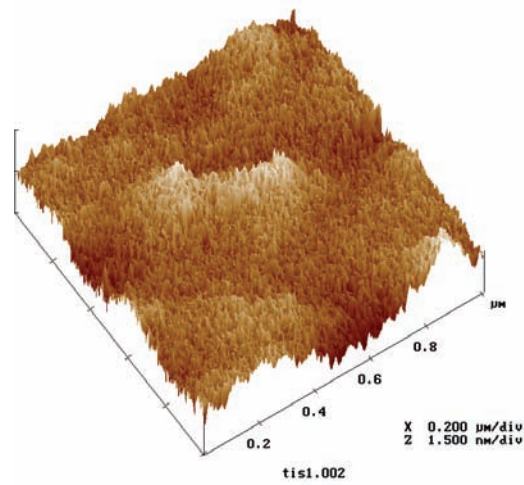
SURFACE CHARACTERIZATION

AFM

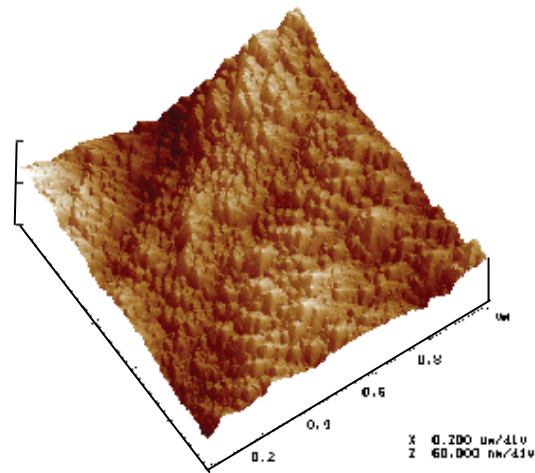
To assess the binding of proteins namely albumin to titanium, surfaces were first characterized for roughness and morphology using AFM.

The surface roughnesses of the various Ti surfaces are presented in Table 1. When treated with hydrogen peroxide a slight increase in surface roughness at the nanometric scale (Ra, Rq and Rmax values) was observed, leading to a more uniform surface. This effect is probably related to the strong oxidizing effect of H₂O₂. Figure 1 shows the 3D AFM images of all Ti surfaces. The increased roughness introduced by hydrogen peroxide treatment is evident in Figure 1b) and c)

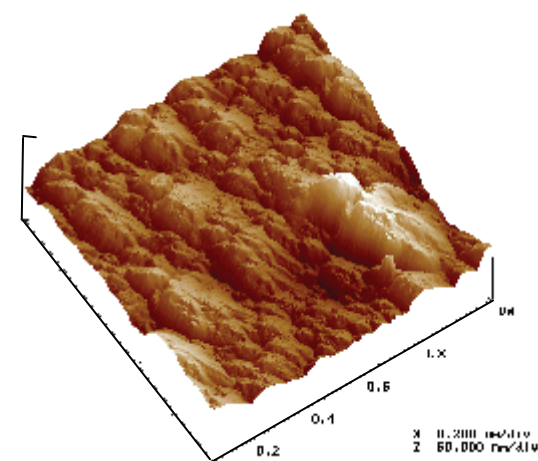
for the cp substrate. The two TiO_2 surfaces have distinct roughnesses, having the TiO_2 sputtered a mean Ra value 14 times smaller than the one for TiO_2 cp (Table 1).



a)



b)



c)

Figure 1 – 3 D AFM images of a) TiO_2 sputtered surface, b) Ti cp surface (before H_2O_2 treatment) and c) TiO_2 cp surface (after H_2O_2 treatment).

Table 1 – R_a , R_q , R_{max} values for all the Ti substrates studied.^a

Substrate	R_a , nm	R_q , nm	R_{max} , nm
TiO ₂ sp	0.23 (0.22,0.29)	0.29 (0.29,0.36)	2.75 (2.7, 3.7)
Ti cp (before H ₂ O ₂ treatment)	4.75 (4.4, 6.3)	6.50 (5.7, 8.1)	55.1 (53, 59)
TiO ₂ cp (after H ₂ O ₂ treatment)	5.15 (5.0, 6.6)	6.53 (6.3, 8.7)	52.9 (52, 67)

^aThe first and the third quartile are shown in parenthesis.

XPS

Titanium surfaces were characterised by XPS to explore the detailed surface chemistry after different surface modifications.

To check the presence of additional elements than those expected (Ti, O and contaminant C), wide scans were performed in all Ti samples before protein adsorption. N and Ca were detected as traces, arising from the manufacture of titanium. For the TiO₂ cp substrates N results from the HNO₃ initial treatment.

The spectra of all the elements detected are identical for TiO₂ sp and TiO₂ cp surfaces.

Analysis of fitted XPS high resolution spectra allows characterization of the chemical environment of the different elements.

To obtain a regular and uniform layer of TiO₂ several surface treatments of Ti cp were tried: exposure to air (48 h), immersion in H₂O₂ 10 mM and in H₂O₂ 100 mM for two different periods, 24 and 48 h. Two angles of escape, 0 and 60° (XPS) were used.

The high resolution O1s spectra obtained from TiO₂ cp with several surface treatments showed a shoulder on the higher binding energy side, justifying the separation of O1s spectrum into two components. The binding energy (BE) assigned to titanium oxide (O_M) (530.4 eV)^{16,17} appears in the low-energy range followed by other components at higher BE, namely hydroxide/hydroxyl groups plus chemisorbed water (O_H) (532.2 eV).^{17,18} Armstrong et al.^{19,20} found that binding energy differences between two major transitions may indicate the chemical composition of a metal oxide. The BE difference between the O1s and the Ti2p3 peaks is $\Delta BE = O(1s) - Ti$

(2p/3)= 71.5 eV, which, according to the data obtained by the same authors, corresponds to TiO_2 .

The Ti2p peak obtained at 60° was a doublet peak, Ti2p_{3/2} at 458.9 eV and Ti2p_{1/2} at 464.4 eV for Ti cp surfaces exposed to air, revealing a Ti^{4+} oxide state^{21,22}, whereas that obtained at 0° additionally showed metallic-state titanium (453.7 eV)²¹⁻²³. This suggests that the surface film is not thick enough for photoelectrons from the bulk metal to reach the surface and that the Ti oxide film surface consists essentially on TiO_2 , without sub-oxides, without TiO and Ti_2O_3 .

After 24 h of 10 mM H_2O_2 treatment, Ti (0) is still observed at takeoff angle of 0° and at 60° , TiO_2 constitutes the oxide with a layer of hydration groups as it was observed for the O1s spectra at 532.2 eV.

Both hydrogen peroxide treatments (48 h 10 mM and 100 mM) lead to the formation of a thicker TiO_2 layer harbouring hydration groups, as indicated from both 0° and 60° measurements.

The Ti/ O_M ratio, 0.55 and 0.52 at takeoff angle of 0° and 60° , respectively, for the H_2O_2 10 mM treatment, was closer to the theoretical value for TiO_2 . Therefore this treatment was selected to be applied in all the Ti cp specimens.

The C1s spectra can be resolved in two different peaks: one at 285.0 eV representing carbons in a hydrocarbon-type environment ($-\text{CH}_3$, $-\text{CH}_2-$) and other at 288.9 eV representing carbons in a $-\text{C-F}-$ bond or $-\text{C=O}$, originated by the chemical etching.²⁴

Nitrogen is always present as a trace element with one peak centred at 400.9 eV characteristic of $-\text{NH}-$ bonds.²⁵

The Ca spectra present a doublet peak with Ca2p_{3/2} at 347.2 eV and Ca2p_{1/2} at 350.7 eV. The BE suggest that a CaO or CaCO_3 ^{26,27} compound may be present at the surface. Ca is a trace element of Ti.

Wettability Studies

To obtain further data on the surface characteristics, the surface free energy was evaluated.

The values of the contact angles of water and diiodomethane for the TiO_2 substrates (Table 2) were obtained by extrapolation of time-dependent experimental results to $t=0$ s.

The surface free energy components (polar and dispersive) were calculated through the geometric mean approach⁷ (based on Young's equation and eq 2). Table 2 presents the values of the surface free energy of Ti substrates (γ_{SV}), and the dispersive, (γ_{SV}^d) and polar (γ_{SV}^p) component as well as the polarity. The polarity is defined as the ratio between polar component divided by the total surface free energy ($\gamma_{SV}^p / \gamma_{SV}$).

Table 2 – Contact angles and their standard deviations of water and diiodomethane (n=10) on TiO₂ surfaces, at t=0 s.^a

	TiO ₂ sputtered	Ti cp (before H ₂ O ₂ treatment)	TiO ₂ cp (after H ₂ O ₂ treatment)
θ_{water} , degrees	84 ± 2	74 ± 3	71 ± 3
$\theta_{\text{diiodomethane}}$, degrees	52 ± 2	53 ± 2	49 ± 3
γ_{SV} , mN/m	36.3	39.5	42.7
γ_{SV}^p , mN/m	3.53	7.30	8.17
γ_{SV}^d , mN/m	32.8	32.1	34.5
$\gamma_{SV}^p / \gamma_{SV}$	0.10	0.18	0.19

^a The surface free energy of TiO₂ substrates, its dispersive and polar components and the polarity, are also shown

All substrates are predominantly dispersive although the Ti substrates after H₂O₂ treatment have a higher contribution of the polar component due to the presence of ionic species. The surface free energy increases after H₂O₂ treatment as there are more reactive sites exposed from the oxide. Considering that polarity is a quantitative indicator of hydrophilicity, we can say that the TiO₂ sputtered substrate is the more hydrophobic substrate.

ALBUMIN ADSORPTION

XPS

Wide scans were performed on TiO₂ cp and TiO₂ sp surfaces, and the composition and morphology of the spectra are identical for both surfaces. For illustration only, the data on the TiO₂ cp surfaces are shown (Figure 2 – 4).

Ti, O, C, N, P and Ca were always present. S was present for HSA concentrations > 0.30 mg/mL. Ca and P have their origins in the electrolyte and C, N, O and S in the protein.

The surface composition of Ti samples has been extensively reported,^{20,22,28-30} but only a few have deconvoluted and assigned each peak (C1s or N1s) to the protein bonds.³¹⁻³³ In this work, we also followed the atomic % of each element during kinetics of adsorption as well as adsorption isotherms.

High resolution spectra were acquired for Ti2p, O1s, C1s, N1s, S2p, Ca2p and P2p providing an indication of the chemical composition of the titanium oxide and protein film formed at the surfaces.

The C1s spectrum of albumin is significantly different from that of TiO₂ surface. A C1s peak broadening was observed after protein adsorption. It was considered that the C1s peak had a contribution of four different peaks corresponding to carbon atoms in different environments, that is, saturated hydrocarbon groups (-CH₃, -CH₂-) with a BE of 285.0 eV, to amine groups (-C-NH-) with BE of 286.6 eV, to the peptide bond (-C=O-NH-) with BE of 288.2 eV and to acidic groups (-C-COO-) with BE of 289.6 eV³². Figure 2a shows an envelope for C1s deconvolution peak after HSA adsorption ([HSA]=0.20 mg/mL), being the -CH₂- groups the major contribution to the C1s intensity.

The kinetics of albumin adsorption on titanium samples could be followed by the C1s peak intensity. Figure 3a shows that as the time of albumin incubation increases the peak intensities also increase.

Figure 4a presents the variation of % C, assigned to each component, as a function of albumin concentration in solution. A plateau is attained between 0.30 and 1.0 mg/mL of HSA in solution.

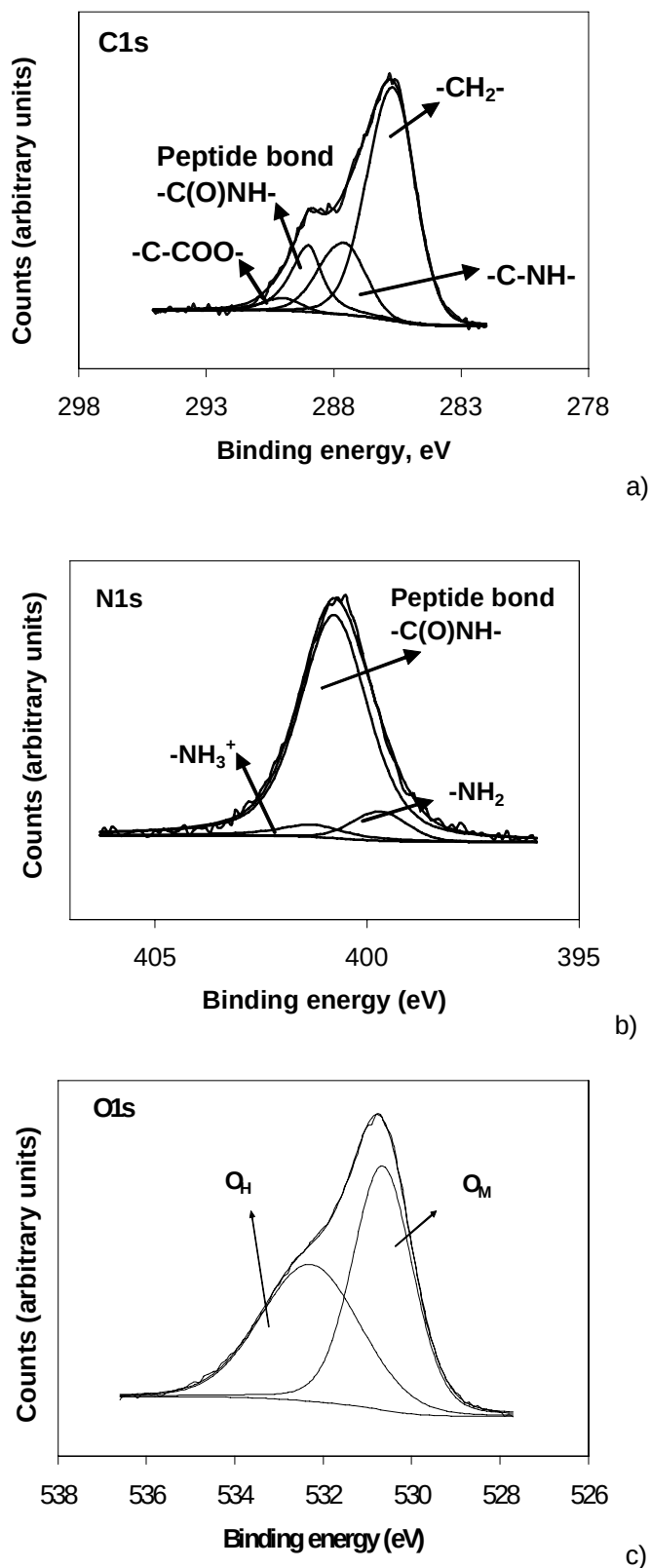


Figure 2 - Representative deconvolution of XPS envelope for a) C1s, b) N1s and c) O1s peaks (after 60 min of HSA 0.20 mg/mL adsorption at TiO_2 cp surface) at 0°.

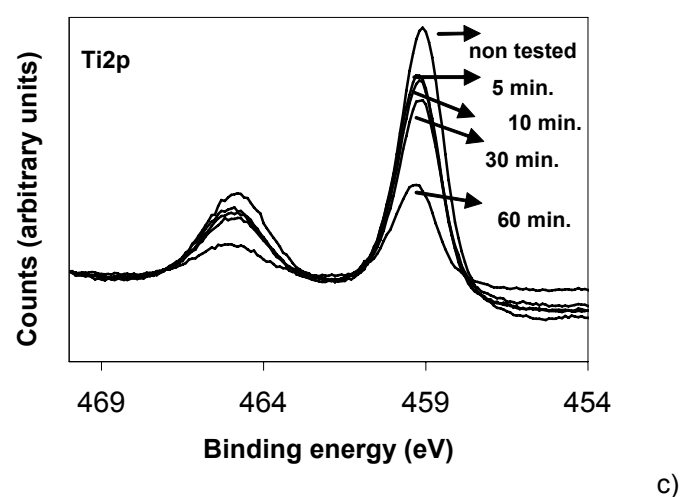
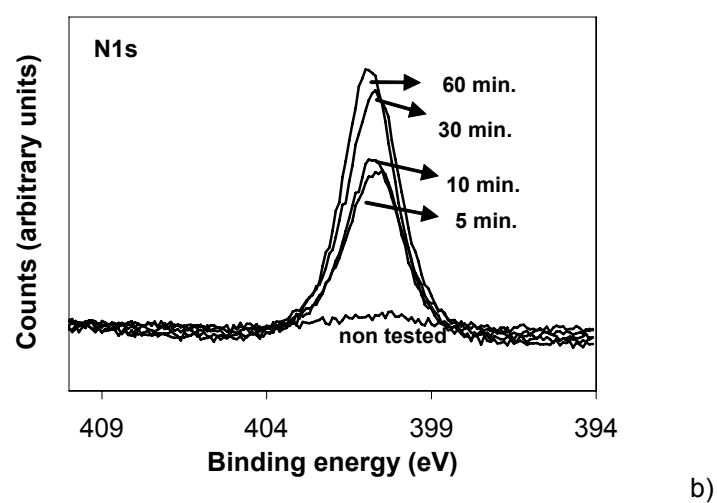
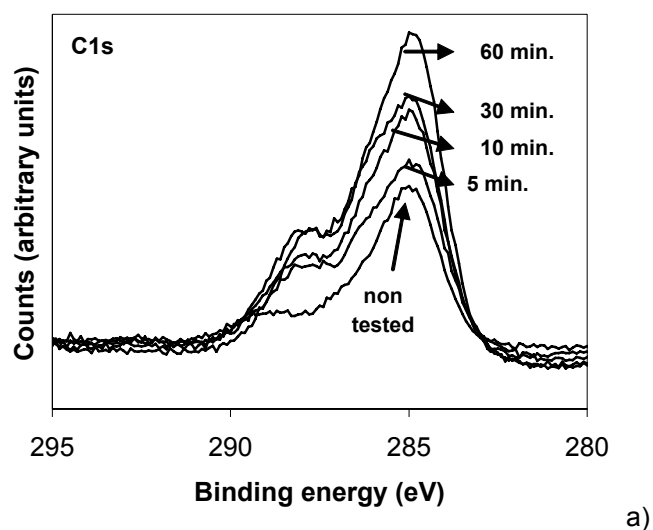


Figure 3 – Evolution of XPS element envelope with increased time of albumin immersion ($[HSA]=0.20$ mg/mL).

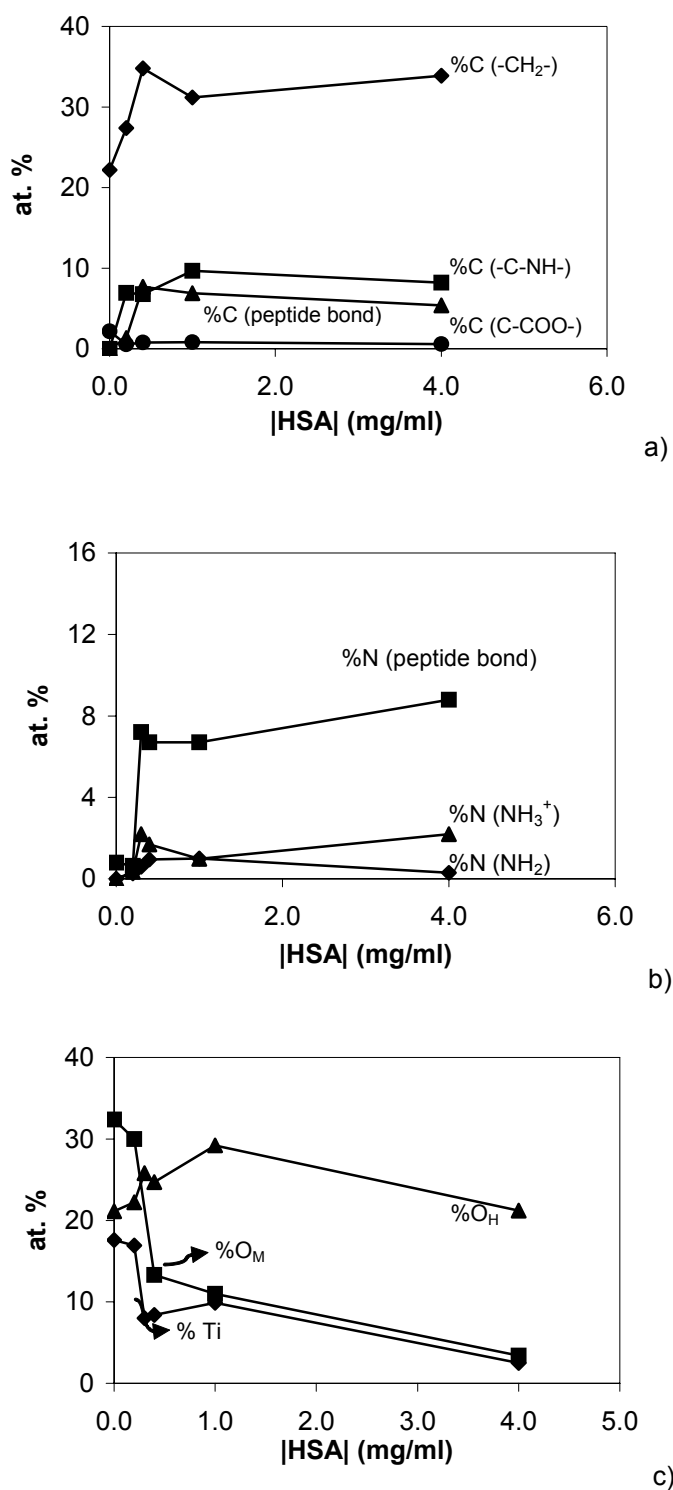


Figure 4 – Changes in atomic % with [HSA] in solution after 60 min of adsorption at TiO_2 cp surface; a) C%, b) N% and c) Ti% and O%.

The N1s spectra, after albumin adsorption, was considered to have a contribution of three different peaks corresponding to -NH_3^+ with BE of 401.8 eV, to the peptide bond (-C=O-NH-) with BE of 400.1 eV and to the NH_2 groups with BE between 389.9-399.9 eV^{32,33} (Figure 2b). The neutral peptide nitrogen peak dominates the N1s spectrum, contributing more than 90% to the total amount. Figure 3b shows that the intensity of N1s peak clearly increases due to albumin adsorption on TiO_2 cp after 5 min. of protein incubation. As the time of incubation increases the quantity of albumin adsorbed to the TiO_2 cp surface also increases attaining a maximum value for 60 min. of adsorption. The N % (peptide bond) reaches a semi-plateau for lower HSA concentrations (0.30 mg/mL), with the peptide bond being the main contribution for the total N (Figure 4b).

The shape of the O1s peak after protein adsorption is similar to the one without protein, suggesting that the contributions from the carboxyl groups and the phosphate from electrolyte are superimposed on the O_H peak. Upon adsorption, the O_M % decreases and the O_H % increases, indicating that a film of protein is covering the titanium oxide (Figure 4c).

As expected, the increasing exposure to albumin resulted in a protein layer covering the TiO_2 as indicated by the decrease of Ti signal (Figure 3c) as well as by the increase of N1s and C1s intensity.

The atomic percentages of Ti, O_M and O_H vs [HSA] are shown in Figure 4c. As the concentration of albumin solution increases the Ti % and the O_M % decreases and the O_H % increases, attaining a semi-plateau between 1.0 and 4.0 mg/mL, due to the existence of a protein layer.

Only one peak is observed at the high resolution S2p spectra, resulting from the sulphur atoms coming from the disulfide groups (-S-S-) in protein molecules at 164.1 eV.³²

P is present as a trace element arising from electrolyte solution.

Wettability Studies

To have further data on protein adsorption, wettability studies were performed and the work of adhesion between the protein solutions and the surfaces was calculated from the values of the contact angles and the surface tensions of the protein solutions, through Eq. 2.

A decrease in the contact angle vs time is observed for the different albumin solutions at the TiO₂ surfaces (Figure 5, for the TiO₂ cp). The variation is more pronounced for the plasma solutions. These solutions are complex mixtures of several proteins and ions, and therefore, the migration of these species to the drop boundary are delayed relative to the single protein solution.

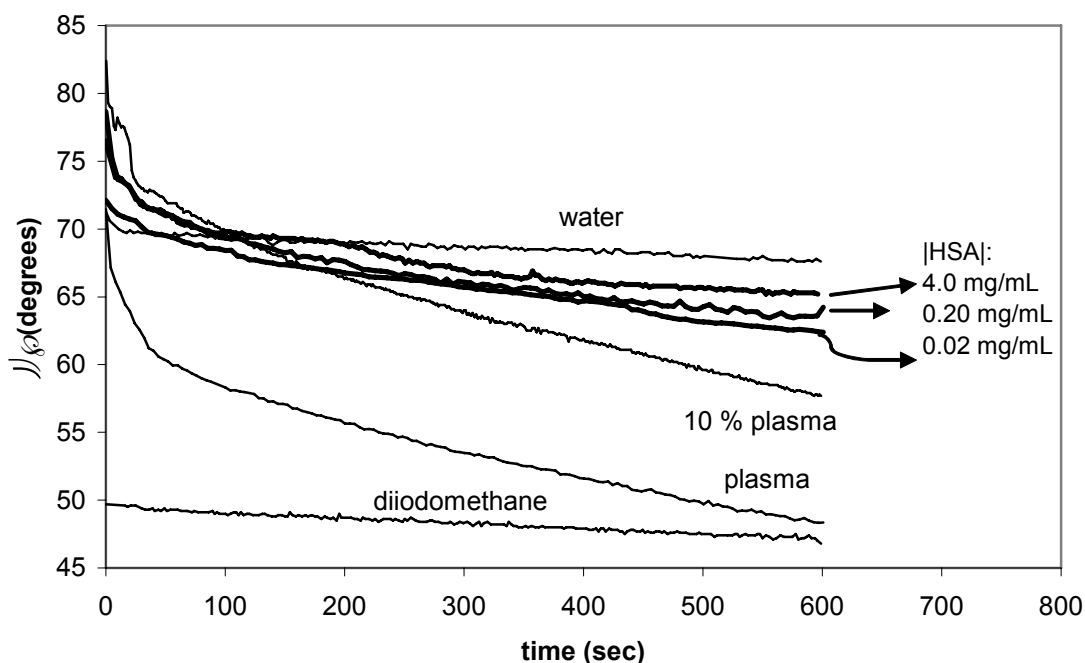


Figure 5 – Time dependence contact angle of several solutions at TiO₂ cp surface.

The values of the contact angles of protein solutions for the TiO₂ substrates, given in Table 3 were obtained by extrapolation of time-dependent experimental results to $t=600$ s.

The contact angle of HSA with TiO₂ cp are not dependent of albumin concentration (Table 3), since they are not statistically different. Comparing the two surfaces, the contact angle of a HSA solution with 4 mg/ml follows an identical trend as the contact angle between the surfaces and water. When a plasma solution was assayed a higher affinity of the solution to the TiO₂ cp surface is also observed.

The surface tension of the different solutions was measured. The surface tension of PBS stays constant during the time of the experiment. The protein solutions have lower surface tensions, which decrease rapidly within the first 100-200 s, reaching then a plateau or a semi-plateau

(data not shown). Several authors attribute this behaviour to adsorption of protein molecules to the air-solution interface.^{34,35} This is clearer for the plasma solutions, as a consequence of the higher concentration of proteins. The surface tensions of different protein solutions ($t=600$ s) are shown in Table 3.

Table 3 - Contact angles and their standard deviation of PBS and different protein solutions on TiO_2 surfaces, at $t=600$ s.^a

Solution	γ_{LV} (mN/m)	θ , degrees	
		TiO_2 sputtered	TiO_2 cp (after H_2O_2 treatment)
PBS	72.9 ± 0.20	77 ± 3	76 ± 4
HSA 0.02 mg/mL	58.0 ± 0.48	64 ± 2	62 ± 3
HSA 0.20 mg/mL	56.0 ± 0.48	65 ± 3	63 ± 4
HSA 4.0 mg/mL	53.4 ± 0.70	70 ± 2	65 ± 4
plasma 10 %	50.1 ± 0.49	52 ± 2	57 ± 3
plasma	47.0 ± 0.33	42 ± 4	49 ± 3

^a The surface tension, γ_{LV} , of the liquids is also shown.

Measurements of the time-dependent contact angles and liquid surface tensions combined with the knowledge of the solid surface free energy, assumed to be time independent, may be used to estimate dynamic solid-liquid interfacial tensions according to Young's equation. However, since the application of Young's equation to a nonequilibrium process is not straightforward, care should be taken in the interpretation of the results.^{36,37} The time dependence of the work of adhesion between each protein and TiO_2 was then evaluated through the Young-Dupré equation, from the correspondent values of γ_{LV} and θ . The advantage of measuring the work of adhesion relies on the fact that this parameter can be measured at any instant of time, whether the system is in equilibrium or not, and correctly interpreted as the force needed to separate the liquid from the substrate.³⁸ To understand the influence of the presence of a protein on the behaviour of the solid/liquid interface, we compared the work of adhesion of the water with the work of adhesion of albumin and of plasma solution (Figure 6).

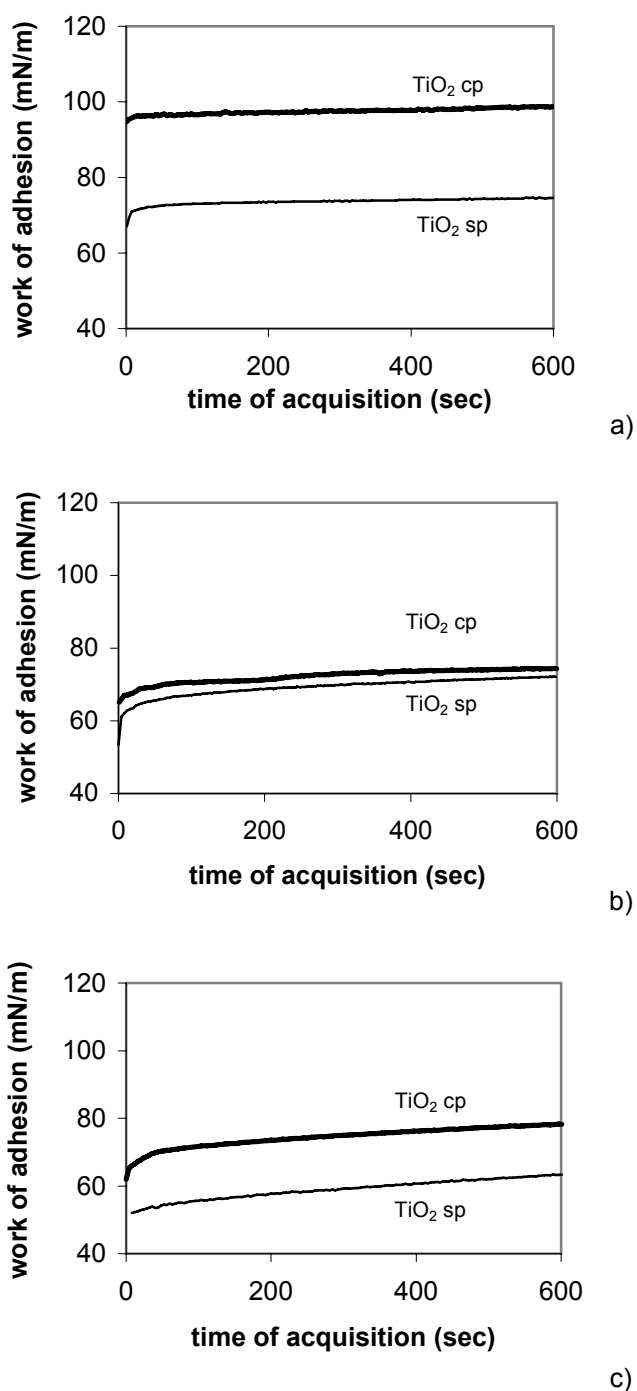


Figure 6 – Work of adhesion between TiO₂ substrates and a) water, b) |HSA|=4.0 mg/mL and c) plasma.

As expected from the surface free energy values, the work of adhesion of water is higher on the TiO₂ cp substrate than on the TiO₂ sp substrate (Figure 6a). Analysis of the three figures shows that the work of adhesion of the protein solutions follows the same trend. The cp surface is the

one presenting the highest value of work of adhesion for all solutions (Figure 6), implying a high solid – liquid interaction.³⁸

Adsorbed macromolecules may use several adsorption sites depending on their structure and molecular mass. At low concentrations, adsorbed proteins are in an unfolded state. At higher protein concentration the adsorption layer becomes more compressed and molecules with different degrees of unfolding will coexist at the interface.³⁵ These phenomena can perhaps explain the highest work of adhesion for the lowest HSA concentration (Figure 7).

Although the work of adhesion cannot be seen as a direct measure of protein adsorption, it quantifies the decrease in surface free energy due protein adsorption.³⁷ Our results indicate a linear relation between the work of adhesion and the logarithm of the albumin concentration (Figure 8). The work of adhesion between plasma and TiO₂ cp is opposite to the one found for the albumin solutions (Figure 7), that is, higher for the undiluted solution.

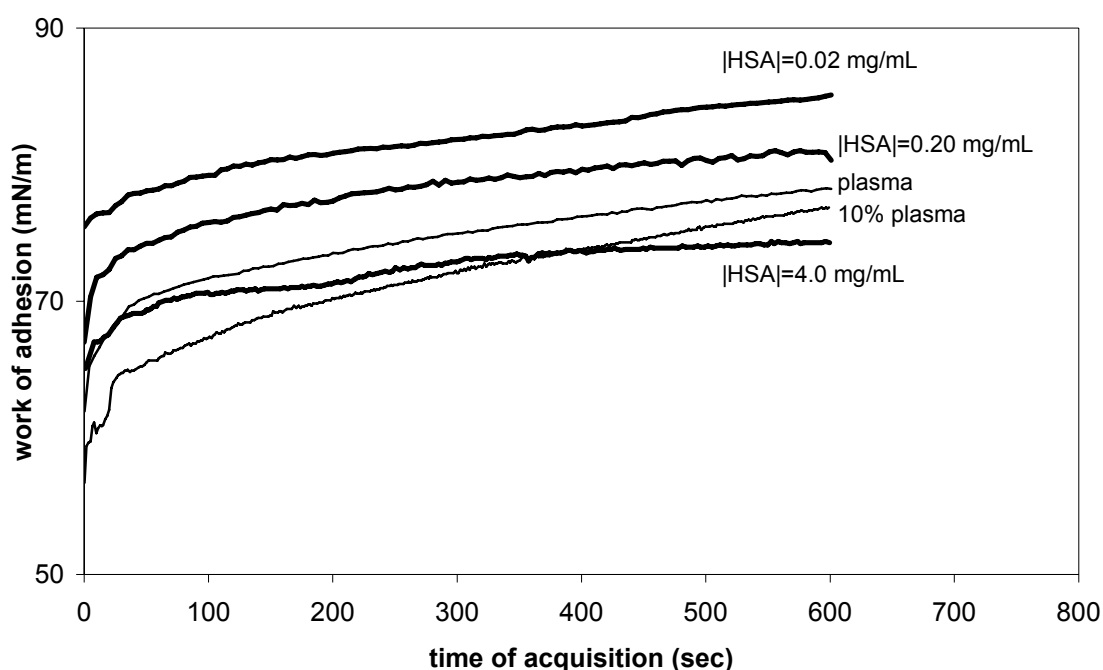


Figure 7 – Work of adhesion between TiO₂ cp and HSA at different concentrations and between TiO₂ cp and plasma solutions.

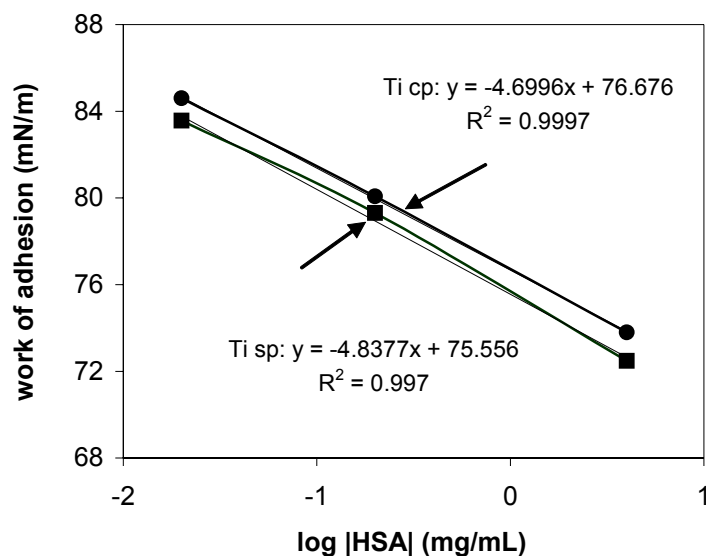


Figure 8 – Work of adhesion between HSA molecules and the TiO₂ substrates, as a function of HSA concentration in solution (t=600 s).

¹²⁵I-HSA Radiolabelling

The amount of iodide ion adsorbed to a titanium surface after exposure to unlabelled protein plus labelled free iodide was found to be ~ 0.2 %. This amount is negligible, considering that in our solution we only have ~ 2% of free iodide ion, instead of 100 %, and that only 0.2 % of free iodide can adsorb to our substrate. This result justifies the use of a buffer without non-labelled iodide ion, PBS.

Preferential adsorption of the ¹²⁵I-labelled albumin did not occur on TiO₂ cp surface as determined by varying the ratio of labelled to unlabelled protein in a series of control experiments. The total HSA concentration was 1.0 mg/mL as determined by the bicinchoninic acid (BCA) assay. The amount of HSA adsorbed in all experiments to TiO₂ cp was 5.0 mg/m².

Using HSA concentrations between 0 and 10 mg/mL for TiO₂ sp and between 0 and 4.0 mg/mL for TiO₂ cp surfaces, adsorption isotherms were performed (Figure 9). The adsorbed amount increased with HSA concentration, and a plateau is reached for concentrations of ~ 1.0 mg/mL for TiO₂ cp and ~ 4.0 mg/mL for TiO₂ sp.

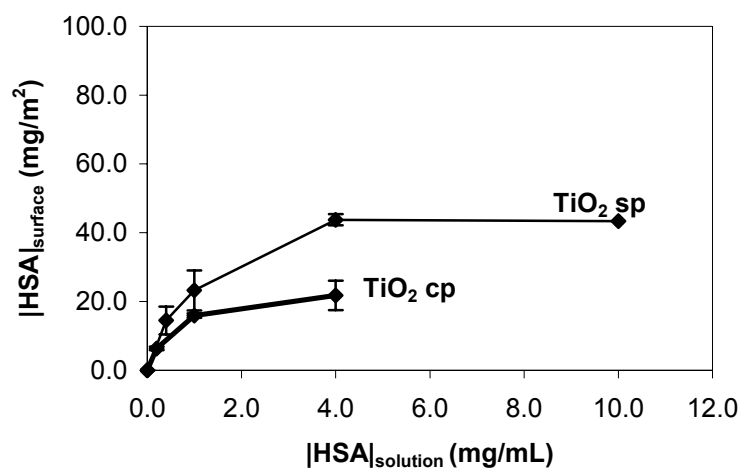


Figure 9 –HSA adsorption isotherms for TiO₂ substrates after 1 h of incubation.

After the adsorption step from single HSA solutions, desorption was performed with HSA (10.0 mg/ml) washes during 24 and 72 h, in order to evaluate the exchangeability of adsorbed HSA molecules by HSA in solution. The HSA molecules adsorbed on TiO₂ sp seem to be more easily exchanged by HSA itself (Figure 10) than those adsorbed on TiO₂ cp after 24 h, since 60-65% of HSA is eluted from TiO₂ sp and only 20-30 % of adsorbed protein was desorbed from TiO₂ cp surface. In contrast, after 72 h, nearly all the albumin effectively exchange with other albumin molecules from TiO₂ cp surface.

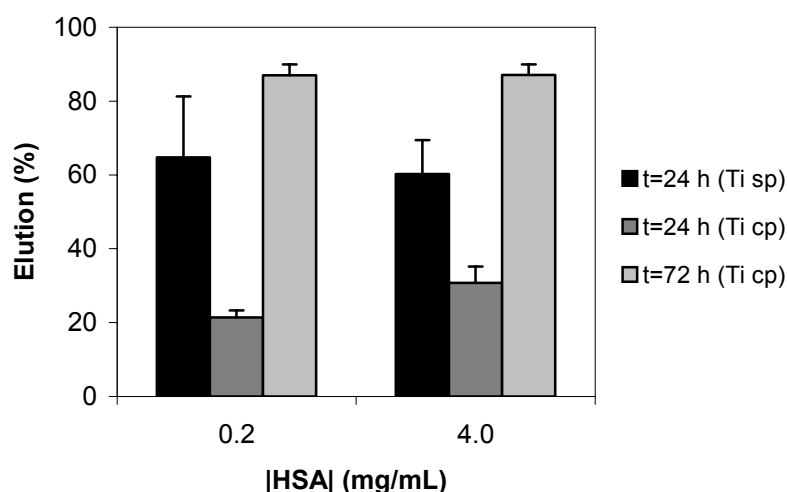


Figure 10 – Elution of HSA by other HSA molecules from TiO₂ cp and TiO₂ sp, as a function of HSA concentration in solution. The elution time was 24 h or 72 h for the TiO₂ cp substrate, and 24 h for the TiO₂ sp.

The initial protein concentration in solution does not influence HSA exchangeability, for both substrates, since identical elutions are obtained for 0.20 and 4.0 mg/mL (after 24 and 72 h).

The kinetics of albumin adsorption was studied on TiO₂ surfaces. Figure 11 and 12 show that a rapid HSA adsorption takes place on both surfaces.

Nevertheless for the time period studied, the albumin adsorption on both TiO₂ surfaces increased steadily to ~ 60 min of incubation. Figure 11 and Figure 12 shows the kinetics of HSA adsorption at two different concentrations 0.20 and 4.0 mg/ml and subsequent desorption with HSA. For the lower HSA concentration in solution as the time of incubation increases the albumin desorbed also increases. For the higher concentration in solution after 60 min. of immersion, desorption was at a minimum. For both albumin concentrations there are practically no exchanged HSA molecules.

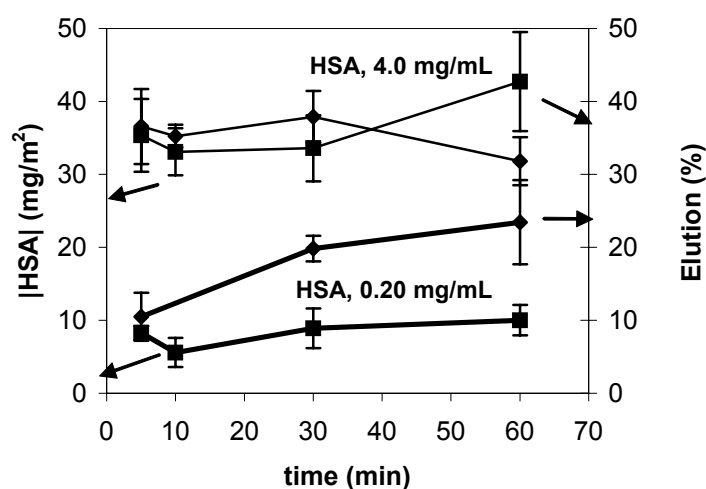


Figure 11 – Comparison of kinetics of HSA adsorption (0.20 mg/mL and 4.0 mg/mL) and desorption (with HSA) from TiO₂ cp surface.

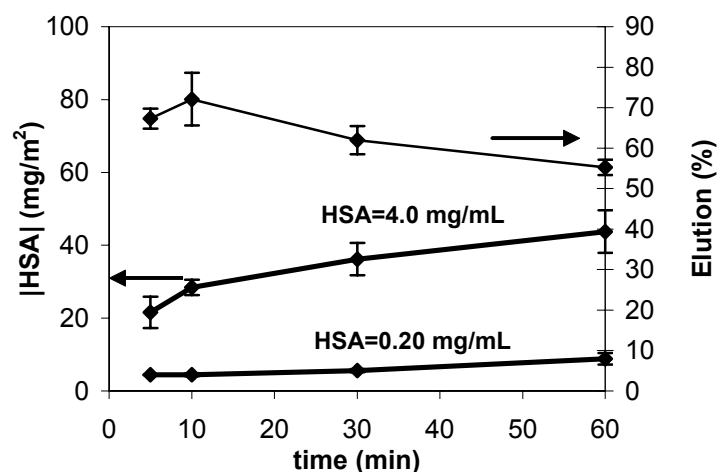


Figure 12 – Comparison of kinetics of HSA adsorption (0.20 mg/mL and 4.0 mg/mL) on TiO₂ sp and desorption (with HSA).

DISCUSSION

The influence of the nature of titanium oxide and of its hydrated state, as well as of the surface on biocompatibility, has become an important area of research.^{4,39-41} It has been shown that the method of preparation of titanium oxide has an effect on the dissolution behaviour of this material in biological environment^{29,42,43} and consequently on its *in vivo* performance.⁴⁴

In this study we prepared two simple titanium oxides, one sputtered on Si and another by Ti (TiO₂ sp) immersion in H₂O₂ 10 mM solution during 48 h (TiO₂ cp). The latter treatment conditions were selected among other hydrogen peroxide concentrations and time of immersions. XPS reveals that both treatments led to a regular and uniform layer of TiO₂, without sub-oxides like TiO and Ti₂O₃, sufficiently thick to prevent the emission of photoelectrons from the bulk material to the surface. Regarding the Ti/O ratio data, the Ti/O_M assumes values near the theoretical (1/2). As expected the Ti/O_H assumes higher values near the interface for the TiO₂ cp substrate due to the existence of a hydrated film. Tengvall et al.⁴ reported that titanium surfaces treated with H₂O₂ (1-30 %) showed that surface Ti/O_M ratio decreases when immersed in solutions of up to 10 mM H₂O₂, that is, in agreement with the data obtained in this work. In addition the TiO₂ film is thicker in 10 mM H₂O₂ than in 100 mM H₂O₂,⁴¹ which corroborates our choice of the peroxide concentration.

The two TiO₂ surfaces have distinct roughnesses with the TiO₂ sp having a mean Ra value 14 times smaller than the one for TiO₂ cp (Figure 1). Wenzel was one of the first investigators to propose a model relating contact angles and surface roughness⁴⁵ Roughening a substrate usually decreases its wettability,⁴⁶ which is in agreement with the results obtained here. Moreover the increased surface energy observed in H₂O₂ treated surfaces is also indicative of higher wettability.⁴¹ All substrates are predominantly dispersive, although the Ti substrates after H₂O₂ treatment have a higher contribution of the polar component due to the presence of ionic species. The surface free energy increases after H₂O₂ treatment as a result of the existence of more reactive sites exposed on the oxide layer. Considering that polarity is a quantitative indicator of hydrophilicity, we can say that the TiO₂ sputtered substrate is the more hydrophobic one.

Concerning protein adsorption, contact angle determination revealed a decrease in the contact angles as time increased for the different albumin solutions at the both TiO₂ surfaces (Figure 5 for TiO₂ cp). The variation is more pronounced for the plasma solutions. These solutions are complex mixtures of several proteins and ions, and therefore, the migration of these species to the drop boundary is delayed relative to the single protein solution. Rakhorst et al.⁴⁷ showed that plasma did not influence the initial contact angle at HDPE, PDMS, PTFE, and PU. The more accentuated decrease of the contact angles was correlated with higher levels of albumin and fibrinogen adsorption as well as platelet adhesion. These authors refer to the biocompatibility properties of the tested materials that might be related to the time behaviour of the contact angles in time, rather than to the initial hydrophobic or hydrophilic state. However, some precaution on the interpretation of biocompatibility based on hydrophilic/hydrophobic interactions should be taken. If the above hypothesis were always true, it would be expected that TiO₂ cp presents higher levels of albumin adsorption than TiO₂ sp, since the former surface shows higher change in contact angle of plasma ($\Delta\theta = -23^\circ$ (t=600 s)) than the latter surface ($\Delta\theta = -18^\circ$ (t=600 s)). This conclusion as well as the contact angle after 600 s (Table 3) is apparently in disagreement with the results obtained by ¹²⁵I-HSA radiolabelling. The radiolabelling data was expressed as the ratio between the amount of adsorbed protein and the geometric area. If the real area was considered, a higher difference would be observed, since the TiO₂ cp is rougher than the TiO₂ sp surface. The quantity of albumin adsorbed is higher on

the more hydrophobic surface (TiO₂ sp) which is in agreement with the hypothesis defended in the literature by many authors⁴⁸⁻⁵¹ that hydrophobic surfaces generally adsorb more albumin than hydrophilic surfaces. The hydrophobicity of a metal surface could be an important factor in determining protein adsorption, but there may also be other factors relevant for metal surfaces, which are not important for polymer surfaces, namely, surface charge and chemical binding between the metal and the protein.⁵² Both TiO₂ surfaces have almost the same surface composition but, the chemically polished titanium after peroxide treatment presents more OH⁻ groups than the sputtered titanium oxide (Ti/O_H = 0.77 or 1.1, respectively), conferring to the former surface a higher negative charge. At physiological pH, albumin has also a net negative charge that repels the TiO₂ cp surface.

Although the work of adhesion cannot be seen as direct measure of protein adsorption, it quantifies the decrease in interfacial free energy (γ_{SL}), due to protein adsorption.³⁷ The wettability results also showed that the work of adhesion of HSA to TiO₂ cp decreased linearly as the albumin concentration increased. Macromolecules adsorbed can use several adsorption sites depending on their structure and molecular mass. At low concentrations, adsorbed proteins are in a unfolded state with more located binding sites to adsorption. It is known^{53,54} that the driving forces for adsorption of “soft” proteins such as HSA, are related to structural rearrangements in the molecules that enable them to overcome the unfavourable conditions offered by an electrostatically repelling surface. At higher protein concentration, the adsorption layer becomes more compressed and molecules with different degrees of unfolding will coexist at the interface,³⁵ being more difficult or maybe impossible to some molecules to overcome the unfavourable conditions to adsorption. These phenomena can perhaps explain the highest work of adhesion obtained for the lowest HSA concentration observed (Figure 4). The opposite correlation was found for the plasma and TiO₂ cp substrate (Figure 7). This behaviour may result from a simultaneous adsorption of other plasma proteins with albumin (namely adhesion proteins like fibronectin and vitronectin) or exchanged with albumin molecules that are in a higher concentration at undiluted plasma and have a higher affinity to the surface.

The work of adhesion of both HSA 4.0 mg/ml and plasma is higher for TiO₂ cp than for TiO₂ sp which may be correlated with the higher polar component of the surface free energy of TiO₂ cp.

Radiolabelling of albumin has shown that the adsorbed amount increases with HSA concentration and a plateau is reached for concentrations of ~ 1.0 mg/ml for TiO₂ cp. As shown in Figure 13, the same conclusion may be drawn from the evolution of the N % determined by XPS. Thus, XPS can be successfully used as an alternative technique to study isothermal adsorption of albumin, correlating well with ¹²⁵I-labelled HSA data.

Although proteins often adsorb as monolayers, multilayer adsorption is not uncommon, namely, for high concentration solutions.^{55,56} Knowing the size of the protein molecule and the molecular mass it is possible to estimate the surface occupied by the molecules lying perpendicular or parallel to the surface and to convert it in mass of albumin per unit surface (m²). The theoretical values for closely packed monolayers formed by side-on adsorption and end-on adsorption are: 2.14 mg/m² and 7.90 mg/m², respectively. Comparing with the data obtained by ¹²⁵I-HSA (Figure 9), these values suggest that the albumin adsorption on both TiO₂ substrates is a multilayer coverage.

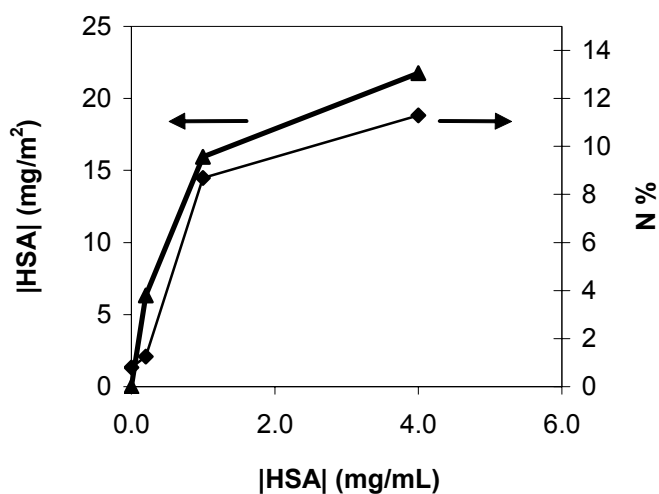


Figure 13 – Comparison between adsorption of albumin onto TiO₂ cp surface by ¹²⁵I-HSA radiolabelling and XPS measurements of N %.

Regarding the exchangeability with other albumin molecules, the HSA molecules adsorbed on TiO₂ sp seem to be more easily exchanged (Figure 10) than those adsorbed on TiO₂ cp after 24 h, since 60-65% of HSA is eluted from TiO₂ sp surface and only 20-30 % of adsorbed protein

was desorbed from TiO₂ cp surface. This result is expected if we look for the work of adhesion for both substrates; that is, HSA are less tightly bound at hydrophobic surfaces than at the hydrophilic surfaces. It is expected that, at least in aqueous media, non-polar interactions are stronger than polar interactions;⁵⁷ however, the binding strength seems to be at a maximum if both polar and non-polar interactions are involved, as is the case of TiO₂ cp substrate. Besides, Kurrall et al.⁵⁸ and Martins et al.⁴⁸ suggested that the main mode of interaction of HSA onto hydroxylated metals was via hydrogen bonds. Although hydrogen bonds are individually weak, the probability of breaking many hydrogen bonds simultaneously is low. This can be responsible for changing the adsorption behavior of the protein from reversible to “irreversible”. However after 72 h nearly all the albumin effectively exchanges with other albumin molecules at the TiO₂ cp surfaces. According to Brash et al.⁵⁹ the exchange of albumin is slow, ~ 40 h and as the surface-protein interactions are stronger for the TiO₂ cp substrate, a longer time is necessary for the total exchange.

For the higher concentration in solution and for both substrates, as the time of incubation increases it is notorious that albumin exchangeability diminishes perhaps due to conformational changes.

CONCLUSIONS

Two simple titanium oxides were prepared, one sputtered on Si (TiO₂ sp) and another obtained by Ti immersion in H₂O₂ 10 mM solution for 48 h (TiO₂ cp). XPS revealed that both treatments led to a regular and uniform layer of TiO₂, without sub-oxides like TiO and Ti₂O₃, sufficiently thick to prevent the emission of photoelectrons from the bulk material to the surface.

The two TiO₂ surfaces have distinct roughnesses, with the TiO₂ sputtered has a mean Ra value 14 times smaller than the one for TiO₂ cp.

TiO₂ cp adsorbs less albumin than the TiO₂ sp as shown by the radiolabelling data, but the adherent molecules are strongly attached to the former surface, as indicated by the work of adhesion and by the desorption studies. The data obtained by ¹²⁵I-HSA also suggests that albumin adsorbs as a multilayer.

Isothermal and kinetics of albumin adsorption on titania surfaces can be successfully studied by XPS and by ¹²⁵I-HSA radiolabelling measurements, and both techniques correlate well.

ACKNOWLEDGEMENTS:

The authors would like to thank Doctor Paulo Freitas and Doctor Susana Freitas (INESC) for the titanium oxide sputtered sample preparation, Doctor Carlos Sá (CEMUP) for assistance in the XPS investigation and the Portuguese Institute of Blood (IPS) for donating the human plasma samples.

REFERENCES

1. Nakabayashi N, Iwasaki Y. Biomaterials: Evolution. European White Book on fundamental research in materials science. Stuttgart: Max Plank Institut fur Metallforschung; 2000. p 70-71.
2. Windler M, Klabunde R. Titanium for hip and knee prostheses. In: Brunette DM, Tengvall P, Textor M, Thomsen P, editors. Titanium in Medicine: Material Science, Surface Science, Engineering, Biological Responses and Medical Applications. Berlin: Springer-Verlag; 2001. p 703-746.
3. Larsson C, Esposito M, Liao H, Thomsen P. The titanium-bone interface *in vivo*. In: Brunette DM, Tengvall P, Textor M, Thomsen P, editors. Titanium in Medicine: Material Science, Surface Science, Engineering, Biological Responses and Medical Applications. Berlin: Springer-Verlag; 2001. p 587-648.
4. Tengvall P, Lundstrom I. Physico-chemical considerations of titanium as a biomaterial. Clinical Materials 1992;9(2):115-134.
5. Ostumi E, Chapman RG, Liang MN, Meluleni G, Pier G, Ingberg DE, Whitesides GM. Langmuir 2001;17:6336-6343.
6. Gehanno V, Freitas PP, Ferreira J, Almeida B, Sousa JB, Kling A, Soares JC, Silva MF. Ion beam deposition of Mn-Ir spin valves. IEEE Trans Magn 1999;35:4361-4367.

7. Owens DK, Wendt RC. Estimation of Surface Free Energy of Polymers. *Journal of Applied Polymer Science* 1969;13(8):1741-1747.
8. Kerkeb ML, González-Caballero F, Chibowski E. Changes in cholesterol surface free energy components due to its phase transition. *Journal of Colloid and Interface Science* 1993;159:439-443.
9. Iodine-125. A guide to radioiodination techniques. Little Chalfont, Buckinghamshire, U.K.: Amersham Life Science; 1993. p 64.
10. Lima J, Sousa SR, Ferreira A, Barbosa MA. Interactions between calcium, phosphate, and albumin on the surface of titanium. *J Biomed Mater Res* 2001;55(1):45-53.
11. Williams DF, Askill IN, Smith R. Protein Adsorption and Desorption Phenomena on Clean Metal-Surfaces. *Journal of Biomedical Materials Research* 1985;19(3):313-320.
12. Sheardown H, Cornelius RM, Brash JL. Measurement of protein adsorption to metals using radioiodination methods: a caveat. *Colloids and Surfaces B-Biointerfaces* 1997;10(1):29-33.
13. Du YJ, Cornelius RM, Brash JL. Measurement of protein adsorption to gold surface by radioiodination methods: suppression of free iodide sorption. *Colloids and Surfaces B-Biointerfaces* 2000;17(1):59-67.
14. Yu XJ, Brash JL. Measurement of protein adsorption to solid surfaces in relation to blood compatibility using radiolabelling methods. In: Dawids S, editor. *Test procedures for the blood compatibility of biomaterials*. Dordrecht, The Netherlands: Kluwer Academic Publishers; 1993. p 287-330.
15. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. Measurement of Protein Using Bicinchoninic Acid. *Analytical Biochemistry* 1985;150(1):76-85.
16. Shirkhanzadeh M. XRD and XPS Characterization of superplastic TiO₂ coatings prepared on Ti6Al4V surgical alloy by an electrochemical method. *Journal of Materials Science-Materials in Medicine* 1995;6(4):206-210.
17. Kilpadi DV, Raikar GN, Liu J, Lemons JE, Vohra Y, Gregory JC. Effect of surface treatment on unalloyed titanium implants: Spectroscopic analyses. *Journal of Biomedical Materials Research* 1998;40(4):646-659.

18. Lausmaa J, Kasemo B, Mattsson H. Surface spectroscopic characterization of titanium implant materials. *Applied Surface Science* 1990;44(2):133-146.
19. Armstrong NR, Quinn RK. Auger and X-Ray Photoelectron Spectroscopic and Electrochemical Characterization of Titanium Thin-Film Electrodes. *Surface Science* 1977;67(2):451-468.
20. Leita E, Barbosa MA, deGroot K. XPS characterization of surface films formed on surface-modified implant materials after cell culture. *Journal of Materials Science-Materials in Medicine* 1997;8(7):423-426.
21. Ramqvist L, Hamrin K, Johansson G, Fahlman A, Nordling C. Charge Transfer in Transition Metal Carbides and Related Compounds Studied by ESCA. *Journal of Physics and Chemistry of Solids* 1969;30(7):1835-&.
22. Hanawa T, Ota M. Characterization of Surface-Film Formed on Titanium in Electrolyte Using XPS. *Applied Surface Science* 1992;55(4):269-276.
23. Kuznetsov MV, Zhuravlev JF, Gubanov VA. XPS Analysis of Adsorption of Oxygen Molecules on the Surface of Ti and Thin Films in Vacuum. *Journal of Electron Spectroscopy and Related Phenomena* 1992;58(3):169-176.
24. Ikeo N, Iijima Y, Niimura N, Sigematsu M, Tazawa T, Matsumoto S, Kojima K, Nagasawa Y. *Handbook of X-ray photoelectron spectroscopy*: JEOL; 1991.
25. Porte-Durrieu MC, Labrugere C, Villars F, Lefebvre F, Dutoya S, Guette A, Bordenave L, Baquey C. Development of RGD peptides grafted onto silica surfaces: XPS characterization and human endothelial cell interactions. *Journal of Biomedical Materials Research* 1999;46(3):368-375.
26. Wagner CD, Riggs WM, Davis LE, Moulder JF. *Handbook of X-ray photoelectron spectroscopy*; 1979.
27. Barr TL. Recent Advances in X-ray Photoelectron-Spectroscopy Studies of Oxides. *Journal of Vacuum Science & Technology A-Vacuum Surfaces and Films* 1991;9(3):1793-1805.
28. Wälivaara B, Aronsson BO, Rodahl M, Lausmaa J, Tengvall P. Titanium with Different Oxides - in-Vitro Studies of Protein Adsorption and Contact Activation. *Biomaterials* 1994;15(10):827-834.

29. Oji MO, Wood JV, Downes S. Effects of surface-treated cpTi and Ti6Al4V alloy on the initial attachment of human osteoblast cells. *Journal of Materials Science-Materials in Medicine* 1999;10(12):869-872.
30. Pan J, Liao H, Leygraf C, Thierry D, Li J. Variation of oxide films on titanium induced by osteoblast-like cell culture and the influence of an H₂O₂ pretreatment. *Journal of Biomedical Materials Research* 1998;40(2):244-256.
31. Ratner BD, Horbett TA, Shuttleworth D, Thomas HR. Analysis of the Organization of Protein Films on Solid-Surfaces by ESCA. *Journal of Colloid and Interface Science* 1981;83(2):630-642.
32. Lindberg B, Maripuu R, Siegbahn K, Larsson R, Golander CG, Eriksson JC. ESCA Studies of Heparinized and Related Surfaces .1. Model Surfaces on Steel Substrates. *Journal of Colloid and Interface Science* 1983;95(2):308-321.
33. Auditore A, Satriano C, Coscia U, Ambrosone G, Parisi V, Marletta G. Human serum albumin adsorption onto a-SiC : H and a-C : H thin films deposited by plasma enhanced chemical vapor deposition. *Biomolecular Engineering* 2002;19(2-6):85-90.
34. Serro APV, Fernandes AC, Saramago BDV. Dynamic interfacial behaviour of bovine serum albumin solutions on titanium surfaces. *Colloids and Surfaces* 1997;125(2-3):209-219.
35. Miller R, Treppo S, Voigt A, Zingg W, Neumann AW. Contact-Angle Kinetics of Human Albumin Solutions at Solid- Surfaces. *Colloids and Surfaces* 1993;69(4):203-208.
36. Lopes M, Monteiro F, Santos J, Serro A, Saramago B. Hydrophobicity, surface tension, and zeta potential measurements of glass-reinforced hydroxyapatite composites. *J Biomed Mater Res* 1999;45(4):370-5.
37. Ferraz MP, Monteiro FJ, Serro AP, Saramago B, Gibson IR, Santos JD. Effect of chemical composition on hydrophobicity and zeta potential of plasma sprayed HA/CaO-P₂O₅ glass coatings. *Biomaterials* 2001;22(23):3105-3112.
38. Vogler EA. Interfacial chemistry in biomaterials science. In: Berg JC, editor. *Surfactant science series 49 (wettability)*. N Y: Marcel Dekker; 1993. p 183-250.
39. Feng B, Weng J, Yang BC, Qu SX, Zhang XD. Characterization of surface oxide films on titanium and adhesion of osteoblast. *Biomaterials* 2003;24(25):4663-70.

-
40. Morra M, Cassinelli C, Cascardo G, Cahalan P, Cahalan L, Fini M, Giardino R. Surface engineering of titanium by collagen immobilization. Surface characterization and *in vitro* and *in vivo* studies. *Biomaterials* 2003;24(25):4639-54.
 41. Wälivaara B, Lundstrom I, Tengvall P. An in-vitro study of H₂O₂-treated titanium surfaces in contact with blood plasma and a simulated body fluid. *Clin Mater* 1993;12(3):141-48.
 42. Lincks J, Boyan BD, Blanchard CR, Lohmann CH, Liu Y, Cochran DL, Dean DD, Schwartz Z. Response of MG63 osteoblast-like cells to titanium and titanium alloy is dependent on surface roughness and composition. *Biomaterials* 1998;19(23):2219-32.
 43. Ong JL, Carnes DL, Cardenas HL, Cavin R. Surface roughness of titanium on bone morphogenetic protein-2 treated osteoblast cells in vitro. *Implant Dent* 1997;6(1):19-24.
 44. Ricci JL, Spivak JM, Blumenthal NC, al e. Modulation of bone ingrowth by surface chemistry and roughness. In: Davis JE, editor. *The bone biomaterial interface*. Toronto: University of Toronto Press; 1991. p 334-349.
 45. Busscher HJ, Vanpelt AWJ, Deboer P, Dejong HP, Arends J. The effect of surface roughening of polymers on measured contact angles of liquids. *Colloid Surface* 1984;9(4):319-331.
 46. Hitchcock SJ, Carroll NT, Nicholas MG. Some effects of substrate roughness on wettability. *Journal of Materials Science* 1981;16:714-732.
 47. Rakhorst G, Van der Mei HC, Van Oeveren W, Spijker HT, Busscher HJ. Time-related contact angle measurements with human plasma on biomaterial surfaces. *International Journal of Artificial Organs* 1999;22(1):35-39.
 48. Martins MCL, Ratner BD, Barbosa MA. Protein adsorption on mixtures of hydroxyl- and methylterminated alkanethiols self-assembled monolayers. *Journal of Biomedical Materials Research Part A* 2003;67A(1):158-171.
 49. Sigal GB, Mrksich M, Whitesides GM. Effect of surface wettability on the adsorption of proteins and detergents. *J. Am. Chem. Soc.* 1998;120(14):3464-3473.
 50. Prime KL, Whitesides GM. Self-Assembled Organic Monolayers - Model Systems for Studying Adsorption of Proteins at Surfaces. *Science* 1991;252(5009):1164-1167.

51. Vandulm P, Norde W. The Adsorption of Human-Plasma Albumin on Solid-Surfaces, with Special Attention to the Kinetic Aspects. *J. Colloid Interf. Sci.* 1983;91(1):248-255.
52. Williams RL, Williams DF. The Effect of Albumin on the Wettability of Pure Metal and Metal-Oxide Surfaces. *Journal of Colloid and Interface Science* 1988;126(2):596-603.
53. Norde W, Favier JP. Structure of Adsorbed and Desorbed Proteins. *Colloids and Surfaces* 1992;64(1):87-93.
54. Serro APVA, Fernandes AC, Saramago BDV, Norde W. Bovine serum albumin adsorption on titania surfaces and its relation to wettability aspects. *Journal of Biomedical Materials Research* 1999;46(3):376-381.
55. Young BR, Pitt WG, Cooper SL. Protein Adsorption on Polymeric Biomaterials .2. Adsorption-Kinetics. *Journal of Colloid and Interface Science* 1988;125(1):246-260.
56. Feng L, Andrade JD. Protein adsorption on low temperature isotropic carbon. III. Isotherms, competitiveness, desorption and exchange of human albumin and fibrinogen. *Biomaterials* 1994;5(15):323-333.
57. Norde W. Adsorption of Proteins from Solution at the Solid-Liquid Interface. *Advances in Colloid and Interface Science* 1986;25(4):267-340.
58. Kurrat R, Prenosil JE, Ramsden JJ. Kinetics of human and bovine serum albumin adsorption at silica-titania surfaces. *Journal Colloid Interface Science* 1997;185(1):1-8.
59. Brash JL, Uniyal S, Samak Q. Exchange of albumin adsorbed on polymer surfaces. *Trans Am Soc Artif Intern Organs* 1974;20A:69-76.

TiO₂ type influences fibronectin adsorption

S. R. Sousa^{1,2,3}, P. Moradas Ferreira^{4,5}, and M. A. Barbosa^{1,2}

¹ INEB, Instituto de Engenharia Biomédica, Laboratório de Biomateriais, R. do Campo Alegre, 823, 4150-180 Porto, Portugal

² Universidade do Porto, Faculdade de Engenharia, Departamento de Engenharia Metalúrgica e de Materiais, R. Dr. Roberto Frias, s/n, 4200 - 465 Porto, Portugal

³ ISEP, Instituto Superior de Engenharia do Porto, Departamento de Engenharia Química, R. Dr. Bernardino de Almeida, 431, 4200 - 072 Porto, Portugal

^{4, 5} ICBAS, IBMC, Instituto de Biologia Molecular e Celular, Grupo de Microbiologia Celular Aplicada, R. do Campo Alegre, 823, 4150-180 Porto, Portugal

ABSTRACT

Human fibronectin (FN) plays a key role in the biointegration of implants as the success depends on adsorption of proteins like FN.¹ Indeed FN can be an intermediary between the biomaterial surface and cells.

The adsorption of human fibronectin (FN) on commercially pure titanium with a titanium oxide layer formed in a H₂O₂ solution (TiO₂ cp) and TiO₂ sputtered on Si (TiO₂ sp) was studied. Adsorption isotherms and the work of adhesion were assessed by wettability studies, X-ray photoelectron spectroscopy (XPS), and by radiolabelling of FN with ¹²⁵I, ¹²⁵I-FN. Exchangeability of bound FN by free FN, was also evaluated by the radiolabelling technique.

Contact angle determinations have shown that FN displays higher affinity for the TiO₂ cp surface than for the TiO₂ sp. As expected from the surface free energy values, the work of

adhesion of FN is higher for the TiO₂ cp substrate, the more hydrophilic one, and lower for the TiO₂ sp substrate, the more hydrophobic one.

The adsorption isotherms were evaluated by two different techniques: radiolabelling of FN (¹²⁵I-FN) and XPS. TiO₂ cp adsorbs more FN than the TiO₂ sp surfaces as shown by the radiolabelling data. FN molecules are also more strongly attached to the former surface as indicated by the work of adhesion and by the exchangeability studies. Results using ¹²⁵I-FN also suggests that FN adsorbs as a multilayer for FN concentrations in solution higher than 100 µg/mL.

INTRODUCTION

FN is a product of most mesenchymal and epithelial cells as well as osteoblasts, and is present in the extracellular matrix (ECM) and plasma. The main function of FN is related to cellular migration during wound healing and development, regulation of cell growth and differentiation and haemostasis/thrombosis. Human Fibronectin (FN) is a glycoprotein that promotes attachment of cells² to the biomaterial surface through its central-binding domain RGD sequence. Indeed, this protein plays a key role in the biointegration of implants. Therefore the study of FN conformation adsorbed on biomaterials is of high relevance.

FN (MW ~500 KD) is a dimer and each chain has 60 nm in length and 2.5 nm in diameter, connected by flexible disulfide bonds near one end. Soluble FN has a compact structure stabilized by intermolecular ionic interactions between the two subunits. The binding sites for cells, the RGD sequences, used as a hinge region, are inaccessible in the soluble form.^{3,4} A more extended conformation may be induced, depending upon environmental pH and salt concentration, and the domains involved in cell binding become exposed.⁵ An identical conformation response may be induced by adsorption to a suitable surface. FN may bind to a number of substrates, exhibiting different conformations. Conformational changes upon adsorption of FN are due to considerable flexibility that permits FN to bind to inert surfaces leading to different surface interactions.⁶

The present work analyses the adsorption of human fibronectin (FN) on commercially pure titanium with a titanium oxide layer formed in a H₂O₂ solution (TiO₂ cp) and TiO₂ sputtered on Si (TiO₂ sp). These surfaces were characterized elsewhere.⁷

Adsorption isotherms, exchangeability of bound FN by free FN, and work of adhesion of FN to titanium oxide surfaces were assessed by wettability studies, X-ray photoelectron spectroscopy (XPS), and by radiolabelling of FN with ¹²⁵I, ¹²⁵I-FN.

MATERIALS AND METHODS

TiO₂ substrates

Commercially pure titanium sheets, 1 mm thick, were used. The nominal composition (wt %, max.) was the following: 0.20 Fe, 0.05 N, 0.08 C and 0.015 H.

Surface was roughened by a chemical polishing (cp) with an etching acid solution (2:3 parts by volume, 40 % of HF + 60 % of HNO₃ (v/v)), for 30 s. The substrates were pre-incubated in H₂O₂ 10 mM for 48 h to obtain a regular titanium oxide film.

TiO₂ sputtered substrates were prepared using an automated, load locked ion beam deposition system (Nordiko N3000), described elsewhere.⁸

The TiO₂ films (50 nm) were deposited on Si wafers (polished/etched, crystal orientation <100>, from AUREL GmbH) by ion beam sputtering from a Ti cp target, in the presence of a mixed Ar-O₂ beam coming from the assist gun (15.02 % O₂ in Ar). The deposition rate was 0.038 nm/s. More deposition details have been reported in a previous study.⁷

The wafers were diced into pieces (5X5 μm²) using a DISCO DAD321 automated saw. Before dicing, all wafers were coated with 1.5 μm of photoresist (reference PFR7790EG, from JSR Electronics), to protect the film surface.

Just before being used, TiO₂ sputtered films were cleaned twice in acetone and immersed in a “piranha” solution (7 parts of 98 % H₂SO₄ and 3 parts of 30 % H₂O₂ (v/v)) for 90 s (*Caution: this solution reacts violently with many organic materials and should be handled with great care*).

Substrates were washed in ethanol, water (distilled and deionized) and again in ethanol for 2 min. in an ultrasonic bath.

Protein Solutions

Human plasma fibronectin 0.1 wt% solution, FN, (Sigma, ref. F-0895) solutions were prepared by dilution in phosphate buffered saline, PBS (Sigma, pH=7.4, ref. P-3813) at concentrations of 0, 5.0, 10.0, 20.0, 100.0 and 200.0 µg/mL. The latter concentration is the normal human serum FN concentration.

X-ray photoelectron spectroscopy, XPS

XPS measurements were carried out on a VG Scientific ESCALAB 200A (UK) spectrometer using MgK α (1253.6 eV) as a radiation source. The photoelectrons were analysed at a take off angles of 0°.

Survey XPS spectra were acquired from 0 to 1100 eV, before and after protein adsorption. High-resolution spectra for Ti 2p, O1s, C1s, N1s and S1s were also obtained. Elemental atomic percentages of Ti, O, C, N and S were determined after deconvolution of high-resolution spectra, using an XPS peak-fitting program (XPS PEAK Version 4.1). To take into account shifts caused by charging of the sample surface, all spectra were adjusted taking the C1s peak at 285.0 eV as reference for the adventitious carbon contamination.

The elemental atomic percentages (atomic %) of Ti, O, C, N and S were determined after 60 min of FN adsorption with concentrations in the range of 0 - 200 µg/mL (adsorption isotherms).

Wettability Studies

Contact angle measurements were performed with a contact angle measuring system from Data Physics, model OCA 15, equipped with a video charge-coupled device (CCD) camera and SCA

20 software. The equipment incorporates an electronic syringe unit with a gas tight 500 µl dosing syringe (Hamilton).

Contact angles were obtained by the sessile drop method, at 25 °C in a chamber saturated with the liquid sample. The drop was recorded as soon as stabilisation was achieved, and images were recorded every 2 s up to 600 s. Digital images of the drop were acquired by the CCD-camera and used for the calculation of the contact angle. For the contact angle determination, an ellipse function was fitted to the measure contour line obtaining the best accuracy. The contact angle value was extrapolated to t=600 s for protein solutions, using a curve-fitting program. In each experiment, at least 10 drops of ~ 4 µL were used.

Surface tension of the protein solution in equilibrium with its own vapour (γ_{LV}) was obtained analysing the change in the shape of a pendant drop kept at 25 °C in a chamber saturated with the liquid sample. As drop stabilization was achieved, images were taken every 2 s during 600 s. In each experiment at least 10 drops of ~ 20 µL were analysed. The liquid densities at 25 °C were determined using an Anton Paar Density meter.

The work of adhesion (the negative part of the Gibbs energy of adhesion) for a liquid and a solid in contact (W) may be obtained by the Young-Dupré equation:

$$W = \gamma_{LV} + \gamma_{SV} - \gamma_{SL} = \gamma_{LV} (1 + \cos\theta) \quad (1)$$

Protein quantification by radioactivity

Quantification of FN adsorbed on the TiO₂ surface was performed using ¹²⁵I labelled FN.

Human plasma fibronectin (Sigma) was labelled with ¹²⁵I using the Iodogen method.^{9,10} ¹²⁵I-FN was passed through a Sephadex G-25 M (PD-10 column Amersham Pharmacia Biotech) to remove unbound ¹²⁵I. The yield of iodination was always higher than 98 %, as determined by precipitating the ¹²⁵I-labelled FN with 20% trichloroacetic acid (TCA method).

The contribution of free ¹²⁵I to the total radioactivity found on the metal surfaces was estimated using unlabelled FN solutions at a concentration of 1.0 mg/mL, and an amount of free ¹²⁵I ion equivalent to that present as ¹²⁵I ion in the labelled protein solutions.

In all studies in which radiolabelling methods are used it must be kept in mind that the behaviour of the labelled protein may or may not reflect the behaviour of unlabelled protein.¹¹ To assess if preferential adsorption of ¹²⁵I labelled FN occurred on TiO₂ surfaces, a series of control experiments were performed varying the ratio of labelled to unlabelled FN (10 - 50 %). The final concentration of the FN solutions (1.0 mg/mL) was confirmed by the bicinchoninic acid (BCA) assay using BCA assay kit from Pierce and bovine serum albumin (BSA) as standard.¹²

Protein solutions for adsorption experiments were prepared adding ¹²⁵I-FN to unlabelled FN solution in order to obtain a final activity of $\sim 10^8$ cpm/mg.

FN adsorption tests were performed placing the Ti substrates in a 24-well tissue plate (Sarsted). A drop of 10 μ l ¹²⁵I-FN solution was added onto each surface. In order to avoid evaporation, PBS was added to the wells of the periphery. Adsorption tests were carried out at 25 °C over a period of time. After this period the drop was absorbed from the surface with absorbent paper and the surface was washed three times with PBS. The samples were transferred to radioimmunoassay tubes and surface activity was measured using a gamma counter. All experiments were done in triplicate. The counts from each sample were averaged, and the surface concentration was calculated by the equation:

$$FN (\mu g/m^2) = \frac{counts(cpm) * |FN|_{solution} (\mu g / mL)}{A_{solution} (cpm / mL) * SA(m^2)} \quad (2)$$

where the counts measure the radioactivity of the samples, $|FN|_{solution}$ is the FN concentration solution, $A_{solution}$ is the specific activity of the FN solution and SA is the surface area.

To understand the FN adsorption on titanium oxide substrates adsorption isotherms were investigated in a FN concentration range of 0 – 200 μ g/mL after 60 min. of adsorption.

Displacement tests were carried out by immersing the labelled Ti surface over 24 h in an unlabelled FN pure solution (400 μ g/mL). The substrates were washed three times with PBS and the residual activity counted.

Statistical analysis of the experimental results was performed using the Kurskall-Wallis, non-parametric tests.

RESULTS

XPS

Wide scans were performed on TiO₂ sp surfaces after FN adsorption, at increasing FN concentrations. Ti, O, C, N, and S were detected and C, N, O, and S arise mainly from protein. High resolution spectra were acquired for Ti2p, O1s, C1s, N1s, and S2p, providing an indication of the chemical composition of the protein film formed at the surfaces. The peaks were deconvoluted and C1s or N1s peaks were assigned to FN. We also followed the atomic % of each element during adsorption isotherms. The surface composition of the substrate is presented elsewhere.⁷

The C1s spectrum of FN is significantly different from that one of TiO₂ surface. A C1s peak broadening was observed after protein adsorption. It was considered that the C1s peak after FN adsorption resulted from four different peaks. The peaks correspond to carbon atoms in different environments, i.e., saturated hydrocarbon groups (-CH₃, -CH₂-) with a BE of 285.0 eV, to amine groups (-C-NH-) with BE of 286.6 eV, to the peptide bond (-C=O-NH-) with BE of 288.2 eV and to acidic groups (-C-COO⁻) with BE of 289.6 eV¹³. Figure 1a shows an envelope for C1s deconvolution peak after FN adsorption ([FN]=200 µg/mL), being the -CH₂- groups the major contribution to the C1s intensity.

The N1s spectra, after FN adsorption, was considered to have a contribution of three different peaks corresponding to -NH₃⁺ with BE of 401.8 eV, to the peptide bond (-C=O-NH-) with BE of 400.1 eV and to the NH₂ groups with BE between 389.9-399.9 eV^{13,14} (Figure 1 b). The neutral peptide nitrogen peak dominates the N1s spectrum, contributing with more than 50% for the total amount.

The shape of O_H peak is not altered after protein adsorption⁷ suggesting that the contributions from the carboxyl groups and the phosphate from electrolyte are superimposed to the O_H peak. The atomic % of Ti, O_M and O_H vs [FN] are shown in Figure 2.

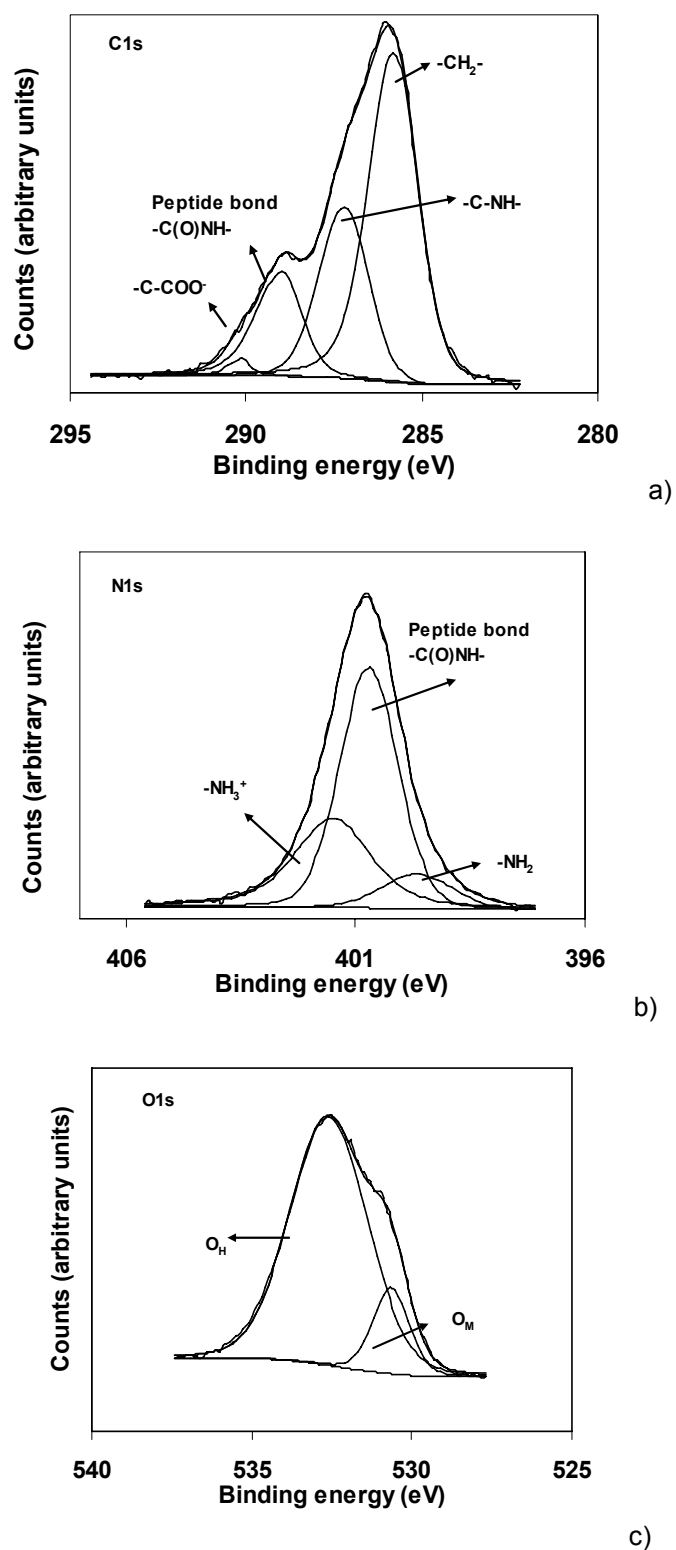


Figure 1 - Representative deconvolution of XPS envelope for a) C1s, b) N1s and c) O1s peak (after 60 min of FN 200 $\mu\text{g/ml}$ adsorption at TiO_2 sp surface) at 0° .

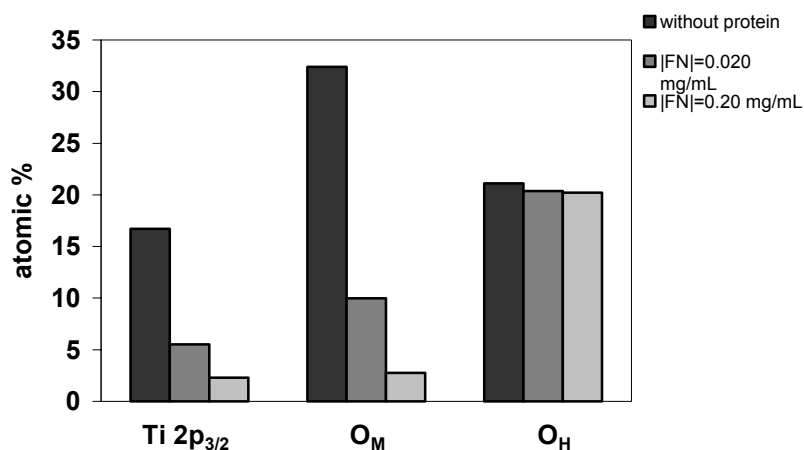


Figure 2 – Atomic % of Ti2p_{3/2}, O_M and O_H as a function of [FN] in solution, after 60 min. of adsorption, on a TiO₂ cp surface.

Upon adsorption the Ti% and the O_M % decreases, indicating that a film of protein is covering the titanium oxide (Figure 2). The decrease is a function of FN concentration.

Only one peak is observed at the high resolution S2p spectra, resulting from the sulphur atoms coming from the disulfide groups (–S–S–) in protein molecules at 164.1 eV.¹³

Wettability studies

To have further information on protein adsorption, wettability studies were performed and the work of adhesion between the protein solutions and the surfaces was calculated from the values of the contact angles and the surface tensions of the protein solutions, through Eq. 1. The values of the contact angles of several solutions, given in Table 1, were obtained by extrapolation of time-dependent experimental results to t=0 s for PBS⁷ and to t=600 s for protein solutions.

Comparison of contact angles between native and coated titanium surfaces revealed that the presence of a protein coating resulted in a more hydrophilic surface. The contact angle of FN with the two TiO₂ surfaces is dependent of FN concentration (Table 1), since they are statistically different. When a FN solution was assayed it is observed a higher affinity of the

solution to the TiO₂ cp surface. When a plasma solution was assayed it is observed a higher affinity of the solution to the TiO₂ sp surface.

Table 1 – Contact angle values of different solutions at TiO₂ surface (n=10).

Solution	γ_{LV} (mN/m)	θ , degrees	
		TiO ₂ sputtered	TiO ₂ cp (after H ₂ O ₂ treatment)
PBS ⁷	72.9 ± 0.20	77 ± 3	76 ± 4
FN 20 µg/ml	58.3 ± 0.72	42 ± 4	35 ± 3
FN 200 µg/ml	56.6 ± 0.82	67 ± 2	62 ± 2
plasma 10 % ⁷	50.1 ± 0.49	52 ± 2	57 ± 3
plasma ⁷	47.0 ± 0.33	42 ± 4	49 ± 3

The surface tension of two FN solutions was measured and compared with the one of PBS and of plasma solutions, measured in a previous work.⁷ The surface tension of PBS stays constant during the time of the experiment. The protein solutions have lower surface tensions, which decrease rapidly within the first 100-200 s reaching then a plateau or a semi-plateau (data not shown). Several authors attribute this behaviour to adsorption of protein molecules to the air-solution interface.^{15,16} The surface tensions of FN and plasma solutions (t=600 s) are shown in Table 1.

The time dependence of the work of adhesion between FN and TiO₂ surfaces was evaluated through the Young-Dupré equation, from the correspondent values of γ_{LV} and θ . Figure 3 shows the curves for FN (|FN|=200 µg/mL) and the two surfaces studied. Although the work of adhesion can not be seen as direct measure of protein adsorption, it quantifies the decrease in surface free energy due protein adsorption. The cp surface is the surface that presents the higher values of work of adhesion, indicating a more intense solid-liquid interaction.

The analysis of Figure 4 showed that the increased of protein concentration lead lower values for the work of adhesion. Adsorbed macromolecules may use several adsorption sites depending on their structure and molecular mass. At low concentrations, adsorbed proteins are in an unfolded state. At higher protein concentration the adsorption layer becomes more

compressed and molecules with different degrees of unfolding will coexist at the interface.¹⁵ These phenomena could account for the highest work of adhesion for the lowest FN concentration (Figure 4).

The work of adhesion between plasma and TiO₂ cp is opposite to the one found for the FN solutions (Figure 4).

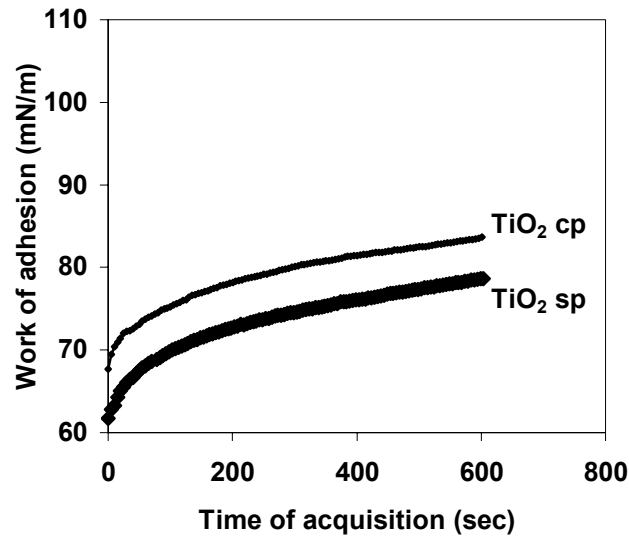


Figure 3 – Work of adhesion between TiO₂ substrates and FN ($[FN]=200\ \mu\text{g/mL}$).

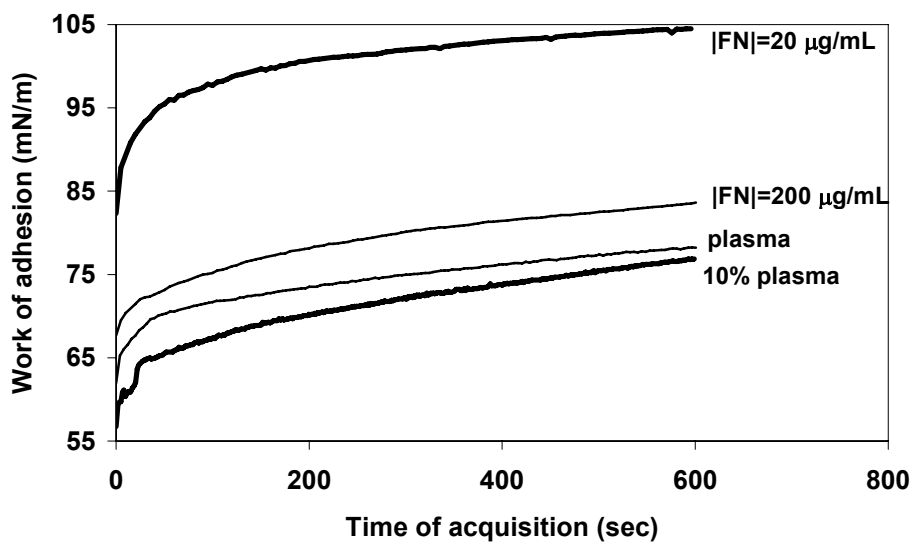


Figure 4 – Work of adhesion between TiO₂ cp and protein solutions.

Protein adsorption from single protein solutions

The amount of iodide ion adsorbed to a titanium surface after exposure to unlabelled FN plus labelled free iodide was found to be ca. 0.2 %. This amount is negligible considering that in our solution we only have ca. 2% of free iodide ion, instead of 100 %, and that only 0.2 % of free iodide can adsorb to our substrate. This result justifies the use of a buffer without non-labelled iodide ion, PBS.

Preferential adsorption of the ^{125}I -labelled FN did not occur on TiO_2 sp surface as determined by varying the ratio of labelled to unlabelled protein in a series of control experiments. The total FN concentration was 100 $\mu\text{g/mL}$ as determined by the bicinchoninic acid (BCA) assay. The amount of FN adsorbed in all experiments to TiO_2 sp was $2870 \pm 2.0 \mu\text{g/m}^2$.

Adsorption isotherms were performed using FN concentrations between 0 and 200 $\mu\text{g/mL}$, on both TiO_2 surfaces, and Figure 5 shows average curves.

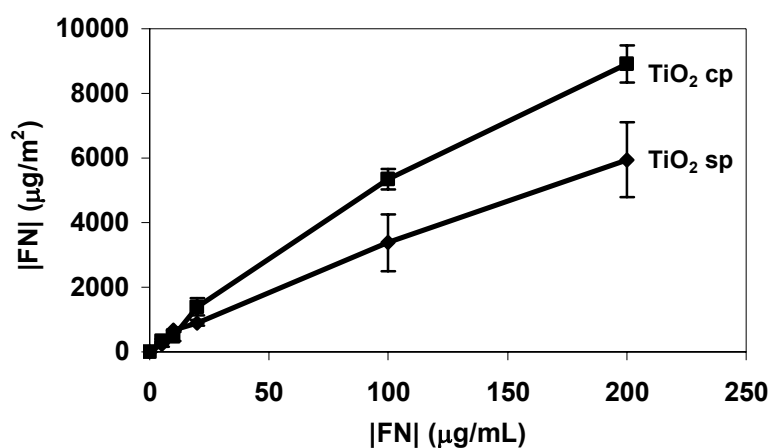


Figure 5 –Adsorption isotherm of FN on TiO_2 .

The adsorbed amount increased as the FN concentration increased in solution and a plateau was never attained for both surfaces.

After the adsorption step from single FN solutions, desorption was performed with FN washes during 24 h, in order to evaluate the exchangeability of FN molecules. In Figure 6 it can be seen

that for the FN concentrations studied, 60-70 % of FN molecules are eluted from TiO₂ surface sp and only 10-55 % of adsorbed protein was desorbed from TiO₂ cp surface.

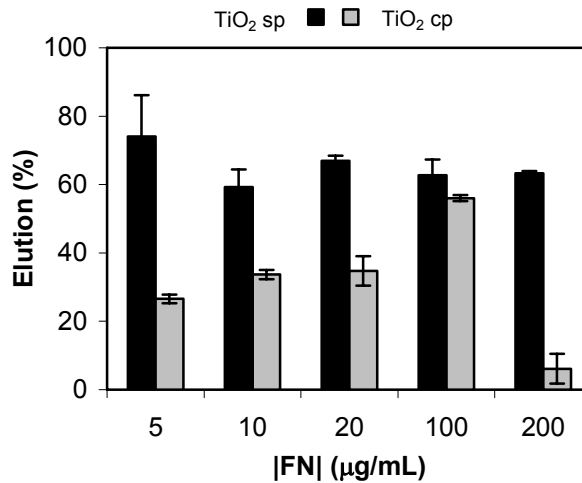


Figure 6 – Elution of FN molecules by other FN molecules from TiO₂ sp and from TiO₂ cp as a function of FN concentration in solution. The elution time was 24 h.

DISCUSSION

The importance of human fibronectin (FN) arises mainly from its ability to be an intermediary between the biomaterial surface and cells. It is believed that the biointegration of implants depends on adsorption of proteins like FN.^{17,18}

Although the work of adhesion cannot be considered as direct measure of protein adsorption, it quantifies the decrease in interfacial free energy, γ_{SL} , due to protein adsorption.¹⁹ The wettability results showed that the work of adhesion of FN to TiO₂ cp decreased when FN concentration increased. Macromolecules adsorbed can use several adsorption sites depending on their structure and molecular mass. At low concentrations, adsorbed proteins are in an unfolded state with more located binding sites to adsorption. It is known,^{20,21} that the driving forces for adsorption of “soft” proteins like FN and HSA, are related to structural rearrangements in the molecules that enable them to overcome the unfavourable conditions offered by an

electrostatically repelling surface. At higher protein concentration the adsorption layer becomes more compressed and molecules with different degrees of unfolding will coexist at the interface,¹⁵ being more difficult or maybe impossible for some molecules to overcome the unfavourable conditions to adsorption. This fact can explain the highest work of adhesion obtained for the lowest FN concentration observed (Figure 4). The opposite correlation was found for the plasma and TiO₂ cp substrate (Figure 4). This behaviour may result from a simultaneous adsorption of other plasma proteins with FN or exchanged with FN molecules that are in a higher concentration at undiluted plasma and have a higher affinity to the surface.

The adsorption isotherms show that the amount adsorbed on both types of surfaces increased as the FN concentration increased in solution and a plateau was never attained. The latter values can be compared with the theoretical values for FN adsorbed on TiO₂ surface knowing the size and molecular weight of the protein. For closely packed monolayers formed by side-on and end-on adsorption the concentrations are 1750 $\mu\text{g}/\text{m}^2$ and 41000 $\mu\text{g}/\text{m}^2$, respectively, demonstrating that the maximum adsorbed amount determined by radiolabelled protein is insufficient for end-on monolayer formation.²² These results may correspond to more than one monolayer in the side-on position only for concentrations higher than 100 $\mu\text{g}/\text{mL}$.

The FN molecules adsorbed on TiO₂ sp seem to be more easily exchanged (Figure 6) than those adsorbed on TiO₂ cp after 24 h, since 60-70% of FN is eluted from TiO₂ sp and only 10-55 % of adsorbed protein was desorbed from TiO₂ cp surface. This result is expected if the work of adhesion for both substrates, is evaluated, thus putting in evidence that, FN is less tightly bound at hydrophobic surfaces than at the hydrophilic surfaces. It is expected that, at least in aqueous media, non-polar interactions are stronger than polar interactions.²³ However, the binding strength seems to be at a maximum if both polar and non-polar interactions are involved, as it is the case of TiO₂ cp substrate. The desorption results also show that elution seems to be more sensitive to the nature of the surface than to the FN concentration in solution. Besides to FN conformation to control cell adhesion, minimum FN concentration seems to be necessary to achieve.¹⁸ Tamada, Y. et al²⁴ referred that the minimum amount of FN required for fibroblast cell adhesion was 500 $\mu\text{g}/\text{m}^2$. After elution, and for FN concentration ≥ 20 $\mu\text{g}/\text{mL}$ for TiO₂ cp, and ≥ 100 $\mu\text{g}/\text{mL}$ for TiO₂ sp, surface retains more than the referred FN amount for cell adhesion. While the type and amount of protein adsorbed on a substrate will influence cell spreading and

growth, the biological activity of the adsorbed proteins may have a more significant impact on cellular interactions. Modulation of the biological activity of adsorbed proteins by the substrate may result from differences in protein conformation which can affect the availability of binding domains. Differences in the biological activity of adsorbed proteins may also be related to levels in the binding strength of an adsorbed. Additionally, it can affect the ability of an adsorbed protein to be displaced by other proteins. To facilitate cellular ECM deposition, adsorbed serum proteins must be held loosely to allow for displacement by cell derived FN.¹⁸ In the present study, FN is more weakly held (higher elutability shown in Figure 6, and lower work of adhesion shown in Figure 3) at the TiO₂ sp surface. This suggests that FN is less resistant to displacement by other serum or cell derived proteins during initial cell attachment periods and later during cell spreading and growth phases.

CONCLUSIONS

It has been demonstrated that when a FN solution was assayed it is observed a higher affinity of the solution to the TiO₂ cp surface. In contrast, when a plasma solution was assayed a higher affinity of the solution to the TiO₂ sp surface occurred. TiO₂ cp adsorbs also more FN than the TiO₂ sp surfaces as shown by the radiolabelling data. Besides FN molecules are more strongly attached to the former surface as indicated by the work of adhesion and by the exchangeability studies. The more loosely FN adsorption to TiO₂ sp surface, may allow displacement by cell-derived FN in order to facilitate cellular ECM deposition. Comparing the theoretical amount for monolayer formation and the data obtained by ¹²⁵I-FN, it can be suggested that FN adsorbs as a multilayer in the side-on position, only for concentrations higher than 100 µg/mL.

This work provided novel data on the relevance of TiO₂ surface for the binding of FN and thus for the formation of a protein layer.

REFERENCES

1. Horbett TA. The role of adsorbed proteins in animal cell adhesion. *Colloids and Surfaces B: Biointerfaces* 1994;2(1-3):225-240.
2. Amaral IF, Lamghari M, Sousa SR, Sampaio P, Barbosa MA. Rat bone marrow stromal cell osteogenic differentiation and fibronectin adsorption on chitosan membranes: The effect of the degree of acetylation. *Journal of Biomedical Materials Research Part A* 2005;75A(2):387-397.
3. Bergkvist M, Carlsson J, Oscarsson S. Surface-dependent conformations of human plasma fibronectin adsorbed to silica, mica, and hydrophobic surfaces, studied with use of Atomic Force Microscopy. *Journal of Biomedical Materials Research Part A* 2003;64A(2):349-356.
4. Johnson KJ, Sage H, Briscoe G, Erickson HP. The compact conformation of fibronectin is determined by intramolecular ionic interactions. *Journal of Biological Chemistry* 1999;274(22):15473-15479.
5. Ugarova TP, Zamarron C, Veklich Y, Bowditch RD, Ginsberg MH, Weisel JW, Plow EF. Conformational Transitions in the Cell-Binding Domain of Fibronectin. *Biochemistry* 1995;34(13):4457-4466.
6. Sukenik CN, Balachander N, Culp LA, Lewandowska K, Merritt K. Modulation of Cell-Adhesion by Modification of Titanium Surfaces with Covalently Attached Self-Assembled Monolayers. *Journal of Biomedical Materials Research* 1990;24(10):1307-1323.
7. Sousa SR, Moradas-Ferreira P, Melo LV, Saramago B, Barbosa MA. Human Serum Albumin Adsorption on TiO₂ from Single Protein Solutions and from Plasma. *Langmuir* 2004;20(22):9745-9754.
8. Gehanno V, Freitas PP, Ferreira J, Almeida B, Sousa JB, Kling A, Soares JC, Silva MF. Ion beam deposition of Mn-Ir spin valves. *IEEE Trans Magn* 1999;35:4361-4367.
9. Iodine-125. A guide to radioiodination techniques. Little Chalfont, Buckinghamshire, U.K.: Amersham Life Science; 1993. p 64.

10. Lima J, Sousa SR, Ferreira A, Barbosa MA. Interactions between calcium, phosphate, and albumin on the surface of titanium. *J Biomed Mater Res* 2001;55(1):45-53.
11. Yu XJ, Brash JL. Measurement of protein adsorption to solid surfaces in relation to blood compatibility using radiolabelling methods. In: Dawids S, editor. *Test procedures for the blood compatibility of biomaterials*. Dordrecht, The Netherlands: Kluwer Academic Publishers; 1993. p 287-330.
12. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. Measurement of Protein Using Bicinchoninic Acid. *Analytical Biochemistry* 1985;150(1):76-85.
13. Lindberg B, Maripuu R, Siegbahn K, Larsson R, Golander CG, Eriksson JC. ESCA Studies of Heparinized and Related Surfaces .1. Model Surfaces on Steel Substrates. *Journal of Colloid and Interface Science* 1983;95(2):308-321.
14. Auditore A, Satriano C, Coscia U, Ambrosone G, Parisi V, Marletta G. Human serum albumin adsorption onto a-SiC : H and a-C : H thin films deposited by plasma enhanced chemical vapor deposition. *Biomolecular Engineering* 2002;19(2-6):85-90.
15. Miller R, Treppo S, Voigt A, Zingg W, Neumann AW. Contact-Angle Kinetics of Human Albumin Solutions at Solid- Surfaces. *Colloids and Surfaces* 1993;69(4):203-208.
16. Serro APV, Fernandes AC, Saramago BDV. Dynamic interfacial behaviour of bovine serum albumin solutions on titanium surfaces. *Colloids and Surfaces* 1997;125(2-3):209-219.
17. Matsuura T, Hosokawa R, Okamoto K, Kimoto T, Akagawa Y. Diverse mechanisms of osteoblast spreading on hydroxyapatite and titanium. *Biomaterials* 2000;21(11):1121-1127.
18. Tidwell CD, Ertel SI, Ratner BD, Tarasevich BJ, Atre S, Allara DL. Endothelial cell growth and protein adsorption on terminally functionalized, self-assembled monolayers of alkanethiolates on gold. *Langmuir* 1997;13(13):3404-3413.
19. Ferraz MP, Monteiro FJ, Serro AP, Saramago B, Gibson IR, Santos JD. Effect of chemical composition on hydrophobicity and zeta potential of plasma sprayed HA/CaO-P₂O₅ glass coatings. *Biomaterials* 2001;22(23):3105-3112.
20. Norde W, Favier JP. Structure of Adsorbed and Desorbed Proteins. *Colloids and Surfaces* 1992;64(1):87-93.

21. Serro APVA, Fernandes AC, Saramago BDV, Norde W. Bovine serum albumin adsorption on titania surfaces and its relation to wettability aspects. *Journal of Biomedical Materials Research* 1999;46(3):376-381.
22. Rouxhet L, Duhoux F, Borecky O, Legras R, Schneider YJ. Adsorption of albumin, collagen, and fibronectin on the surface of poly(hydroxybutyrate-hydroxyvalerate) (PHB/HV) and of poly(epsilon-caprolactone) (PCL) films modified by an alkaline hydrolysis and of poly(ethylene terephthalate) (PET) track-etched membranes. *Journal of Biomaterials Science-Polymer Edition* 1998;9(12):1279-1304.
23. Norde W. Adsorption of Proteins from Solution at the Solid-Liquid Interface. *Advances in Colloid and Interface Science* 1986;25(4):267-340.
24. Tamada Y, Ikada Y. Effect of Preadsorbed Proteins on Cell-Adhesion to Polymer Surfaces. *Journal of Colloid and Interface Science* 1993;155(2):334-339.

Dynamics of fibronectin adsorption on TiO₂ surfaces

S. R. Sousa^{1,2,3}, M. Manuela Brás¹, P. Moradas-Ferreira^{4,5}, and M. A. Barbosa^{1,2}

¹ INEB, Instituto de Engenharia Biomédica, Laboratório de Biomateriais, R. do Campo Alegre, 823, 4150-180 Porto, Portugal

² Universidade do Porto, Faculdade de Engenharia, Departamento de Engenharia Metalúrgica e de Materiais, R. Dr. Roberto Frias, s/n, 4200 - 465 Porto, Portugal

³ ISEP, Instituto Superior de Engenharia do Porto, Departamento de Engenharia Química, R. Dr. Bernardino de Almeida, 431, 4200 - 072 Porto, Portugal

^{4, 5} ICBAS, IBMC, Instituto de Biologia Molecular e Celular, Grupo de Microbiologia Celular Aplicada, R. do Campo Alegre, 823, 4150-180 Porto, Portugal

ABSTRACT

The present work analysis the dynamics of fibronectin, FN, adsorption on two different stable titanium oxides, with varied surface roughness, and chemically similar to those used in clinical practice. The two types of titanium oxide surfaces used were: TiO₂ sputtered on Si (TiO₂ sp) and TiO₂ formed on commercially pure titanium after immersion in H₂O₂ (TiO₂ cp). Surface characterization was previously carried out using different techniques.¹ Imaging and roughness analysis before and after FN adsorption used Atomic Force Microscopy (AFM) in Tapping® mode, in air, and in Magnetic Alternating Current (MAC) mode, in liquid (water). FN adsorption as a function of time was followed by X-ray photoelectron spectroscopy (XPS), by radiolabelling

of FN with ^{125}I , ^{125}I -FN, and by ellipsometry. Exchangeability studies were performed using FN and HSA.

AFM roughness analysis revealed that, before FN adsorption, both TiO_2 surfaces exhibited a lower root-mean-squared (R_q) and maximum peak with the depth of the maximum valley (R_{max}) roughness in air than in water, due to TiO_2 hydration. After protein adsorption, the same behaviour was observed for the TiO_2 sp substrate, while R_q and R_{max} roughness values in air and in water were similar in the case of the TiO_2 cp substrate, for the higher FN concentration used. Surface roughness was always significantly higher on the TiO_2 cp surfaces. AFM led to directly visualize adsorbed FN on both surfaces tested, indicating that after 10 min of FN incubation the TiO_2 sp surface was partially covered by FN. The adsorbed protein seems to form globular aggregates or ellipsoids, and FN aggregates coalesce forming clusters as the time of adsorption and the concentration increases.

Radiolabelling of FN revealed that a rapid adsorption occurs on both surfaces and the amount adsorbed increased with time, reaching a maximum after 60 min. of incubation. Time dependence is also observed for the evolution of the %N determined by XPS and by the increase of the thickness by ellipsometry. TiO_2 cp adsorbs more FN than the TiO_2 sp surfaces, after 60 min. of adsorption, as shown by the radiolabelling data. FN molecules are also more strongly attached to the former surface as indicated by the exchangeability studies.

The overall results suggest that FN spontaneously adsorbs as self-assembly at TiO_2 surfaces as a function of time. The aggregate structure is an intermediate feature shared by some protein fibrillar assemblies at interfaces, which is believed to promote cell adhesion, and cytoskeleton organization.^{2,3}

INTRODUCTION

The surface properties of the material, including composition and morphology, play a key role in the onset of the adaptative response of the body to the foreign material implantation. One of the events is the coating of the surface with protein film.⁴ Protein orientation will be dependent on the characteristics of the binding surface, that may modify their biological activity in relation to subsequent cell attachment.^{5,6}

Titanium, biocompatible metal commonly used in medical implants, is known to form a stable oxide layer with thicknesses between 0.5 and 7 nm.⁷⁻¹⁰ The interaction of cells with this layer is mediated by the extracellular matrix proteins. A number of evidences indicated that fibronectin, one of the earliest cell-binding proteins produced by osteoblasts, plays an important role in the interactions of implants with their surrounding matrix.^{11,12} Fibronectin is a member of the family of high molecular weight glycoproteins found in both plasma and cell surface, under compact form, and at the extracellular matrix (ECM) under extended form. In vivo, the compact-to-extended transition is partly triggered by cell applied mechanical forces through cell membrane receptors, the integrins.¹²⁻¹⁵ FN extension may also be triggered by adsorption onto hydrophilic surfaces.^{15,16} Fibronectin is responsible for key functions such as provision of a structural framework for cell attachment, migration, and differentiation, cell-cell and cell-substrate adhesion through integrin receptors.¹⁷⁻²⁰

Data on the characteristics of the adsorbed protein layer have been obtained using different technical approaches, namely X-ray photoelectron spectroscopy (XPS), Fourier transformed infrared (FTIR), ELISA, circular dichroism spectroscopy, ellipsometry, and protein radiolabelling.²¹⁻³⁴ These methods provide information on the coverage, thickness, conformation and identification of adsorbed proteins. In the last few years atomic force microscopy (AFM) has provided reliable data on imaging the protein in aqueous environments i.e., in physiological conditions. A number of studies using AFM imaging have focused on fibrinogen and IgG on different substrates such as mica and oxidized silicon^{15,35,36} as well as on titanium.^{37,38} However, very few have addressed on FN adsorbed on Ti and other surfaces,^{4,15,39} and so far none in liquid, using the magnetic AC mode (MAC). The MAC mode was specifically designed for imaging soft samples in fluid environments at very high resolution, overcoming the resolution limitations and difficulty to set up of the TappingTM mode in liquid. In MAC mode a magnetically driven oscillating probe with an oscillation amplitude significantly smaller than that of fluid TappingTM mode is used.⁴⁰⁻⁴²

This work was aiming at characterizing FN adsorption on two TiO₂ with different roughness using AFM in air and in liquid. This is the first report on the use of MAC mode to address the adsorption of FN on Ti. The FN adsorption as a function of time was followed by X-ray photoelectron microscopy (XPS), radiolabelling of FN (¹²⁵I-FN), and by ellipsometry.

MATERIALS AND METHODS

TiO₂ substrates

Two types of TiO₂ surfaces were used: commercially pure titanium with a titanium oxide layer formed in an H₂O₂ solution and TiO₂ sputtered on Si. The preparation of the two types of titanium oxides has already been described in a previous work.¹ Briefly, commercially pure titanium sheets, 1 mm thick, were used. TiO₂ cp substrates were obtained roughening the surface by a chemical polishing, cp, with an etching acid solution (2:3 parts by volume, 40 % of HF + 60 % of HNO₃ (v/v)), for 30 s. The substrates were pre-incubated in H₂O₂ 10 mM for 48 h to obtain a regular titanium oxide film.

TiO₂ sputtered substrates were prepared using an automated, load locked ion beam deposition system (Nordiko N3000), described elsewhere.⁴³ The TiO₂ films (50 nm) were deposited on Si wafers (polished/etched, crystal orientation <100>, from AUREL GmbH) by ion beam sputtering from a commercially pure Ti target, in the presence of a mixed Ar-O₂ beam coming from the assist gun (15.02% O₂ in Ar). The deposition rate was 0.038 nm/s. More details of the deposition have been reported in a previous study.¹

The wafers were diced into pieces (5X5 μm²) using a DISCO DAD321 automated saw. Before dicing, all wafers were coated with 1.5 μm of photoresist (JSR Electronics), to protect the film surface.

Before use, TiO₂ sputtered films were cleaned twice in acetone and immersed in a “piranha” solution ((7:3 parts by volume, 98 % H₂SO₄ + 30 % H₂O₂ (v/v)) for 90 s (caution: *this solution reacts violently with many organic materials and should be handled with great care*). Substrates were washed in ethanol, water (distilled and deionized) and again in ethanol for 2 min in an ultrasonic bath.

Protein Solutions

Human plasma fibronectin 0.1 % solution, FN, (Sigma, ref. F-0895) solutions were prepared by dilution in phosphate buffered saline, PBS (Sigma, pH=7.4, ref. P-3813) at concentrations of 20.0 µg/mL, for the XPS, radiolabelling and ellipsometry studies. For Atomic Force Microscopy (AFM) analysis FN concentrations of 50.0 and 75.0 µg/mL were used. Desorption was also evaluated using FN and HSA solutions of 400 µg/mL.

Atomic Force Microscopy, AFM

The Atomic Force Microscopy (AFM) measurements were taken with a PicoPlus Scanning Probe Microscope interfaced with a Picoscan Controller 2500 (both from Molecular Imaging, USA). Each sample was imaged with a 100x100 µm² piezoscanner. Imaging and roughness analysis were performed before and after FN adsorption in Tapping® mode, in air, and in Magnetic Alternating Current (MAC) mode, in liquid (water), at room temperature. A silicon cantilever (type NCH from Molecular Imaging, USA), was used with a spring constant 25-50 N/m, in Tapping® mode for the substrates without protein. For the titanium oxide substrates with protein adsorbed, a silicon cantilever (type FM from Molecular Imaging, USA), was used with a spring constant 1.2-3.5 N/m, in Tapping® mode. A silicon cantilever (type IV MAClever from Molecular Imaging, USA), was used with a spring constant of 0.10 N/m, in MAC mode. Three samples of each type were analysed at three randomly chosen locations.

A drop of FN solution of 50 or 75 µg/mL in PBS was added during 10 and 30 min. Any salts and nonadsorbed FN molecules were washed off with distilled water, immediately before AFM imaging. Lower concentrations of FN and less time of incubation have been tried, but the FN quantity at the surface was insufficient for detection.

The rinsed surface was dried with a flow of nitrogen and immediately imaged or used directly without drying and imaged in water, depending on the analysis being performed in air or in liquid, respectively.

The surface roughness before and after FN adsorption was obtained from scanned areas of 1000x1000 nm² using the SPIP software. To characterize the roughness, the root mean square roughness (R_q) was obtained as well as the maximum peak with the depth of the maximum valley (R_{max}).

X-ray photoelectron spectroscopy, XPS

XPS measurements were carried out on a VG Scientific ESCALAB 200A (UK) spectrometer using $MgK\alpha$ (1253.6 eV) as a radiation source. The photoelectrons were analysed at take off angles of 0°.

Survey XPS spectra were acquired from 0 to 1100 eV before and after hydrogen peroxide treatment, as well as after protein adsorption. High-resolution spectra for Ti 2p, O1s, C1s, N1s and S1s were also obtained. Elemental atomic percentages of Ti, O, C, N and S were determined after deconvolution of high-resolution spectra, using a XPS peak-fitting program (XPS PEAK Version 4.1). To take into account shifts caused by charging of the sample surface, all spectra were adjusted taking the C1s peak at 285.0 eV as reference for the adventitious carbon contamination.

The elemental atomic percentages of Ti, O, C, N and S were determined for the adsorption times of 5, 30, and 60 min for FN concentrations of 20 µg/mL.

Protein quantification by radioactivity

Quantification of FN adsorbed on the TiO₂ surface was performed using ¹²⁵I labelled FN. Human plasma fibronectin (Sigma) was labelled with ¹²⁵I using the Iodogen method.^{45,46} ¹²⁵I-FN was passed through a Sephadex G-25 M (PD-10 column Amersham Pharmacia Biotech) to remove unbound ¹²⁵I. The yield of iodination was always higher than 98 %, as determined by precipitating the ¹²⁵I-labelled FN with 20% trichloroacetic acid (TCA method).

The contribution of free ¹²⁵I to the total radioactivity found on the metal surfaces was estimated using unlabelled FN solutions at a concentration of 1.0 mg/mL, and an amount of free ¹²⁵I ion equivalent to that present as ¹²⁵I ion in the labelled protein solutions.

In all studies in which radiolabelling methods are used it must be kept in mind that the behaviour of the labelled protein may or may not reflect the behaviour of unlabelled protein.³⁴ To assess if preferential adsorption of ¹²⁵I labelled FN occurred on TiO₂ surfaces, a series of control experiments were performed varying the ratio of labelled to unlabelled FN (10 - 50 %). The final concentration of the FN solutions (1.0 mg/mL) was confirmed by the bicinchoninic acid (BCA) assay using BCA assay kit from Pierce and bovine serum albumin (BSA) as standard.⁴⁷

Protein solutions for adsorption experiments were prepared adding ¹²⁵I-FN to unlabelled FN solution in order to obtain a final activity of ~ 10⁸ cpm/mg.

FN adsorption tests were performed placing the Ti substrates in a 24-well tissue plate (Sarstedt). A drop of 10 µl ¹²⁵I-FN solution was added onto each surface. In order to avoid evaporation, PBS was added to the wells of the periphery. Adsorption tests were carried out at 25 °C over a period of time. After this period the drop was absorbed from the surface with absorbent paper and the surface was washed three times with PBS. The samples were transferred to radioimmunoassay tubes and surface activity was measured using an automatic gamma counter, model 1470 Wizard™, from Wallac (USA). All experiments were done in triplicate. The counts from each sample were averaged, and the surface concentration was calculated by the equation:

$$FN (\mu g/m^2) = \frac{counts(cpm) * |FN|_{solution} (\mu g / mL)}{A_{solution} (cpm / mL) * SA(m^2)} \quad (1)$$

where the counts measure the radioactivity of the samples, $|FN|_{solution}$ is the FN concentration solution, $A_{solution}$ is the specific activity of the FN solution and SA is the surface area expressed as the area of the surface taking the z height into account.

FN adsorption on titanium oxide substrates was investigated for concentration of 20 µg/mL during 10, 30, and 60 min. Displacement tests were carried out by immersing the labelled Ti

surface over 24 h in an unlabelled FN or HSA pure solution. The substrates were washed three times with PBS and the residual activity counted.

Statistical analysis of the experimental results was performed using the Kurskall-Wallis, non-parametric tests.

Ellipsometry

TiO₂ cp samples could not be evaluated by ellipsometry due a combined effect: high surface roughness and to nonreflecting surfaces causing nonspecular scattering of the incident light.

The samples of Si/TiO₂ were monitored *in situ* (in PBS) as a function of time, with an Imaging Null Ellipsometer, model EP3, from Nanofilm Surface Analysis (Germany). The ellipsometer was operated in a polarizer-compensator-sample-analyzer (PCSA) mode at a wavelength of 532 nm and at an angle of incidence of 60°. The measurements were made in four zones in continuous mode, to correct any instrument misalignment, due to optical component imperfections. The refractive index (n) and the extinction coefficient (k) of Si/TiO₂, were determined using the ellipsometric angles delta and psi when the system reached equilibrium. After the optical analysis of the bare substrate surface, the FN solution of 20 µg/mL was introduced at a constant rate of 30 mL/h. Adsorption of FN was followed by the change of the ellipsometric angles delta (Δ) and psi (ψ) with time. These values were used to determine an equivalent thickness (d_{protein}) assuming a refractive index n_{protein} = 1.46 for the protein layer. The adsorbed amount of protein (µg/m²) was calculated by means of the de Feijter's formula:

$$FN (\mu\text{g}/\text{m}^2) = d_{\text{protein}} \frac{n_{\text{protein}} - n_{\text{buffer}}}{dn/dc} \quad (2)$$

where d_{protein} is the average (protein) adlayer thickness probed by the laser beam and dn/dc is the change in refractive index with protein concentration in solution (dn/dc=0.181 mL/g).^{48,49} The refractive indices used were 1.33 and 1.46 for the buffer and adsorbed FN, respectively. The latter is a typical value for hydrated serum proteins.²² Experiments were done in triplicate.

RESULTS

AFM

Titanium oxide surfaces were analysed using Tapping® mode AFM in air and MAC mode AFM, in water. Figure 1a shows MAC mode AFM topographic contour image of 50x50 μm^2 and sectional analyses of the TiO₂ cp substrate before FN adsorption. The correspondent phase image and sectional analyses is shown in Figure 1b.

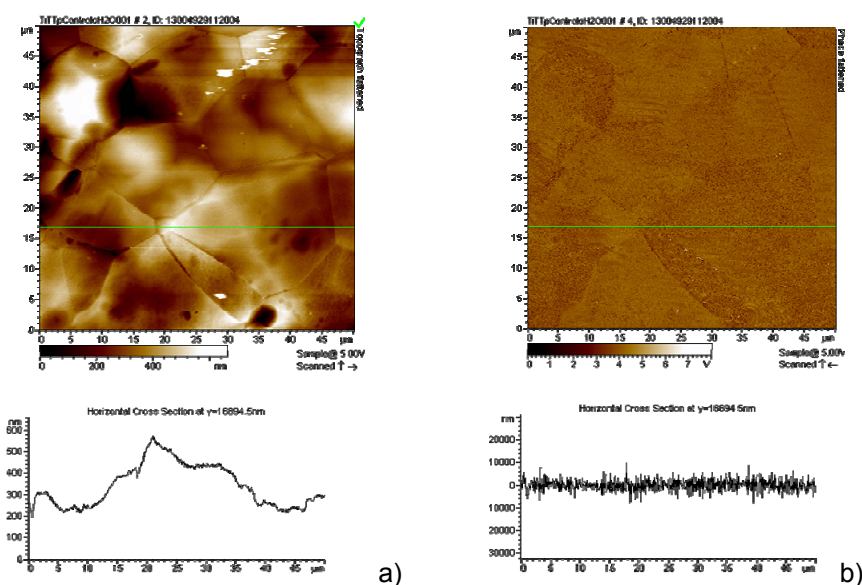


Figure 1 – AFM images and cross sections of a clean TiO₂ cp substrate surface, a) topography and b) phase image. The image was performed using MAC mode in water.

The sample was generally rough on the nanometer scale, as it is shown at the depth profile of Figure 1a. In this figure hexagonal grain and grain boundary of Ti cp are clearly visible. This morphology is typical after acid etching treatment since Ti surfaces that are treated chemically often develop specific surface topographies due to local differences in passivation/dissolution

rate. The surface topography formed is highly dependent on the microstructure with Ti cp developing a grain orientation dependent surface morphology. The phase imaging signal of AFM is a potential solution for imaging rough samples that present differences in material properties of the surface,^{35,37} namely viscoelastic properties, surface energy, etc. As it is shown in Figure 1b the surface is homogeneously covered.

Using AFM, adsorbed FN on both surfaces tested was visualized, in air and in water. Figure 2a-d shows topographic AFM images recorded in air and in water of TiO₂ cp surfaces, before and after protein adsorption (lateral scan size: 2000x2000 nm²). Figure 2e-h shows the correspondent phase images. In the case of TiO₂ cp samples without protein adsorption, the height and phase images (Figure 2a, c, e, and g, respectively) show topographic features, such as globules, characteristic of titanium oxide. These features revealed different morphologies in air and in water that may result from combined effects. The interaction between the tip and the sample changes in liquid. An enhanced resolution is obtained in the MAC mode in water, with lack of adhesion. Also, TiO₂ is expected to swell in water becoming gel-like with a layer of hydration and so reducing perturbation.

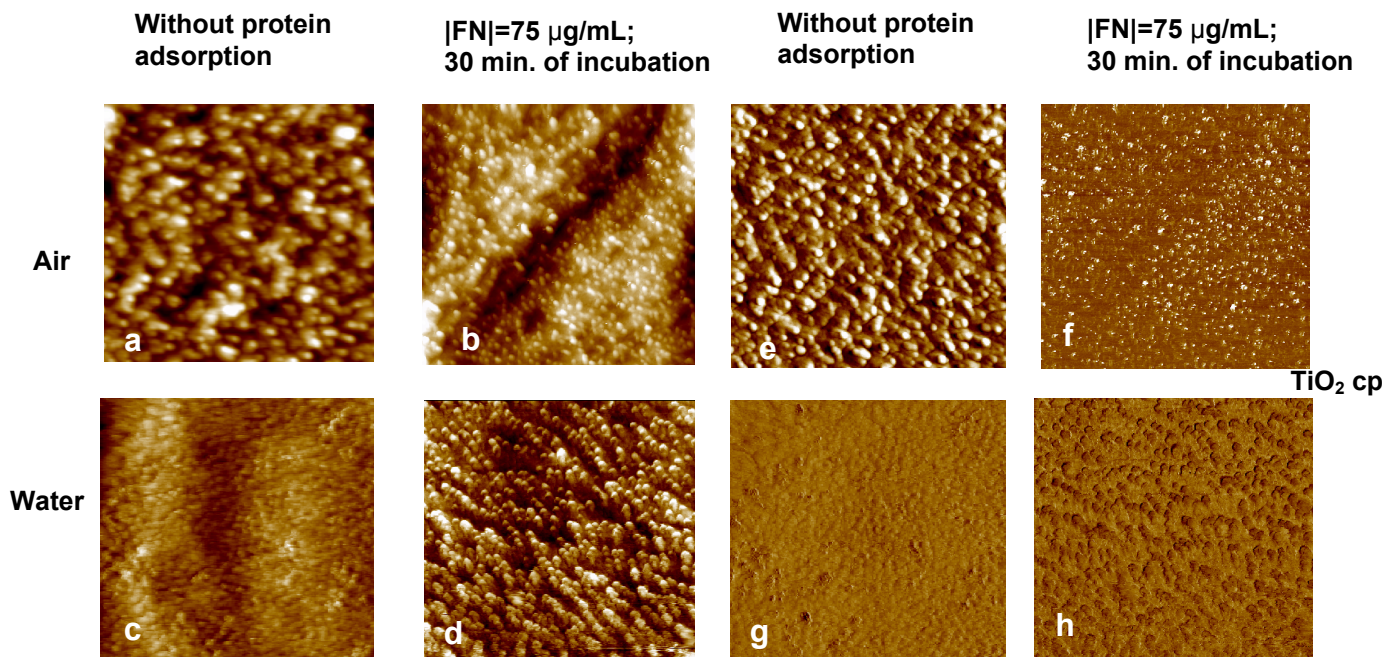


Figure 2 – AFM height and phase images of TiO₂ cp surfaces, before and after protein adsorption recorded in air and in water (lateral scan size: 2000x2000 nm²). The height (a- d) and phase images (e- h) respectively, show that contrast from the surface is high enough to observe protein.

The AFM height images (Figure 2b and d) obtained after FN adsorption on TiO₂ cp were different in morphology to the corresponding images of the surface without adsorbed FN (Figure 2a and c). Protein can be visualized due to the high contrast of the phase images (Figure 2e-h). Analysis over 1000x1000 nm² areas showed that, before FN adsorption, TiO₂ cp surfaces exhibited a lower root-mean-squared (R_q) and maximum peak with the depth of the maximum valley (R_{max}) roughness in air than in water (Figure 3).

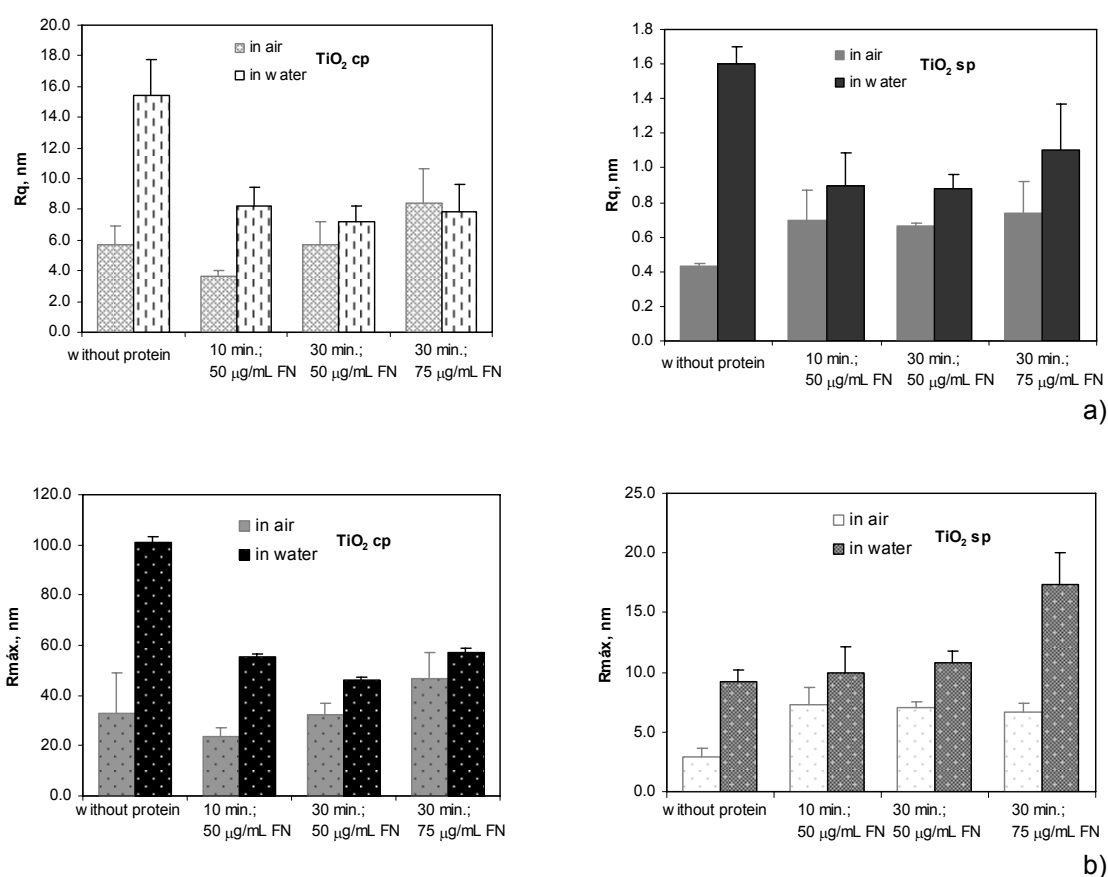


Figure 3 - Surface roughness of TiO₂ cp and TiO₂ sp determined in air and water, before and after protein adsorption (scan area: 1000x1000 nm²), a) R_q and b) R_{max} .

This is probably due to the effect reported before of the formation of a gel-like titanium oxide with a layer of hydration, instead of a dehydrated oxide. A similar trend in air and in water, was

found after 10 min of FN adsorption ($[FN]=50\text{ }\mu\text{g/mL}$). For this adsorption condition it is observed a decrease in the R_q and R_{max} roughness values, suggesting adsorption of FN molecules at the surface valleys. As the time of adsorption and concentration increases the R_q and R_{max} roughness values became similar in air and in water. Regarding the acquisition on air, the R_q and R_{max} increases with time and concentration. AFM performed on water presented R_q and R_{max} lower values after FN adsorption. Surface roughness was always significantly higher on the TiO_2 cp surfaces.

The AFM height image (Figure 4a) revealed that after 10 min of incubation with $50\text{ }\mu\text{g/mL}$ a modest amount of FN can be observed at the TiO_2 sp surface. The sectional analyses of height of Figure 4a and correspondent phase image (Figure 4b) illustrates a TiO_2 sp surface with two types of stiffness suggesting that FN aggregates are growing on top. Most aggregates appear similar in size and form.

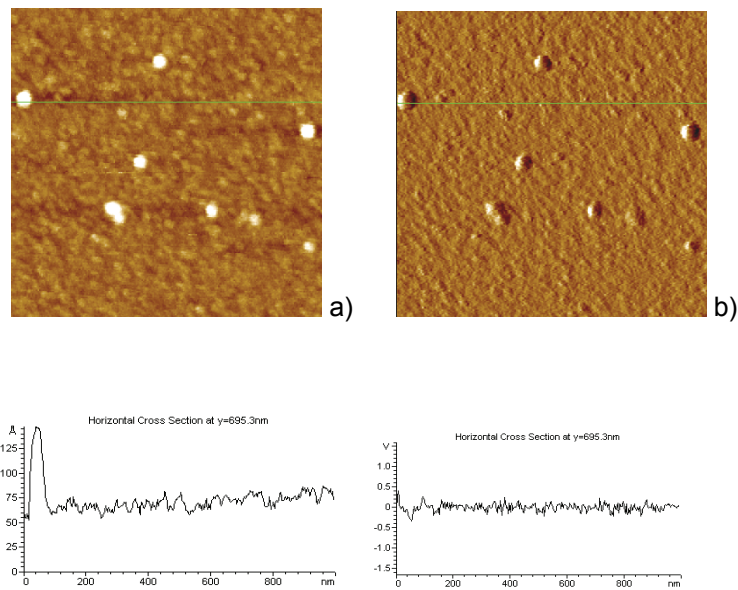


Figure 4 - AFM height (a) and phase (b) images of FN adsorbed on a TiO_2 sp surface after 10 min of incubation with $50\text{ }\mu\text{g/mL}$ of FN and correspondent cross sectional profiles [$1000\times 1000\text{ nm}^2$]. The images were performed using MAC mode in water.

AFM height and phase images obtained after FN adsorption on TiO₂ sp were different in morphology and contrast to the corresponding images of the surface without adsorbed FN. As topography images show direct evidence of individual globules and FN aggregates disperse on the surface (Figure 5a, b, c and d), the phase images are not shown. As for the TiO₂ cp surface, a different topography is observed for the TiO₂ sp surface without FN adsorption, when the image is acquired in air and in water. After 30 min. of incubation of 75 µg/mL of FN, the protein initially adsorbs randomly and then coalesce forming clusters (Figure 5).

A smoother surface (values of R_q in Figure 3) is obtained when the TiO₂ sp surface is observed on air than on water, before FN adsorption. For all the adsorption conditions, R_q and R_{max} values increased after FN adsorption, attaining a constant value, when TiO₂ sp surfaces were observed on air. When the AFM operated on water the R_q and R_{max} values decreased, relatively to those found prior to adsorption.

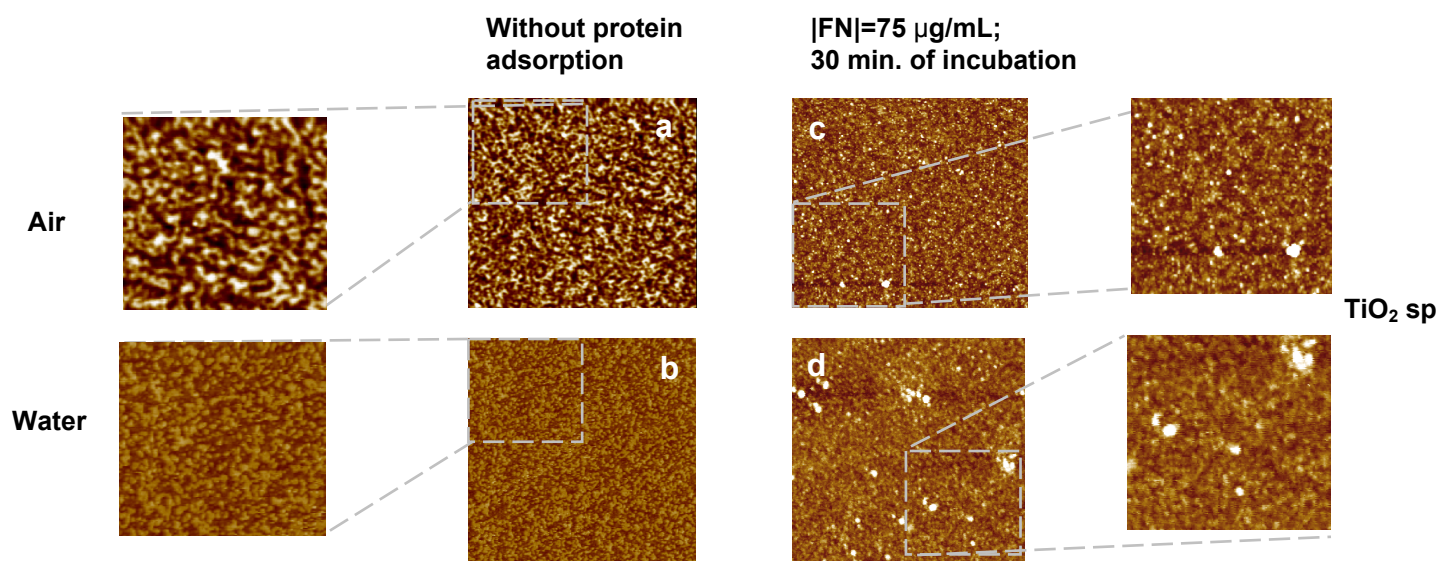


Figure 5 – Topographic AFM images recorded in air (a and c) and in water (b and d) of TiO₂ sp surfaces, before and after 30 min. with 75 µg/mL of FN adsorption (lateral scan size: 2000x2000 nm² and 1000x1000 nm² for the higher magnification images).

Cross sectional analysis of FN globules exhibit an average width of 55 ± 9 nm and height of 4.6 ± 1.6 nm taken on 10 different entities (Table 1), after 10 min. of adsorption with 50 µg/mL of FN. The height of the protein layer increases with the time of incubation maintaining constant

the diameter, indicating that the incoming molecules seem to adsorb on the top of the pre-existing layer.

Table 1 – Particle dimensions of FN adsorbed on TiO₂ sp, under different adsorption conditions.

	50 µg/mL of FN, 10 min. of incubation	50 µg/mL of FN, 30 min. of incubation	75 µg/mL of FN, 10 min. of incubation
Particle width, nm	55 ± 9	55 ± 13	83 ± 17
Particle height, nm	4.6 ± 1.6	7.1 ± 3.2	8.4 ± 5.3

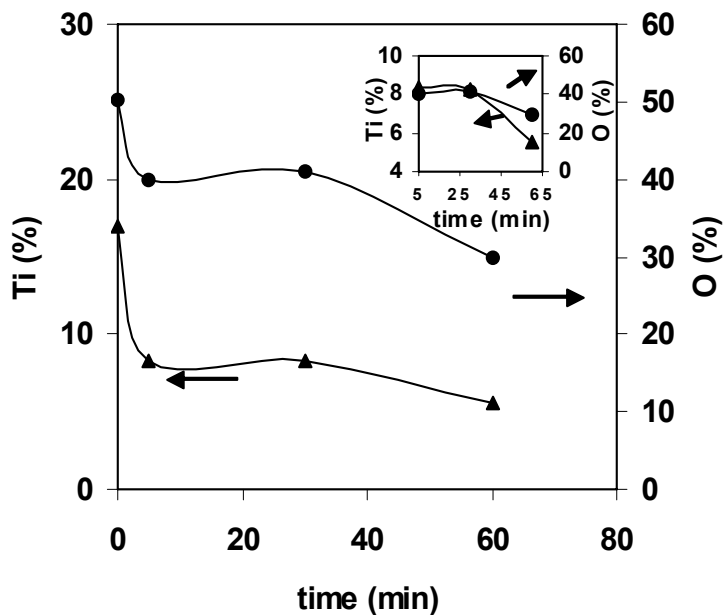
For the higher concentration, Figure 5c and Figure 5d, clearly shows that FN coalesces to form globular clusters. Cross sectional analysis of these globular-like proteins shows a mean width of 83 ± 17 nm and a mean height of 8.4 ± 5.3 nm (Table 1), giving evidence that the aggregates coalesce. These results also suggest that FN adsorbs on TiO₂ surface exhibiting an ellipsoid shape taking into account to the dimensions of aggregates.

XPS

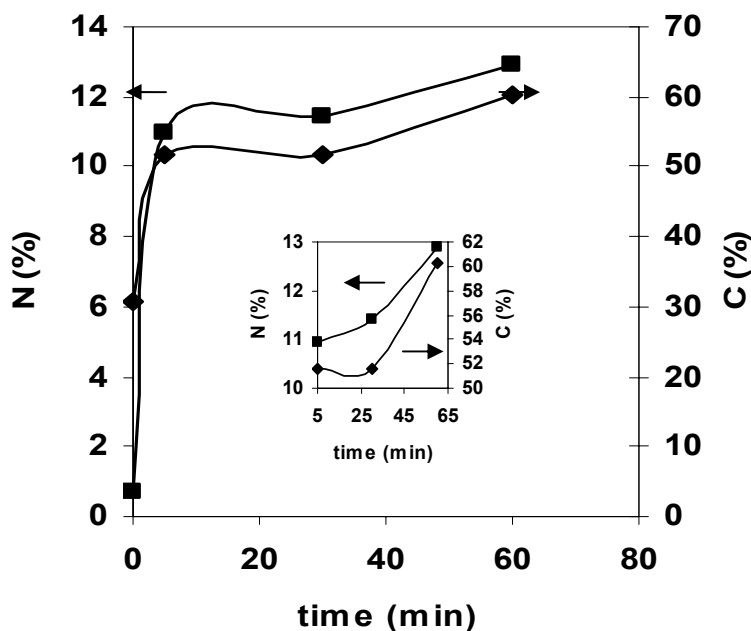
By XPS, the relative atomic composition (atom %) of titanium oxide surfaces, after FN adsorption was calculated. Previously the surface composition of Ti samples without protein was reported.¹ Survey spectra (data not shown) suggested the absence of contaminants since no other elements than the expected ones (Ti, O, C, N, and S) were found. High resolution spectra were acquired for Ti2p, O1s, C1s, N1s, and S2p, on TiO₂ sp surfaces after FN adsorption at a concentration of 20 µg/mL, for 5, 30, and 60 min. of incubation.

Figure 6a shows that after 5 min of FN incubation the Ti and O peak intensity decreases, reaching a plateau upon 30 min of incubation, with a subsequent decrease afterward. As is shown in Figure 6b, after 5 min of FN incubation the peak intensities of N and C increases. A markedly increase is observed after 30 min of incubation. For the time interval tested, a maximum value of N% and C% is attained for 60 min. of adsorption.

A single peak is observed at the high resolution S2p spectra, resulting from the sulphur atoms coming from the disulfide groups (–S–S–) in protein molecules at 164.1 eV.²⁶



a)



b)

Figure 6 – Evolution of XPS element envelope with increased time of FN immersion ($[FN]=20$ $\mu\text{g/mL}$): a) Ti% and O%; b) N% and C%.

Protein adsorption and reversible binding of fibronectin by radiolabelling

Fibronectin adsorption on titanium oxides was evaluated by radiolabelling. Residual free ^{125}I adsorption to the TiO_2 surface was found to be ca. 0.2 %, after exposure of this surface to mixtures with different concentrations of unlabelled FN plus labelled free iodide. This amount is negligible considering that in our solution we only have ca. 2% of free iodide ion, instead of 100 %, and that only 0.2 % of free iodide can adsorb to our substrate. This result justifies the use of a buffer without non-labelled iodide ion, PBS.

Preferential adsorption of the ^{125}I -labelled FN relatively to the unlabelled FN did not occur on TiO_2 cp surface as determined by varying the ratio of labelled to unlabelled protein in a series of control experiments.⁵⁰

The kinetics of FN adsorption was analysed on TiO_2 surfaces. Fig. 7 shows that a rapid adsorption takes place on both surfaces attaining a maximum for 60 min. of incubation. For this time period, TiO_2 cp surfaces adsorb higher amounts of FN, than TiO_2 sp surfaces.

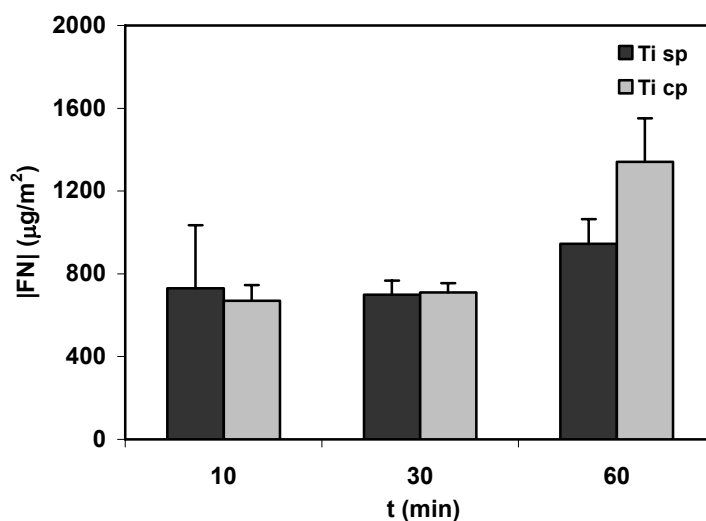


Figure 7 – Kinetics of FN adsorption (20 µg/mL) on TiO_2 surfaces.

Exchangeability of bound FN molecules was evaluated by the exchange of the pre-adsorbed ^{125}I -FN for unlabeled FN or HSA. Figure 8 shows the percentage of ^{125}I -FN eluted from the two

titanium oxides after soaking in FN and HSA, after 60 min. of adsorption a) and in function of time of adsorption b). The elution time was 24 h. It can be seen that TiO_2 sp shows a higher reversibility of FN molecules by other FN molecules as well as by HSA (Figure 8a). For $[\text{FN}] = 20 \mu\text{g/mL}$ in solution and for both substrates, as the time of adsorption increases the FN exchangeability does not change significantly (Figure 8b). These results indicate that the elution seems to depend more on the nature of the surface than to the time of incubation or the type of protein in the elution solution.

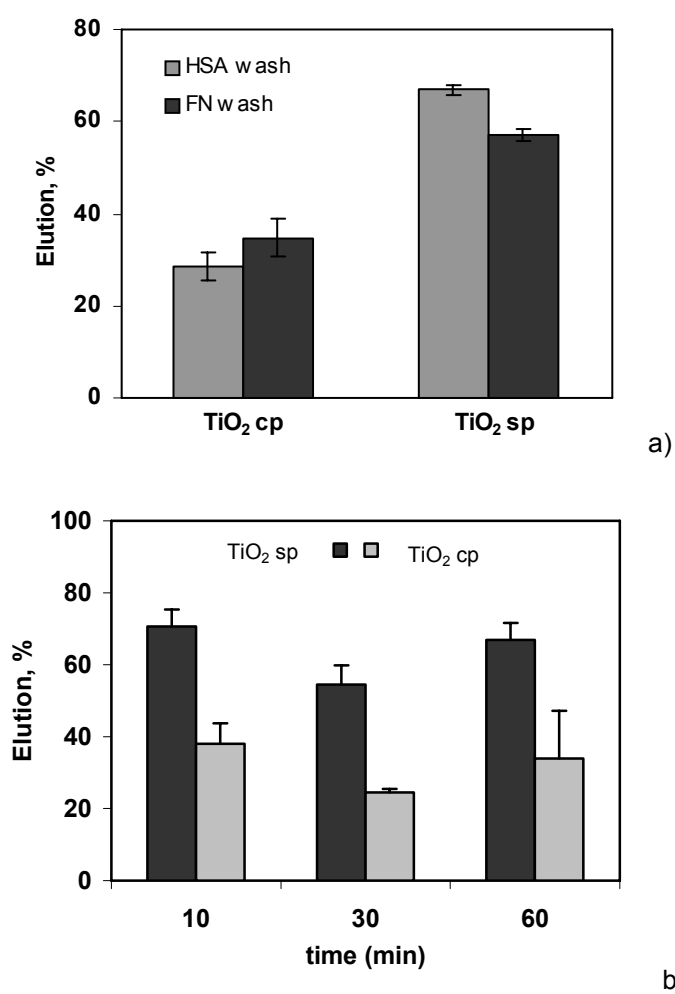


Figure 8 – Elution of FN molecules after adsorption of FN at $20 \mu\text{g/mL}$ (60 min.) from TiO_2 sp and from TiO_2 cp. a) Elution of FN by other FN molecules or by HSA, and b) Elution of FN by other FN in solution as a function of time of adsorption. The elution time was 24 h.

Ellipsometry

The variation of the d_{FN} and $|\text{FN}|_{\text{surface}}$ with time, for $|\text{FN}|=20 \mu\text{g/mL}$ was followed by ellipsometry (data not shown). The adsorbed amount increased abruptly attaining a limiting adsorbed amount of $1.5 \pm 0.40 \text{ mg/m}^2$. The average thickness of the FN layer is $2.1 \pm 0.5 \text{ nm}$.

DISCUSSION

In recent years a number of evidences clearly indicated that atomic force microscopy (AFM) can directly provide image of proteins in physiological conditions.^{4,15,35-38,51} Although, it is quite difficult to achieve good resolution while imaging in liquids, using Tapping™ mode, the use of MAC mode AFM is more suitable for imaging soft samples in fluid environments at very high resolution. It is then possible to operate at very small amplitudes of vibration since the tip is drive directly, rather than using excitations of the fluid.⁴¹ In contrast, the dried titanium oxide is more rigid, and thus, can be imaged by Tapping™ mode AFM with higher resolution. However, the fact that drying is associated with possible artifacts has to be taking into account. A great advantage of operating in fluid is the relative lack of adhesion between the tip and the surface. AFM roughness analysis over $1000 \times 1000 \text{ nm}^2$ areas showed that, before FN adsorption, both TiO_2 surfaces exhibited a lower root-mean-squared (R_q) and maximum peak with the depth of the maximum valley (R_{max}) roughness in air than in water. This can be associated to the effect reported before of a gel-like titanium oxide formation with a layer of hydration in water. After protein adsorption R_q roughness values are identical in air and in water for TiO_2 cp suggesting adsorption of FN molecules at the surface valleys. For TiO_2 sp substrates due to the smoothness of the original surface, proteins stand out of the surface, and R_q values in air, increased after FN adsorption.

Until now AFM imaging has been limited to adsorbed protein on relatively smooth model surfaces such as mica, graphite, or self assembled monolayers (SAMs)^{15,35,36,52-55} on which the proteins can be observed as they can be easily distinguished from the background. The inherent roughness of biomaterials surfaces may unable to visualize adsorbed proteins from

topographic images. However Holland N. B. et al.³⁵ showed that the phase imaging signal of tapping mode AFM provides information on differences in material properties of the surface, and was used to distinguish individual protein molecules from the underlying polymer surface. On the low-density polyethylene surface, characteristic topographical features are of the same magnitude as the protein molecules, so that the protein cannot be distinguished from the surface using topographic images. However, phase images were used to successfully locate and characterize the distribution of the protein.

Topographic and/or phase AFM images enabled the visualization of FN globules onto the two TiO₂ surfaces. The different viscoelastic properties of protein and the surface provide different energy dissipation in the AFM oscillating tip and consequently a different contrast for both.^{35,37} The visualization of FN globules could be achieved and the results suggest that the adsorbed FN molecules do not form a continuous layer at the TiO₂ surface (Figure 2, 4 and 5), revealing an heterogeneous covering. The diameter dimensions of FN globules measured in this work (55 ± 9 to 83 ± 17 nm) are in agreement with the rotary shadowing of FN on mica (a titanium charge equivalent surface), showing also lengths of 60 ± 7 nm to a range of 120-160 nm.⁵⁶ On the contrary, MacDonald, D. E. et al.⁴ found by AFM on air, that globular FN has an average length of 16.5 ± 1.0 nm on Ti cp. This variability of FN dimensions was accounted to the extreme flexibility of the protein.^{4,16} FN has been reported to have globular and filamentous forms depending on substrate and solution conditions.⁵⁷ Factors like temperature, pH, and ionic strength of the local environment also play a role in the final shape of the FN molecule. Electrostatic interactions between regions of opposite net charge within or between chains could hold the molecule in a relatively compact conformation which becomes more open in high salt contents or when pH is shifted more than a few units from neutrality, therefore exposing previously hidden domain such as cell-binding sites.¹⁵ Moreover the size of FN adsorbed depends on the deposition method (spraying or droplet deposition), on the tip shape and capillary forces, and on the surface properties.^{4,36} In plasma or solution under normal physiological conditions as well as at cell surface, FN has been reported to assume a globular or compact form.^{2,14,58,59} The extracellular matrix (ECM) incorporates these FN were they are found in the flexible and extended form, which self-associate into a dense fibrillar network. In vivo, the compact-to-extended transition is partly triggered by cell applied mechanical forces

through cell membrane receptors, the integrins,⁶⁰ or upon the adsorption onto hydrophilic surfaces.^{15,16} Bergkvist et al.¹⁵ observed that on silica surfaces and on mica, which are hydrophilic, most of the molecules had an elongated structure with partial intramolecular chain interactions, compared to molecules adsorbed on hydrophobic, methylated surfaces, where a compact structure predominated. Based on the above findings, and to the fact that TiO_2 cp are more hydrophilic than the TiO_2 sp,¹ it is expected to have more elongated structures on the rough surface.

Kinetics of FN adsorption by radiolabelling of FN has shown that a rapid adsorption takes place (ca. 75 %), reaching a maximum after 60 min of immersion (Figure 7). As shown in Figure 6b, the same conclusion may be drawn from the evolution of the N % determined by XPS. Thus XPS can be successfully used as an alternative technique to study adsorption kinetics of FN, correlating well with ^{125}I -labelled FN data (Figure 9).

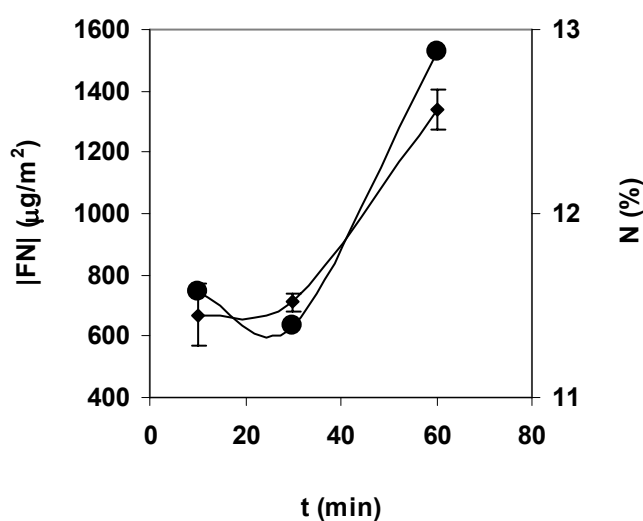


Figure 9 – Comparison between adsorption of FN as a function of time onto a TiO_2 cp surface by ^{125}I -FN radiolabelling and XPS measurements of N%.

The ellipsometric data also shows that at 60 min. of adsorption a maximum amount of FN is attained at the surface. Regarding the amount of FN adsorbed at TiO_2 surface, the results from the ellipsometry ($1500 \pm 400 \mu\text{g}/\text{m}^2$) are comparable to those obtained by the radiolabelling technique ($946 \pm 118 \mu\text{g}/\text{m}^2$), and below, to the theoretical value for a side-on FN monolayer.

The results obtained by AFM also suggest that the adsorbed FN molecules do not form a continuous layer at the TiO₂ surface (Figure 2, 4 and 5), but rather an heterogeneous covering. By AFM cross sectional analysis it was observed that the height of FN globules increases with the time of incubation, while maintaining the diameter constant (Table 1), suggesting that the incoming molecules may adsorb on top of pre-existing protein forming multilayers. For closely packed monolayers concentrations formed by side-on and end-on adsorption are 1750 $\mu\text{g}/\text{m}^2$ and 41000 $\mu\text{g}/\text{m}^2$,⁶¹ respectively, demonstrating that the maximum adsorbed amount determined by radiolabelled protein is insufficient for end-on monolayer formation.

For $[\text{FN}]=20 \mu\text{g}/\text{mL}$ in solution and for both substrates, as the time of adsorption increases the FN exchangeability does not changes significantly (Figure 8b). Nevertheless the desorption results also show that the elution seems to be more dependent to the nature of the surface than to the time of adsorption or to the protein in elution solution. Although some studies suggest that tight protein binding may be conducive to cell attachment and growth,⁶² other studies provide evidence that tight protein binding may not be desirable⁶³ since tight binding of a protein to a surface may indicate that conformation or orientation changes (especially denaturation) have taken place during or after protein adsorption. Even though the type and the amount of protein adsorbed on a substrate will influence cell spreading and growth, the biological activity of the adsorbed proteins may have a more significant impact on cellular interactions.⁶⁴

Tamada, Y. et al.⁶⁵ referred that the minimum amount of FN required for fibroblast cell adhesion was 500 $\mu\text{g}/\text{m}^2$. After elution and for the initial time of adsorption of 60 min of FN (20 $\mu\text{g}/\text{mL}$) TiO₂ cp retains more than the referred FN amount for cell adhesion. TiO₂ sp surface retains the minimum amount necessary for cell adhesion. The ability of TiO₂ type substrate to support MC3T3-E1 adhesion, seems to correlate well with the substrate affinity to adsorb and retain FN. MC3T3-E1 osteoblasts cell adhesion tests where performed on TiO₂ sp and TiO₂ cp after 4 and 24 h of incubation after FN preadsorption.⁶⁶ The data shows that after 4h of incubation statistically higher amounts of adherent cells were obtained for TiO₂ cp surfaces, when compared to the TiO₂ sp surfaces. For the longer period of incubation the differences between the substrates are much lower, but still statically significant. The two TiO₂ differ in surface roughness, being the TiO₂ cp ~14 times rougher than TiO₂ sp. It is unlike that the differences observed for cell growth between the two substrates can be attributed solely to morphological

differences. Similarly, cell growth differs significantly for a given TiO₂ substrate preadsorbed with FN or HSA, yet the surface roughness is equivalent. Although surface wettability might have contributed to higher cell affinity toward TiO₂ cp, it has to be taken into account that these surfaces retained the amount sufficient for cell adhesion and also flexible and elongated FN molecules, exposing cell-binding sites are expected.

CONCLUSIONS

The present work analysis the dynamics of fibronectin, FN, adsorption on two different stable titanium oxides, with varied surface roughness, assessed by radiolabelling FN, XPS, ellipsometry and AFM.

FN spontaneously adsorbs at TiO₂ surfaces as aggregates, visualized by Tapping® mode AFM in air and MAC mode AFM in water, at TiO₂ sp surfaces. Phase imaging AFM detects successfully adsorbed FN on the rougher TiO₂ surfaces, TiO₂ cp, and the roughness parameters, R_q and R_{max} , also confirm the presence of FN on both substrates.

Kinetics of FN adsorption by radiolabelling of FN, XPS and ellipsometry have shown that a rapid adsorption occurs for both substrates and the adsorbed amount increased as the time of incubation also increases, reaching a maximum after 60 min of immersion. TiO₂ cp surfaces retained the amount sufficient for cell adhesion.

Regarding the amount of FN adsorbed at TiO₂ surface, the data obtained by ellipsometry correlate well with those obtained by the radiolabelling technique, showing also that are bellow to the theoretical value for a side-on FN monolayer. AFM data agree with these results as showed by the fact that FN does not adsorbed as a monolayer but instead as islands or clusters.

TiO₂ cp adsorbs more FN than the TiO₂ sp surfaces, after 60 min. of incubation, as shown by the kinetics radiolabelling data. FN molecules are also more strongly attached to the former surface as indicated by the exchangeability studies.

The overall results suggest that FN spontaneously adsorbs as self-assembly at TiO₂ surfaces as a function of time. The aggregate structure is an intermediate feature shared by some protein fibrillar assemblies at interfaces, which is believed to promote cell adhesion, and cytoskeleton organization.^{2,3,67}

Acknowledgements:

The authors kindly acknowledge Mr R. Azevedo for the ellipsometry measurements.

REFERENCES

1. Sousa SR, Moradas-Ferreira P, Melo LV, Saramago B, Barbosa MA. Human Serum Albumin Adsorption on TiO₂ from Single Protein Solutions and from Plasma. *Langmuir* 2004;20(22):9745-9754.
2. Pellenc D, Berry H, Gallet O. Adsorption-induced fibronectin aggregation and fibrillogenesis. *Journal of Colloid and Interface Science* 2006;298(1):132-144.
3. Maheshwari G, Brown G, Lauffenburger DA, Wells A, Griffith LG. Cell adhesion and motility depend on nanoscale RGD clustering *Journal of cell science* 2000;113(10):1677-1686.
4. MacDonald DE, Markovic B, Allen M, Somasundaran P, Boskey AL. Surface analysis of human plasma fibronectin adsorbed to commercially pure titanium materials. *Journal of Biomedical Materials Research* 1998;41(1):120-130.
5. Hederos M, Konradsson P, Liedberg B. Synthesis and self-assembly of galactose-terminated alkanethiols and their ability to resist proteins. *Langmuir* 2005;21(7):2971-2980.
6. Liu LY, Chen SF, Giachelli CM, Ratner BD, Jiang SY. Controlling osteopontin orientation on surfaces to modulate endothelial cell adhesion. *Journal of Biomedical Materials Research Part A* 2005;74A(1):23-31.
7. Kasemo B, Lausmaa J. *Surface Science Aspects on Inorganic Biomaterials*. Crc Critical Reviews in Biocompatibility 1986;2(4):335-380.
8. Lausmaa J. Surface spectroscopic characterization of titanium implant materials. *Journal of Electron Spectroscopy and Related Phenomena* 1996;81(3):343-361.
9. McCafferty E, Wightman JP. An X-ray photoelectron spectroscopy sputter profile study of the native air-formed oxide film on titanium. *Applied Surface Science* 1999;143(1-4):92-100.

10. Sittig CE, Wieland M, Xiao S-J, Textor M. Characterization and Properties of Titanium Implant Surfaces. 1999; Münsterlingen.
11. Horbett TA. The role of adsorbed proteins in animal cell adhesion. *Colloids and Surfaces B: Biointerfaces* 1994;2(1-3):225-240.
12. Hynes RO. Integrins - Versatility, Modulation, and Signaling in Cell-Adhesion. *Cell* 1992;69(1):11-25.
13. Suzuki S, Oldberg A, Hayman EG, Pierschbacher MD, Ruoslahti E. Complete Amino-Acid Sequence of Human Vitronectin Deduced from Cdna - Similarity of Cell Attachment Sites in Vitronectin and Fibronectin. *Embo Journal* 1985;4(10):2519-2524.
14. Baneyx G, Baugh L, Vogel V. Fibronectin extension and unfolding within cell matrix fibrils controlled by cytoskeletal tension. *Proceedings of the National Academy of Sciences of the United States of America* 2002;99(8):5139-5143.
15. Bergkvist M, Carlsson J, Oscarsson S. Surface-dependent conformations of human plasma fibronectin adsorbed to silica, mica, and hydrophobic surfaces, studied with use of Atomic Force Microscopy. *Journal of Biomedical Materials Research Part A* 2003;64A(2):349-356.
16. Baugh L, Vogel V. Structural changes of fibronectin adsorbed to model surfaces probed by fluorescence resonance energy transfer. *Journal of Biomedical Materials Research Part A* 2004;69A(3):525-534.
17. Capadona JR, Collard DM, Garcia AJ. Fibronectin adsorption and cell adhesion to mixed monolayers of tri(ethylene glycol)- and methyl-terminated alkanethiols. *Langmuir* 2003;19(5):1847-1852.
18. Cutler SM, Garcia AJ. Engineering cell adhesive surfaces that direct integrin $\alpha 5 \beta 1$ binding using a recombinant fragment of fibronectin. *Biomaterials* 2003;24(10):1759-1770.
19. Keselowsky BG, Collard DM, Garcia AJ. Surface chemistry modulates fibronectin conformation and directs integrin binding and specificity to control cell adhesion. *Journal of Biomedical Materials Research Part A* 2003;66A(2):247-259.
20. Magnusson MK, Mosher DF. Fibronectin - Structure, assembly, and cardiovascular implications. *Arteriosclerosis Thrombosis and Vascular Biology* 1998;18(9):1363-1370.

21. Francois P, Vaudaux P, Taborelli M, Tonetti M, Lew DP, Descouts P. Influence of surface treatments developed for oral implants on the physical and biological properties of titanium .2. Adsorption isotherms and biological activity of immobilized fibronectin. *Clinical Oral Implants Research* 1997;8(3):217-225.
22. Hook F, Vörös J, Rodahl M, Kurrat R, Boni P, Ramsden JJ, Textor M, Spencer ND, Tengvall P, Gold J and others. A comparative study of protein adsorption on titanium oxide surfaces using in situ ellipsometry, optical waveguide lightmode spectroscopy, and quartz crystal microbalance/dissipation. *Colloids and Surfaces B-Biointerfaces* 2002;24(2):155-170.
23. Ivarsson B, Lundstrom I. Physical Characterization of Protein Adsorption on Metal and Metaloxide Surfaces. *CRC Critical Reviews in Biocompatibility* 1986;2(1):1-96.
24. Lhoest JB, Detrait E, van den Bosch de Aguilar P, Bertrand P. Fibronectin adsorption, conformation, and orientation on polystyrene substrates studied by radiolabeling, XPS, and ToF SIMS. *Journal of Biomedical Materials Research* 1998;41(1):95-103.
25. Liedberg B, Ivarsson B, Lundstrom I. Fourier-Transform Infrared Reflection Absorption-Spectroscopy (Ft-Iras) of Fibrinogen Adsorbed on Metal and Metal-Oxide Surfaces. *Journal of Biochemical and Biophysical Methods* 1984;9(3):233-243.
26. Lindberg B, Maripuu R, Siegbahn K, Larsson R, Golander CG, Eriksson JC. ESCA Studies of Heparinized and Related Surfaces .1. Model Surfaces on Steel Substrates. *Journal of Colloid and Interface Science* 1983;95(2):308-321.
27. Malmsten M. Ellipsometry studies of the effects of surface hydrophobicity on protein adsorption. *Colloids and Surfaces B: Biointerfaces* 1995;3(5):297-308.
28. Ratner BD, Horbett TA, Shuttleworth D, Thomas HR. Analysis of the Organization of Protein Films on Solid-Surfaces by ESCA. *Journal of Colloid and Interface Science* 1981;83(2):630-642.
29. Serro AP, Bastos M, Pessoa JC, Saramago B. Bovine serum albumin conformational changes upon adsorption on titania and on hydroxyapatite and their relation with biomineralization. *Journal of Biomedical Materials Research Part A* 2004;70A(3):420-427.

30. Sundgren JE, Bodo P, Ivarsson B, Lundstrom I. Adsorption of Fibrinogen on Titanium and Gold Surfaces Studied by ESCA and Ellipsometry. *Journal of Colloid and Interface Science* 1986;113(2):530-543.
31. Sundgren JE, Bodo P, Lundstrom I. Auger-Electron Spectroscopic Studies of the Interface between Human-Tissue and Implants of Titanium and Stainless-Steel. *Journal of Colloid and Interface Science* 1986;110(1):9-20.
32. Williams DF, Askill IN, Smith R. Protein Adsorption and Desorption Phenomena on Clean Metal-Surfaces. *Journal of Biomedical Materials Research* 1985;19(3):313-320.
33. Williams RL, Williams DF. The Effect of Albumin on the Wettability of Pure Metal and Metal-Oxide Surfaces. *Journal of Colloid and Interface Science* 1988;126(2):596-603.
34. Yu XJ, Brash JL. Measurement of protein adsorption to solid surfaces in relation to blood compatibility using radiolabelling methods. In: Dawids S, editor. *Test procedures for the blood compatibility of biomaterials*. Dordrecht, The Netherlands: Kluwer Academic Publishers; 1993. p 287-330.
35. Holland NB, Marchant RE. Individual plasma proteins detected on rough biomaterials by phase imaging AFM. *Journal of Biomedical Materials Research* 2000;51(3):307-315.
36. Jandt KD. Atomic force microscopy of biomaterials surfaces and interfaces. *Surface Science* 2001;491(3):303-332.
37. Cacciafesta P, Hallam KR, Watkinson AC, Allen GC, Miles MJ, Jandt KD. Visualisation of human plasma fibrinogen adsorbed on titanium implant surfaces with different roughness. *Surface Science* 2001;491(3):405-420.
38. Cacciafesta P, Humphris ADL, Jandt KD, Miles MJ. Human plasma fibrinogen adsorption on ultraflat titanium oxide surfaces studied with atomic force microscopy. *Langmuir* 2000;16(21):8167-8175.
39. Zenhausern F, Adrian M, Descouts P. Solution Structure and Direct Imaging of Fibronectin Adsorption to Solid-Surfaces by Scanning Force Microscopy and Cryoelectron Microscopy. *Journal of Electron Microscopy* 1993;42(6):378-388.
40. Han WH, Lindsay SM, Jing TW. A magnetically driven oscillating probe microscope for operation in liquids. *Applied Physics Letters* 1996;69(26):4111-4113.

41. Jalili N, Laxminarayana K. A review of atomic force microscopy imaging systems: application to molecular metrology and biological sciences. *Mechatronics* 2004;14(8):907-945.
42. Moreno-Herrero F, Colchero J, Gomez-Herrero J, Baro AM. Atomic force microscopy contact, tapping, and jumping modes for imaging biological samples in liquids. *Physical Review E* 2004;69(3).
43. Gehanno V, Freitas PP, Ferreira J, Almeida B, Sousa JB, Kling A, Soares JC, Silva MF. Ion beam deposition of Mn-Ir spin valves. *IEEE Trans Magn* 1999;35:4361-4367.
44. Armstrong NR, Quinn RK. Auger and X-Ray Photoelectron Spectroscopic and Electrochemical Characterization of Titanium Thin-Film Electrodes. *Surface Science* 1977;67(2):451-468.
45. Iodine-125. Little Chalfont, Buckinghamshire, U.K.: Amersham International; 1993. 64 p.
46. Lima J, Sousa SR, Ferreira A, Barbosa MA. Interactions between calcium, phosphate, and albumin on the surface of titanium. *J Biomed Mater Res* 2001;55(1):45-53.
47. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. Measurement of Protein Using Bicinchoninic Acid. *Analytical Biochemistry* 1985;150(1):76-85.
48. Malmsten M. Ellipsometry studies of fibronectin adsorption. *Colloids and Surfaces B: Biointerfaces* 1995;3(6):371-381.
49. Lai C-S, Wolff CE, Novello D, Griffone L, Cuniberti C, Molina F, Rocco M. Solution Structure of Human Plasma Fibronectin under Different Solvent Conditions: Fluorescence Energy Transfer, Circular Dichroism and Light-scattering Studies. *Journal of Molecular Biology* 1993;230(2):625-640.
50. Sousa SR, Moradas-Ferreira P, Barbosa M. TiO₂ type influences fibronectin adsorption. *Journal of Materials Science: Materials in Medicine* 2005;16(12):1173-1178.
51. Zhang HL, Bremmell K, Kumar S, Smart RSC. Vitronectin adsorption on surfaces visualized by tapping mode atomic force microscopy. *Journal of Biomedical Materials Research Part A* 2004;68A(3):479-488.

52. Advincula M, Fan XW, Lemons J, Advincula R. Surface modification of surface sol-gel derived titanium oxide films by self-assembled monolayers (SAMS) and non-specific protein adsorption studies. *Colloids and Surfaces B-Biointerfaces* 2005;42(1):29-43.
53. Li LY, Chen SF, Zheng J, Ratner BD, Jiang SY. Protein adsorption on oligo(ethylene glycol)-terminated alkanethiolate self-assembled monolayers: The molecular basis for nonfouling behavior. *Journal of Physical Chemistry B* 2005;109(7):2934-2941.
54. Lin Y, Wang J, Wan LJ, Fang XH. Study of fibrinogen adsorption on self-assembled monolayers on Au(111) by atomic force microscopy. *Ultramicroscopy* 2005;105(1-4):129-136.
55. Wang MS, Palmer LB, Schwartz JD, Razatos A. Evaluating protein attraction and adhesion to biomaterials with the atomic force microscope. *Langmuir* 2004;20(18):7753-7759.
56. Engel J, Odermatt E, Engel A, Madri J, Furthmayr H, Rohde H, Timpl R. Shapes, domain organizations and flexibility of laminin and fibronectin, two multifunctional proteins of the extracellular matrix. *J Mol Biol.* 1981;150(1):97-120.
57. Ugarova TP, Zamarron C, Veklich Y, Bowditch RD, Ginsberg MH, Weisel JW, Plow EF. Conformational Transitions in the Cell-Binding Domain of Fibronectin. *Biochemistry* 1995;34(13):4457-4466.
58. Rocco M, Aresu O, Zardi L. Conformational State of Circulating Human-Plasma Fibronectin. *Febs Letters* 1984;178(2):327-330.
59. Rocco M, Infusini E, Daga MG, Gogioso L, Cuniberti C. Models of Fibronectin. *Embo Journal* 1987;6(8):2343-2349.
60. Peters DM, Portz LM, Fullenwider J, Mosher DF. Co-assembly of plasma and cellular fibronectins into fibrils in human fibroblast cultures. *The Journal of Cell Biology* 1990;111(1):249-56.
61. Rouxhet L, Duhoux F, Borecky O, Legras R, Schneider YJ. Adsorption of albumin, collagen, and fibronectin on the surface of poly(hydroxybutyrate-hydroxyvalerate) (PHB/HV) and of poly(epsilon-caprolactone) (PCL) films modified by an alkaline hydrolysis and of poly(ethylene terephthalate) (PET) track-etched membranes. *Journal of Biomaterials Science-Polymer Edition* 1998;9(12):1279-1304.

62. Pettit DK, Horbett TA, Hoffman AS. Influence of the substrate binding characteristics of fibronectin on corneal epithelial cell outgrowth. *J Biomed Mater Res.* 1992;26(Oct 10):1259-1275.
63. Goncalves IC, Martins MCL, Barbosa MA, Ratner BD. Protein adsorption on 18-alkyl chains immobilized on hydroxyl-terminated self-assembled monolayers. *Biomaterials* 2005;26(18):3891-3899.
64. Scotchford CA, Gilmore CP, Cooper E, Leggett GJ, Downes S. Protein adsorption and human osteoblast-like cell attachment and growth on alkylthiol on gold self-assembled monolayers. *Journal of Biomedical Materials Research* 2002;59(1):84-99.
65. Tamada Y, Ikada Y. Effect of Preadsorbed Proteins on Cell-Adhesion to Polymer Surfaces. *Journal of Colloid and Interface Science* 1993;155(2):334-339.
66. Sousa SR, Lamghari M, Moradas-Ferreira P, Barbosa M. Osteoblasts adhesion and morphology on TiO₂ depends on the competitive preadsorption of HSA and FN. *J Biomed Mater Res* 2007:in press.
67. Meadows PY, Walker GC. Force microscopy studies of fibronectin adsorption and subsequent cellular adhesion to substrates with well-defined surface chemistries. *Langmuir* 2005;21(9):4096-4107.

Osteoblast adhesion and morphology on TiO₂ depends on the competitive preadsorption of albumin and fibronectin

S. R. Sousa^{1,2,3}, M. Lamghari¹, P. Sampaio⁴, P. Moradas-Ferreira^{5,6}, and M. A. Barbosa^{1,2}

¹ INEB, Instituto de Engenharia Biomédica, Laboratório de Biomateriais, R. do Campo Alegre, 823, 4150-180 Porto, Portugal

² Universidade do Porto, Faculdade de Engenharia, Departamento de Engenharia Metalúrgica e de Materiais, R. Dr. Roberto Frias, s/n, 4200 - 465 Porto, Portugal

³ ISEP, Instituto Superior de Engenharia do Porto, Departamento de Engenharia Química, R. Dr. Bernardino de Almeida, 431, 4200 - 072 Porto, Portugal

⁴ IBMC, Instituto de Biologia Molecular e Celular, Advance Light Microscopy Facility, R. do Campo Alegre, 823, 4150-180 Porto, Portugal

⁵ ICBAS, Instituto de Ciências Biomédicas Abel Salazar, Largo Prof. Abel Salazar, 2, 4099 - 003 Porto, Portugal

⁶ IBMC, Instituto de Biologia Molecular e Celular, Grupo de Microbiologia Celular Aplicada, R. do Campo Alegre, 823, 4150-180 Porto, Portugal

ABSTRACT

This study aims at assessing the influence of the competitive preadsorption of human serum albumin, HSA and human plasma fibronectin, FN, from binary solutions and 10 % plasma on

MC3T3-E1 osteoblast adhesion and morphology on two types of TiO₂ substrates. One was commercially pure titanium with a titanium oxide layer formed in an H₂O₂ solution and the other TiO₂ sputtered on Si.¹ The strategy applied in the present investigation was to compare osteoblast adhesion to surfaces preadsorbed with HSA, FN, HSA/FN=1, HSA/FN=200 and 10% plasma. The adsorption of proteins was evaluated measuring the amount and the effectiveness of binding with radiolabelled proteins, ¹²⁵I-FN and ¹²⁵I-HSA.

Our results indicated that MC3T3-E1 osteoblast adhesion correlates well with the amounts of FN and HSA adsorbed on TiO₂ surfaces. Also, we found that fewer osteoblasts adhered to both substrates preadsorbed with HSA, HSA/FN=200 and 10% plasma, after 4 and 24h, than to the surfaces preadsorbed with FN and HSA/FN=1. For the latter, FN was able to compensate the inhibitory effect of HSA on osteoblast adhesion. Therefore, the presence of lower amounts of co-adsorbed albumin may improve presentation of FN in a more integrin-recognized conformation, suggesting that some degree of molecular packing prevents loss of integrin-binding activity.

FN reversibility does not seem to be dependent on the HSA/FN adsorption mass ratio in solution, suggesting that FN competitively adsorbs to TiO₂ in a favorable conformation and does not suffers subsequent conformational changes allowing exchange with other FN molecules in solution.

Keywords: osteoblast adhesion, TiO₂, competitive protein adsorption, FN, HSA, protein radiolabelling.

INTRODUCTION

Cell adhesion to synthetic surfaces is involved in a variety of phenomena such as, coagulation, immune response, wound healing, and tissue integration of biomaterials. As cellular attachment, adhesion and spreading belong to the first phase of cell/material interactions, the success of this phase will influence proliferation and differentiation of cells on biomaterial surfaces.² In the case of materials for bone tissue regeneration, osteoblasts are expected to adhere and proliferate. Ultimately, the efficiency of orthopedic or dental implants is achieved if a solid

interface is established with complete attachment between the material's surface and the bone tissue with no fibrous tissue interface.

However protein adsorption from plasma occurs rapidly, rendering the direct recognition of surface functional groups by the cells virtually impossible, thus affecting cell growth.³ The composition of the adsorbed protein layer differs from the fluid phase composition and varies significantly with the type of substrate. It has been demonstrated to undergo conformational and orientation changes with time.⁴ Human serum albumin is known to eliminate non-specific protein adsorption and to prevent cell adhesion, while fibronectin, collagens and other subtle trace proteins (osteopontin, laminin, vitronectin) are adhesive proteins required for integrin-receptor-based cell adhesion and proliferation on surfaces.

Titanium and its alloys have been widely used for more than a decade in dental and orthopedic field as biomaterials. Titanium surface properties can thus affect the amount and type of protein bound as well as orientation, conformation and strength of proteins adsorbed. Additionally, for cells that secrete and assemble an extracellular matrix, surface properties, such as chemical composition, wettability, and topography, may affect the ability of the cell to deposit its extracellular matrix (ECM) by stabilizing the protein deposits or by affecting the orientation of cell-binding domains in the deposits.^{3,5-8} So far, few studies have addressed the effect of TiO₂ surface properties on cell behavior mediated by the protein layer adsorbed from complex solutions such as plasma or serum as well as from protein binary solutions.^{4,9-11}

Adsorbed fibronectin (FN) and human serum albumin (HSA) density and availability on titanium surfaces are key parameters to understanding and improving cellular efficiencies for the clinical use of this material. FN adsorption in competition with albumin is significant to cell recognition ability of ECM components and adhesion. Therefore, the substrate needs to provide sufficient amounts of serum-derived adsorbed adhesion factors to facilitate initial cell adhesion; this subsequently leads the cell to deposit its own ECM. To facilitate cellular ECM deposition adsorbed serum proteins are believed that have to be held loosely to allow for displacement by cell-derived FN maintaining a favorable presentation of binding sites to cells.^{3,12} Loss of FN's secondary structure has been correlated with elimination of integrin-mediated cell responses such as actin stress fiber formation in fibroblasts.^{12,13} Co-adsorbing FN with albumin at some FN-to-HSA ratios "rescues" these cell responses, suggesting that some degree of molecular packing prevents loss of integrin-binding activity.^{10,13,14}

The aim of this study is to investigate the effect of competitive preadsorption of HSA and FN on MC3T3-E1 osteoblasts adhesion and morphology on TiO₂ substrates. The adsorption of proteins was evaluated by measuring the amount and the effectiveness of binding with radiolabelled proteins, ¹²⁵I-FN and ¹²⁵I-HSA.

MATERIALS AND METHODS

TiO₂ substrates

Two types of TiO₂ surfaces were used for protein adsorption studies and osteoblast cell culture: (a) commercially pure titanium with a titanium oxide layer formed in an H₂O₂ solution and (b) TiO₂ sputtered on Si.¹ The preparation of the two types of titanium oxides has already been described in a previous work.¹ Briefly, commercially pure (CP) titanium sheets, 1 mm thick, were used. TiO₂ cp substrates were obtained roughening the surface by a chemical polishing, cp, with an etching acid solution (2:3 parts by volume, 40 % of HF + 60 % of HNO₃ (v/v)), for 30 s. The substrates were pre-incubated in H₂O₂ 10 mM for 48 h to obtain a regular titanium oxide film.

TiO₂ sputtered substrates were prepared using an automated, load locked ion beam deposition system (Nordiko N3000), described elsewhere.¹⁵ The TiO₂ films (50 nm) were deposited on Si wafers (polished/etched, crystal orientation <100>, from AUREL GmbH) by ion beam sputtering from a commercially pure Ti target, in the presence of a mixed Ar-O₂ beam coming from the assist gun (15.02% O₂ in Ar). The deposition rate was 0.038 nm/s. More details of the deposition have been reported in a previous study.¹

The wafers were diced into pieces (5X5 μm²) using a DISCO DAD321 automated saw. Before dicing, all wafers were coated with 1.5 μm of photoresist (JSR Electronics), to protect the film surface.

Before use, TiO₂ sputtered films were cleaned twice in acetone and immersed in a “piranha” solution (7 parts of 98 % H₂SO₄ and 3 parts of 30 % H₂O₂ (v/v)) for 90 s. Substrates were

washed in ethanol, water (distilled and deionized) and again in ethanol for 2 min in an ultrasonic bath.

Protein Solutions

Human serum albumin (Sigma, ref. A – 1653, 96-99 %) solutions were prepared in phosphate buffered saline, PBS (Sigma, ref. P - 3813), at concentrations of 0.20, 1.0 and 4.0 mg/mL. Human plasma fibronectin (0.1 % solution, Sigma, ref. F – 0895, ca. 100 %) solutions were prepared by dilution in PBS at concentrations of 0.020 and 0.20 mg/mL.

The different [HSA]/[FN] mass ratios used for competitive HSA and FN adsorption tests were 1, 10, 50 and 200. The higher ratio of HSA to FN used corresponds to the average ratio found in plasma. The other ratios, although they do not correspond to physiological values, were used in order to investigate the importance of varying concentrations in the pattern for adsorption. For cell culture tests the [HSA]/[FN] mass ratios used were 1 and 200.

Plasma was obtained from whole blood of healthy donors free of medication for at least 10 days, using the CPDA1 anticoagulant solution (Macopharma). Plasma aliquots were frozen to -20 °C and used up to 1 week. In experiments in which surfaces were exposed to 10 % plasma, plasma was diluted using PBS.

Protein quantification by radioactivity

Quantification of HSA and FN adsorbed on the TiO₂ surface was performed using ¹²⁵I labelled HSA or ¹²⁵I labelled FN. Human serum albumin and human plasma fibronectin were labelled with ¹²⁵I using the Iodogen method.¹⁶⁻¹⁸ ¹²⁵I-FN or ¹²⁵I-HSA was combined with nonlabeled protein (HSA or FN) or with solutions of different HSA/FN mass ratio as tracer to obtain a final activity of ~ 10⁸ cpm/mg.

Adsorption tests were performed by placing the Ti substrates in a 24-well tissue culture plate (Sarsted). A drop of 10 µL protein solution was added onto each surface. In order to avoid evaporation, PBS was added to the wells of the periphery. Adsorption tests were carried out at

25 °C during 60 min. After this period the drop was absorbed from the surface with absorbent paper and the surface was washed three times with PBS. The samples were transferred to radioimmunoassay tubes and surface activity was measured using a gamma counter. All experiments were performed in triplicate. The counts from each sample were averaged, and the surface concentration was calculated by the equation:

$$\text{Protein (mg/m}^2\text{)} = \frac{\text{counts(cpm)} * |\text{protein}|_{\text{solution}} \text{ (mg / mL)}}{A_{\text{solution}} \text{ (cpm / mL)} * SA(\text{m}^2)} \quad (1)$$

where the counts measure the radioactivity of the samples, $|\text{protein}|_{\text{solution}}$ is the concentration of HSA or FN solution, A_{solution} is the specific activity of the respective protein solution and SA is the surface area.

Displacement tests were carried out by immersing the labelled Ti surface over 24 h in an unlabelled HSA or FN pure solution as well as 10 % plasma solution. The substrates were washed three times with PBS and the residual activity counted.

Cell adhesion and Morphology

MC3T3-E1 osteoblast cell adhesion and morphology on TiO₂ sp and TiO₂ cp were analyzed by Neutral red ¹⁹ assay and by Confocal microscopy, after 4 and 24 h.

Sample preparation and sterilization

After TiO₂ sample preparation, as previously described, the samples were immersed in ethanol 70 % (v/v) overnight in a sterilized Petri dish and kept in a laminar flux chamber. Just before the adhesion test, samples were washed in sterile PBS five times.

Cell culture

MC3T3-E1 cells, established as an osteoblastic cell line from normal mouse calvaria were grown in alpha minimal medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Invitrogen), 0.5 % (v/v) gentamycine (Invitrogen), 1 % (v/v) fungizone (Invitrogen), 50 µg/mL Vitamin C (Sigma) and 10 mM βglycerophosphate (Sigma). Cells were cultured in 75 cm² plastic culture flasks, and incubated in a humidified incubator (37 °C and 5 % CO₂).

Cell seeding

Freshly confluent MC3T3-E1 osteoblast cells were incubated with trypsin solution (0.25% trypsin, 0.05% EDTA, 0.1% glucose in PBS) for 10 min, and then resuspended in cell culture medium. Before cell seeding a preadsorption step was performed on TiO₂ substrates and on tissue culture polystyrene (TCPS) according to the following conditions. All TiO₂ substrates were pre-adsorbed for 1 h with: (1) PBS, (2) FN at 20 µg/mL, (3) HSA at 4.0 mg/mL (4), HSA/FN mixtures in ratios 1, and 200, and (5) 10% plasma. TCPS was pre-adsorbed with PBS for 1 h. Cells were seeded on TiO₂ sp or TiO₂ cp substrates and on TCPS commercial 24-well cell culture plates (Sarstedt), used as control, at a cell seeding density of 12x10⁴/well and 4x10⁴/well, for Neutral red test and for Confocal microscopy, respectively. Cells were cultured for periods of 4 and 24 h. For each material and culture period, two samples without cells were incubated in the same way and used as blanks in Neutral red assay.

Neutral red

To assess the number of viable adherent cells on TiO₂ substrates, NR assay, was performed. A stock solution of 0.4% Neutral red dye (Sigma) in H₂O was prepared and stored at room temperature. The stock solution was diluted in supplemented culture medium just before addition to cells. At the end of the incubation time (4 or 24 h) at 37°C, the medium was removed and replaced with 200 µL/well of NR solution. After the reaction time, the medium was discarded and the

cultures washed twice with PBS 1x. The cells were lysed with 1 % acetic acid in 50 % ethanol solution for 10 min. to release the dye. The lysates were collected and the optical density was determined using the spectrophotometer equipped with a 540 nm optical filter. A minimum of 3 measurements was used to calculate the mean and standard deviation of the optical density (OD) for control and samples.

Confocal microscopy

The cell-seeded samples were fixed with 4% para-formaldehyde, PFA, for 10 min. After washing with PBS 1x, cells were permeabilized with 0.1% Triton X-100 for 10 min and then washed again three times with PBS. Cell cytoskeletal filamentous actin (F-actin) was visualized by treating the cells with 5 U/mL Alexa Fluor® 488 phalloidin (Molecular Probes) for 20 min in the dark. The cells then were washed 3 times with PBS and cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) dye. Samples were finally mounted in Vectashield® (Vector) solution and cells were examined using a Leica confocal scanning microscope (Leica Heidelberg GmbH).

Statistical analysis

Data were analyzed using the nonparametric Mann-Whitney U test. Two-tailed asymptotic significance levels were considered. Results were considered statistically significant when $p \leq 0.05$. Calculations were performed using SPSS® software for Windows (version 12.0).

RESULTS

MC3T3-E1 osteoblasts adhesion and morphology

The results from the MC3T3-E1 osteoblast cell adhesion tests are shown in Figure 1. A decrease in cell adhesion up to 54 % was observed for all samples compared to control (TCPS, data not shown).

Statistical differences were found for MC3T3-E1 cell adhesion to TiO₂ sp substrate after 4 h, from substrates pre-adsorbed with FN and substrates pre-adsorbed with the HSA, HSA/FN=1, HSA/FN=200 and 10% plasma. When FN is used to cell adhesion assays the levels of adherent cells are higher than when HSA is pre-adsorbed on the surfaces. No statistical difference was observed between substrates pre-adsorbed with FN and those without pre-adsorbed protein.

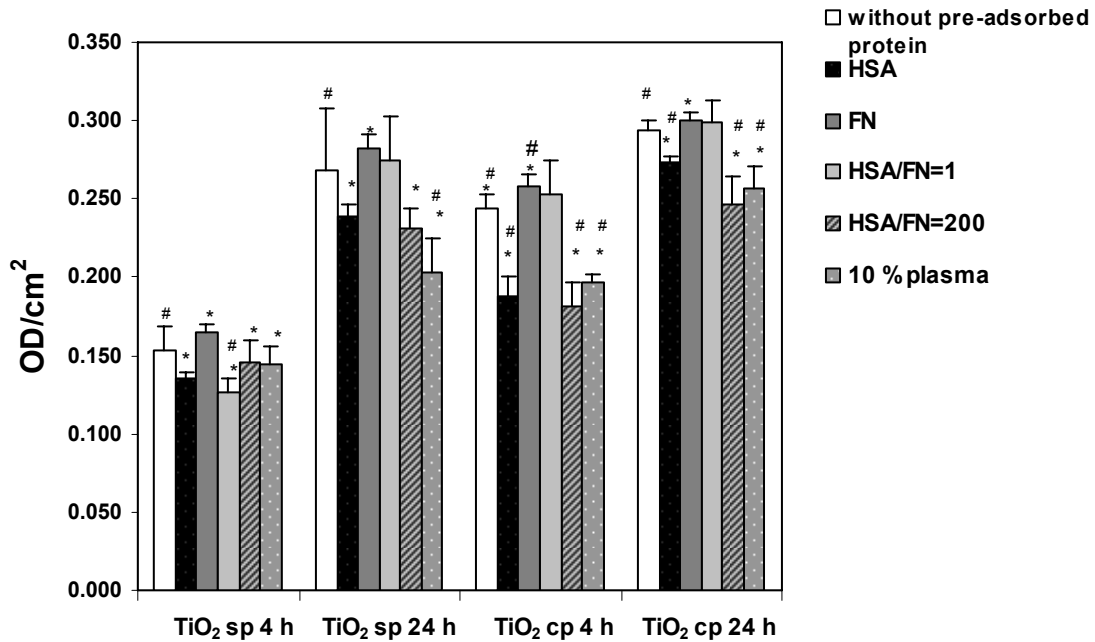


Figure 1 – Effect of protein preadsorption on MC3T3-E1 osteoblast cell adhesion on TiO₂ after 4 and 24 h of incubation, expressed as OD/cm². TCPS was used as a control (data not shown). Values reported are the average $\pm$ SD of four to six cultures. Asterisks (*) indicate a significant difference from a substrate to those pre-adsorbed with FN ($p \leq 0.05$). The hash symbol (#) indicates a significant difference between a substrate and those samples without pre-adsorbed protein ($p \leq 0.05$).

For all the other preadsorption conditions that used HSA, a significant decrease of viable adherent cells was observed, when compared to the substrates pre-adsorbed with FN. When HSA/FN is pre-adsorbed on TiO₂ sp substrates at ratio 1:1 (HSA/FN=1) the amount of adherent cells after 4 h of incubation is significantly lower compared to substrates without pre-adsorbed protein (Figure 1).

The number of adherent cells on TiO₂ sp substrates is dependent on the incubation time. As shown in Figure 1, the values are higher after 24 h than at 4 h of incubation, for all the preadsorption conditions. Enhanced cell adhesion was observed after 24 h of incubation on TiO₂ sp substrates pre-adsorbed with FN, when compared to identical substrates pre-adsorbed with the HSA, HSA/FN=200 and 10% plasma. On the contrary, the presence of competitive preadsorption of HSA/FN=1 did not significantly decreased cell adhesion when compared to substrates pre-adsorbed with FN. A similar observation was made for the 4h of incubation. When 10 % plasma is pre-adsorbed on TiO₂ sp the number of adherent cells after 24 h is significantly lower compared to substrates without pre-adsorbed proteins.

For the TiO₂ cp substrate the same pattern of cell attachment after 4 h of incubation was observed as with TiO₂ sp substrates, that is, the pre-adsorption with FN and HSA/FN=1 promote osteoblasts adhesion (Figure 1). It is also observed that there is a significantly decrease on the number of adherent cells for substrates pre-adsorbed with HSA, HSA/FN=1, HSA/FN=200 and 10% plasma when compared to the substrates without pre-adsorbed protein. As before, the presence of competitive preadsorption of HSA/FN=1 did not significantly decrease cell adhesion, when compared to the substrates pre-adsorbed with FN.

The inhibitory effect of HSA is more evident when TiO₂ cp substrates are used. The Ti surface, when treated with FN, also promotes the adherence of cells when compared to the substrates without pre-adsorbed protein.

As it was observed for TiO₂ sp for all the preadsorption conditions, the number of adherent cells on TiO₂ cp substrates is dependent on the incubation time, as the OD values are higher after 24 h than at 4 h of incubation. FN and HSA/FN=1, increases the osteoblasts attachment to TiO₂ cp when compared to the substrates pre-adsorbed with the HSA, HSA/FN=200 and 10% plasma, as was previously reported for the former substrate. A lower level of cell attachment was observed for the preadsorption of HSA, HSA/FN=200 and 10% plasma when compared to the substrates without pre-adsorbed protein.

After 4h of incubation, statistically higher amounts of adherent cells were obtained for TiO₂ cp surfaces, when compared to the TiO₂ sp surfaces (Figure 1). Statistically higher numbers of adherent cells on TiO₂ cp were observed after preadsorption with FN, HSA and 10% plasma, for 24 h of incubation (Figure 1). Similar numbers of cells were found for the two TiO₂ substrates for the preadsorption conditions: no protein, HSA/FN=1 and HSA/FN=200.

Osteoblast morphology and cytoskeletal organization on TiO₂ substrates upon protein preadsorption is given by confocal microscopy (Figure 2 and 3) and are in accordance with the results shown by NR assay. Since similar morphologies were obtained independently of substrate type, the above Figures referred to TiO₂ sp or TiO₂ cp.

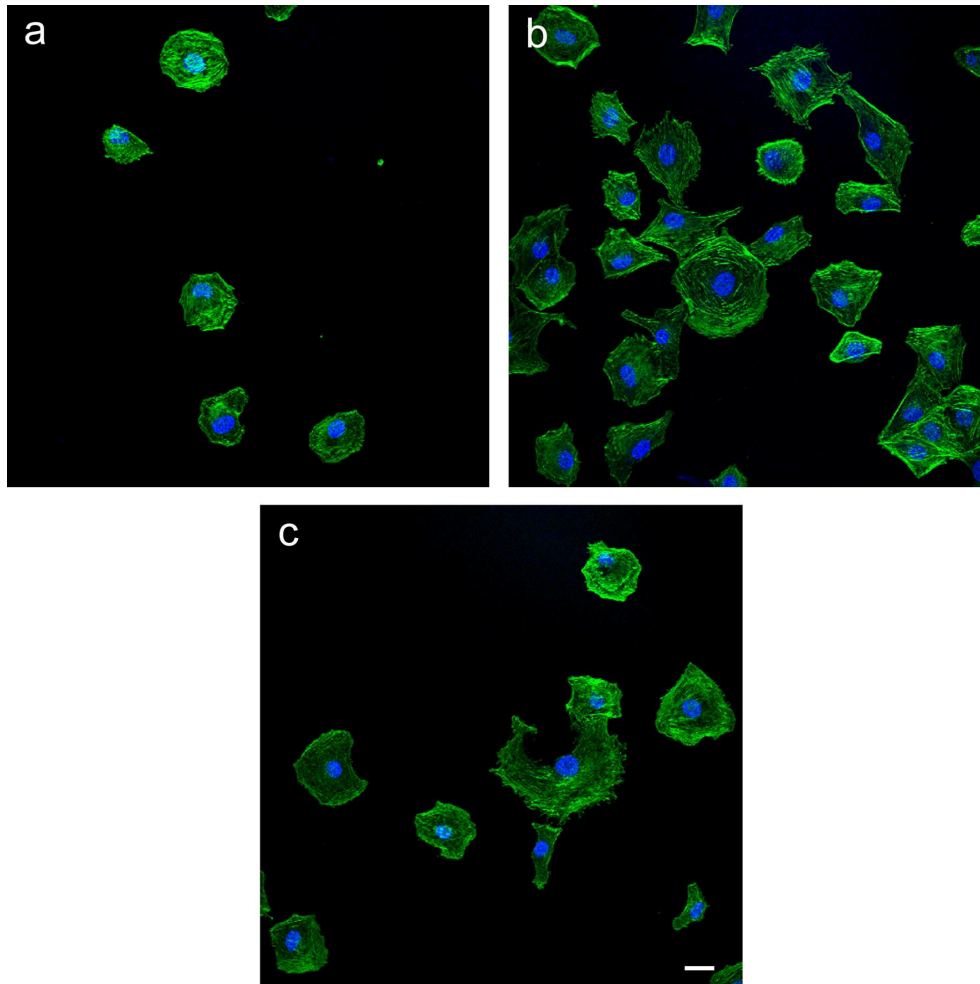


Figure 2 – MC3T3-E1 cell morphology and cytoskeletal organization on TiO₂ sp imaged with confocal microscopy after 4 h, with the preadsorption conditions: a) HSA, b) FN, c) HSA/FN=200. F-actin is indicated in green while cell nuclei were counterstained in blue with DAPI dye. Scale bar = 20 μ m.

The rounded morphology displayed by the osteoblasts, due to the early stages of cell culture, is identical for both substrates and for all the preadsorption conditions tested, after 4 h of cell incubation. However enhanced cell adhesion upon preadsorption of FN was observed (Figure

2b). Also, changes in cell morphology with the appearance of some polygonal cells among spherical ones is observed, when compared to substrates preadsorbed with albumin and with HSA/FN=200 (Figure 2a and 2c). After 24 h of cell incubation, more cells were observed to be attached and spread (Figure 3a, b and c). At this time point there are mainly two types of cell morphology on cp and sp substrates, cells elongated with fusiform fibroblastic appearance and cells with polygonal shape with round nuclei, with numerous filopodia and cell-to-cell contacts. Figure 3b provides evidence for the adhesion-inducing effect of FN, showing a higher number of cells and higher number of cell-to-cell contact, compared to the inhibitory effect of HSA and 10 % plasma, on TiO₂ cp (Figure 3a and c).

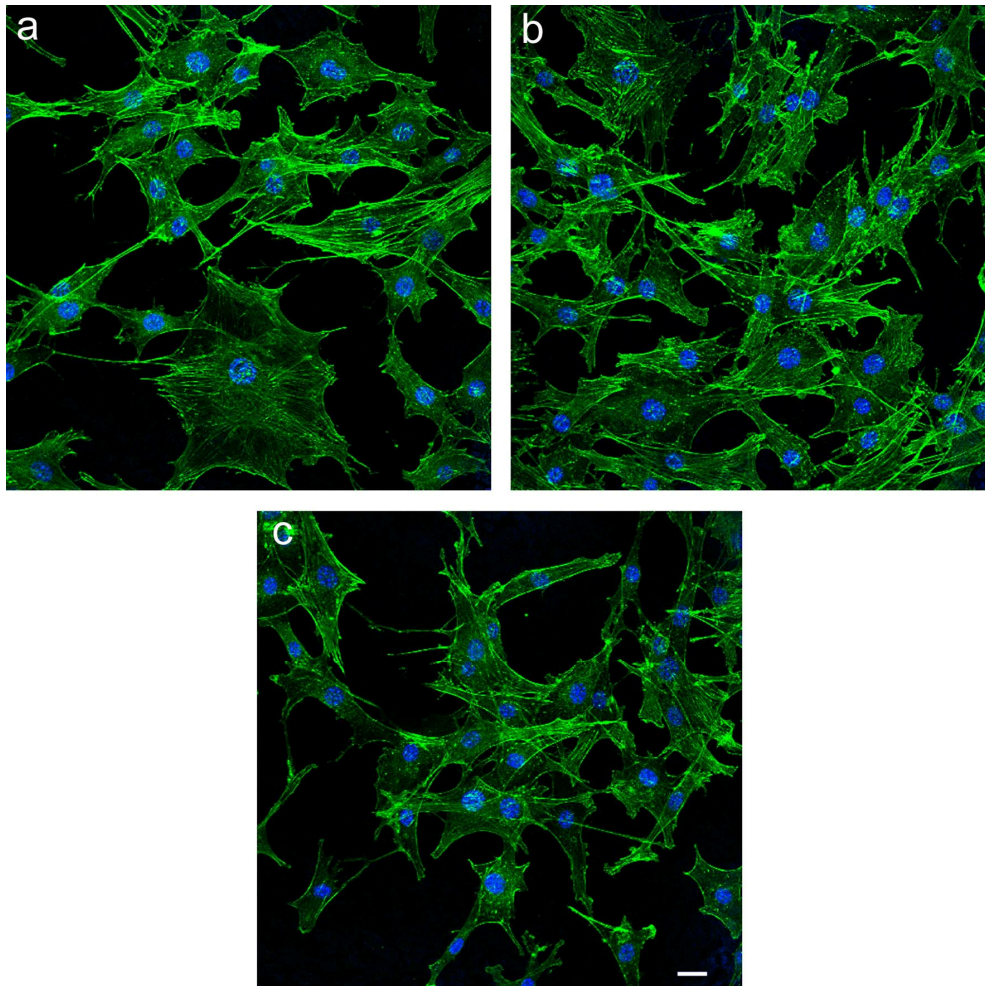


Figure 3 – MC3T3-E1 cell morphology and cytoskeletal organization on TiO₂ cp imaged with confocal microscopy after 24 h, with the preadsorption conditions: a) HSA, b) FN, c) 10 % plasma. F-actin is indicated in green while cell nuclei were counterstained in blue with DAPI dye. Scale bar = 20 μm.

Competitive protein adsorption studies

To investigate the mechanisms underlying the role of protein preadsorption on the differences observed in the number of adhesion of cells on TiO₂ substrates, the adsorption of radiolabelled albumin and FN on TiO₂ sp and on TiO₂ cp, was tested. The amount of HSA and FN competitively adsorbed from several HSA/FN mass ratio solutions to the TiO₂ sp surface is shown in Figure 4. Calculations were performed considering that 100 % adsorption corresponds to the concentration of HSA adsorbed from FN free solution or to FN adsorbed from HSA free solution.

HSA adsorption to TiO₂ sp surface sites is dependent upon the concentration of FN co-adsorbing from solution (Figure 4). When the two proteins are present at 1:1 ratio, the maximum of HSA adsorbed drops drastically when compared to the single protein solution. Maximum HSA adsorbed is found at HSA/FN ratio of 50 and this value remains constant even when this ratio is increased up to the physiological mass ratio.

FN adsorption to TiO₂ surfaces is also dependent on the concentration of HSA coadsorbing from solution. The maximum of FN competitively adsorbed over HSA is always lower when compared to the FN adsorption from the single FN solution. This value decreases as the proportion of FN is decreasing in solution. For the physiological [HSA]/[FN] mass ratio, the HSA adsorption is reduced ca. 36% and FN adsorption is reduced more than 90 % at TiO₂ sp surfaces.

Identical differences were observed in the amount of HSA and FN adsorbed from all the HSA/FN mass ratio solutions to the TiO₂ cp surfaces (data not shown). The differences observed in HSA and FN adsorbed amounts between both TiO₂ surfaces, followed the pattern described for the corresponding pure single solutions. Higher adsorbed amounts of HSA were found on TiO₂ sp surfaces and, conversely, higher amounts of FN were observed on TiO₂ cp substrates.^{1,18}

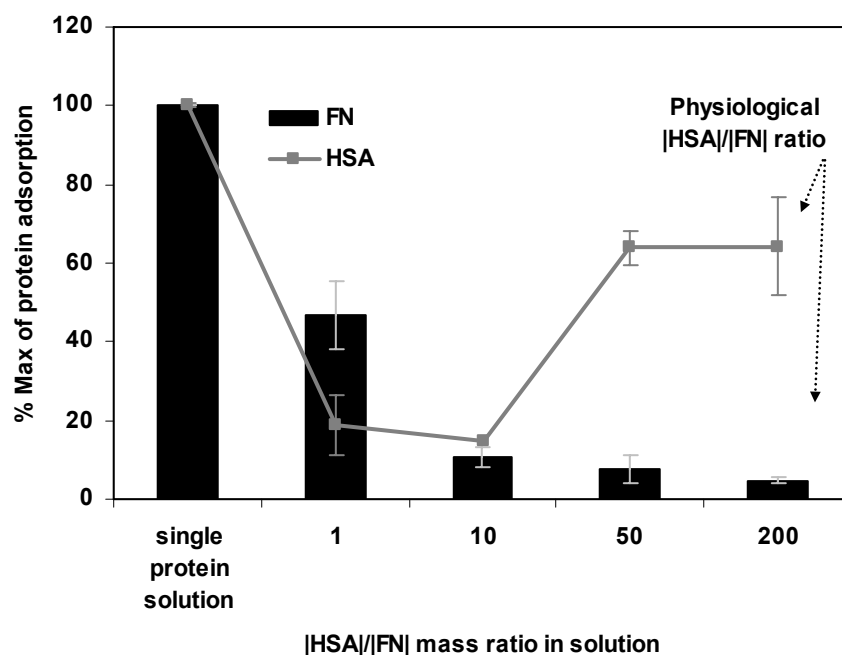


Figure 4 – Competitive adsorption of HSA and FN to TiO_2 sp substrates as a function of HSA/FN mass ratio in solution (1, 10, 50 and 200). Calculations were performed considering as 100 % adsorption the concentration of HSA or FN adsorbed from a pure albumin solution or from a pure fibronectin solution, respectively.

The amount of HSA and FN competitively adsorbed from 10 % plasma to the TiO_2 sp surface is shown in Figure 5.

The maximum HSA adsorption from 10 % plasma is reduced more than 50 % when compared to the maximum HSA adsorption from solution with physiological HSA/FN ratio. On the other hand the maximum FN amount adsorbed from 10 % serum is identical to the maximum FN amount adsorbed from the physiological HSA/FN ratio. On the basis of the present data, HSA adsorption appears to be limited by FN and by other proteins.

A similar pattern was found for albumin and FN adsorption from 10 % plasma solution to the TiO_2 cp surface (data not shown).

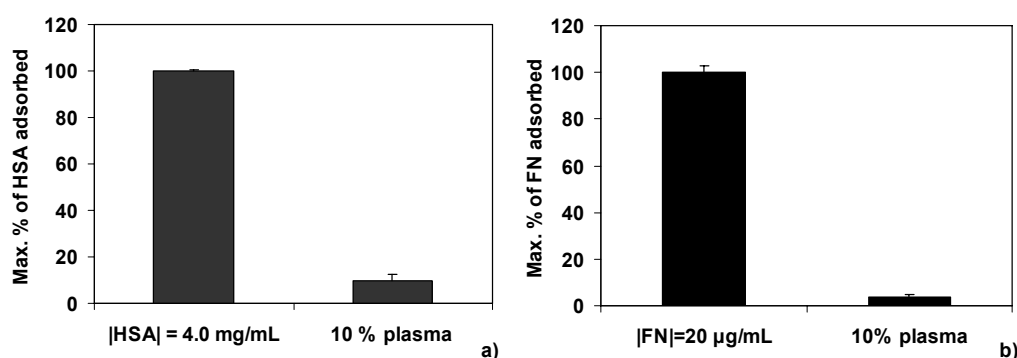


Figure 5 – Relative a) HSA and b) FN amount competitively adsorbed from 10 % plasma solutions to TiO₂ sp substrates. Calculations were performed considering as 100 % adsorption the concentration of HSA or FN adsorbed from a pure albumin solution or from a pure fibronectin solution, respectively.

Reversible binding of HSA and FN

Protein binding strength to a substrate may be correlated with the biological activity of an adsorbed protein that may influence cellular interactions.³

The exchangeability of bound HSA on both substrates was evaluated by the exchange of pre-adsorbed ¹²⁵I-HSA for unlabeled HSA or FN. The percentage of ¹²⁵I-HSA eluted from the TiO₂ surfaces after soaking in HSA or FN solutions for 24 h was calculated. The results indicated that there are more HSA reversibly bound to TiO₂ sp substrates than the TiO₂ cp substrates. No differences were found in the elution properties of HSA response to the washing solution, for both substrates.

The exchangeability of bound FN, on both TiO₂ substrates was evaluated by the exchange of pre-adsorbed ¹²⁵I-FN for unlabeled FN or HSA. The percentage of ¹²⁵I-FN elution onto the TiO₂ surfaces, after soaking in HSA or FN solutions for 24 h was calculated. The results showed that there is more FN reversibly bound to TiO₂ sp substrates than the TiO₂ cp substrates. Almost no differences were found in FN elutability in response to the washing solution, for both substrates. After the HSA adsorption step from HSA and FN binary solutions, exchangeability of bound HSA was evaluated by the exchange of pre-adsorbed ¹²⁵I-HSA for unlabeled HSA. Similarly, after the FN adsorption step from HSA and FN binary solutions, exchangeability of bound FN

was evaluated by the exchange of pre-adsorbed ^{125}I -FN for unlabeled FN. The percentage of ^{125}I -HSA or ^{125}I -FN elution onto the TiO_2 sp surfaces after soaking in HSA or FN solutions for 24 h, as a function of HSA/FN mass ratios is shown in Figure 6.

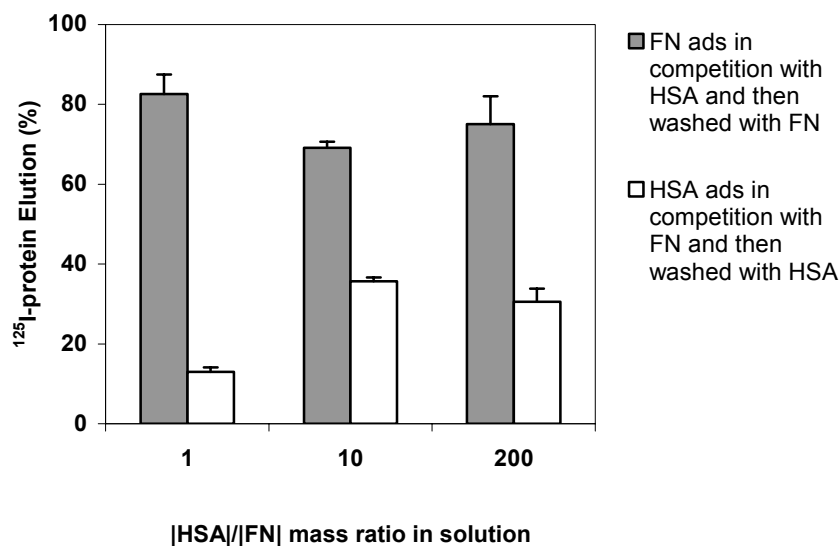


Figure 6 – Exchangeability of HSA and FN competitively adsorbed to TiO_2 sp surfaces, following 24 h exposure to HSA and FN solutions, respectively.

FN molecules (70-80 %) are eluted from the TiO_2 sp surface by other FN molecules and only 10-40 % of albumin molecules are exchanged by other albumin molecules (Figure 6), for the HSA/FN mass ratios studied. FN exchangeability was not sensitive to the HSA/FN ratio, and on the contrary HSA exchangeability varied with HSA/FN mass ratio. The lowest value of HSA exchangeability is observed when both proteins are present in the same proportion. HSA exchangeability was always lower than the FN exchangeability.

DISCUSSION

The tissue response to implants is highly dependent on the initial attachment of cells to the implant surface. This cell adhesion is related to the ability of cells to interact with the protein

layer adsorbed previously on the implant surface and therefore the nature of this protein layer is of key relevance. Indeed, serum or plasma contains proteins such as albumin and IgG that decrease or inhibit cell attachment while FN and vitronectin (VN) can facilitate the adhesion of cells.^{5,20-22} It is believed that the efficiency of cell adhesion and growth is dependent on the balance between adhesion-promoting versus adhesion-inhibiting proteins which competitively adsorb to a surface. The increase impairment of the cell adhesion may be due to the fact that proteins can block the access of the cell to an adhesive protein already adsorbed or may compete with an adhesive protein for binding sites.³ FN was identified as the serum protein primarily responsible for cell attachment and spreading and the functionality of FN is dependent on the surface chemistry and on the cell type.^{9,21,23-26}

So far the experimental data on the role of HSA and FN regarding the cell adhesion is not clear and thus we address this topic by analysing the binding of both proteins in two titanium surfaces.

Our previous studies,^{1,18} have shown that TiO₂ cp and TiO₂ sp rapidly adsorb both albumin and fibronectin from buffer solution, reaching a maximum of adsorption after 60 min. For a given concentration of HSA TiO₂ sp adsorbs more than TiO₂ cp. An average saturation plateau at ca. 40 mg/m² is attained for bulk HSA concentration of 4.0 mg/mL (10 % of the HSA plasma concentration) for the TiO₂ sp surface and ca. 20 mg/m² is attained for bulk HSA concentration of 1.0 mg/mL for the TiO₂ cp surface.¹ In contrast, TiO₂ cp adsorbs more FN than the TiO₂ sp surfaces, nevertheless for FN concentrations between 0 and 200 µg/mL, the FN adsorbed amount increased for both substrates, as the FN concentration increased in solution, and a plateau was never attained.

This report provides further evidence that both HSA and FN adsorbed amounts decrease significantly when HSA/FN solutions are used in comparison to those found for single protein solutions. Albumin competitively adsorbed from binary solutions with FN differs significantly with TiO₂ substrate type, adsorbing higher amounts of HSA at the TiO₂ sp surfaces. Similarly FN competitively adsorbed from binary solutions with albumin also differs with TiO₂ type, adsorbing higher amounts of FN at TiO₂ cp substrates. HSA adsorbed amounts are significantly reduced when other serum or plasma proteins are present (Figure 4). Other authors found that the adsorption of serum FN to different surfaces decreases significantly or is completely blocked by other serum components (e.g., albumin or vitronectin) at serum concentrations greater than 1%.

Therefore small amounts are expected to adsorb onto surfaces at serum concentrations (5-30 %). The lower levels of FN adsorption relative to HSA are expected since plasma contains a 200-fold excess of albumin compared with FN, yet its competitive effectiveness for adsorption is similar to that of albumin.¹⁰ Although the density of FN adsorbed onto TiO₂ substrates was low, it maybe sufficient to support focal contact formation and is in agreement with the findings of McFarland et al.²¹ and Keselowsky et al.²⁵ These authors showed that this process is dependent on the conformation of the adsorbed FN.

As far as the ability of a TiO₂ type substrate to support MC3T3-E1 adhesion is concerned, it seems to correlate well with the substrate affinity to HSA and FN. As previously referred TiO₂ cp adsorbs less albumin and more FN than TiO₂ sp, after 4h of cell adhesion, supporting an expected higher number of adherent cells, for all the pre-adsorbing conditions. After 24 h of incubation TiO₂ sp still harbors fewer cells for substrates pre-adsorbed with HSA, HSA/FN=200 and 10 % plasma. Although surface wettability might have contributed to higher cell affinity toward TiO₂ cp, it has to be taken into account that for osteoblasts, cell attachment and spreading vary with surface chemistry rather than with wettability.²⁷⁻²⁹ The two TiO₂ differ in surface roughness, being the TiO₂ cp ~14 times rougher than TiO₂ sp. It is unlike that the differences observed for cell growth between the two substrates can be attributed solely to morphological differences. While surface roughness of TiO₂ cp is higher than that observed for TiO₂ sp, the number of cells after 24h and not at 4 h of incubation are similar for substrates without pre-adsorbed protein. Similarly, cell growth differs significantly for a given TiO₂ substrate preadsorbed with proteins, yet the surface roughness is equivalent.

Our results indicated that the ability of both substrates to support cell adhesion was negatively affected by the preadsorption of HSA, HSA/FN=200, and 10% plasma, when compared to the positive effect of FN pre-adsorption. For the two latter preadsorption conditions, albumin is found in a larger proportion relatively to FN. Both high albumin concentration and relatively small molecular mass, kinetically favor more rapid mass transport to surfaces compared to larger and less concentrated proteins like FN. The presence of small amounts of co-adsorbed albumin apparently increases adsorbed FN cell binding domain availability, resulting in a more biological active FN molecular conformation, an effect called "albumin rescuing".^{5,10,30} At equal protein ratio, HSA/FN=1, FN was able to compensate the negative effect of HSA with exception of TiO₂ sp after 4 h of MC3T3-E1 incubation. For the short incubation time a combined effect is

probably occurring; the substrate capacity to adsorb more HSA than FN, and the higher albumin diffusivity lead to inhibition of cell adhesion. With time the elution of HSA seems to be more difficult than elution of FN (Figure 6), suggesting that HSA may undergo conformational changes at the surface and FN does not. Although the type and the amount of protein adsorbed on a substrate will influence cell spreading and growth, the biological activity of the adsorbed proteins may have a more significant impact on cellular interactions. Although some studies suggest that tight protein binding may be conducive to cell attachment and growth,³¹ other studies provide evidence that tight protein binding may not be desirable¹¹ since tight binding of a protein to a surface may indicate that conformation or orientation changes (especially denaturation) have taken place during or after protein adsorption.

The preadsorption of serum or plasma components is observed to decrease cell adhesion which is in accordance with other studies.^{10,32}

CONCLUSIONS

Regarding MC3T3-E1 osteoblast cell adhesion for both substrates preadsorbed with HSA, HSA/FN=200 and 10% plasma, it is observed that fewer osteoblasts adhered to both substrates after 4 and 24h than to the surfaces preadsorbed with FN and HSA/FN=1. For the latter protein preadsorption, FN was able to overcome the inhibitory effect of HSA on osteoblast adhesion, with the exception of TiO₂ sp after 4 h of MC3T3-E1 incubation. Therefore, the presence of small amounts of co-adsorbed albumin may improve the exposure of FN in a more integrin-recognized conformation, an effect called “albumin rescuing”.

In addition, we found that MC3T3-E1 osteoblast adhesion correlates well with the amounts of FN and HSA adsorbed on TiO₂ surfaces.

The results suggest that depending on protein ratio, HSA can compete with FN for the TiO₂ surface sites, explaining the lower and identical adhesion levels of osteoblasts observed for the physiological [HSA]/[FN] mass ratio and 10 % plasma. On the contrary at 1:1 concentration ratio, FN density levels are higher than those observed for HSA, which may account for some degree of molecular packing, to the enhanced levels of MC3T3-E1 adhesion.

Regarding the elution properties, it was found that FN molecules exchanged with other FN molecules, after competitive adsorption, between 70 and 80 % and HSA was exchanged to a much lower level with other HSA molecules. FN reversibility does not seem to be dependent on the HSA/FN adsorption mass ratio in solution, suggesting that FN competitively adsorbs to TiO₂ in a favorable conformation and does not suffer subsequent conformational changes allowing exchange with other FN molecules in solution.

REFERENCES

1. Sousa SR, Moradas-Ferreira P, Saramago B, Melo LV, Barbosa MA. Human serum albumin adsorption on TiO₂ from single protein solutions and from plasma. *Langmuir* 2004;20(22):9745-9754.
2. Anselme K. Osteoblast adhesion on biomaterials. *Biomaterials* 2000;21(7):667-681.
3. Tidwell CD, Ertel SI, Ratner BD, Tarasevich BJ, Atre S, Allara DL. Endothelial cell growth and protein adsorption on terminally functionalized, self-assembled monolayers of alkanethiolates on gold. *Langmuir* 1997;13(13):3404-3413.
4. Malmsten M, Lassen B. Competitive Adsorption at Hydrophobic Surfaces from Binary Protein Systems. *Journal of Colloid and Interface Science* 1994;166(2):490-498.
5. Koenig AL, Gambillara V, Grainger DW. Correlating fibronectin adsorption with endothelial cell adhesion and signaling on polymer substrates. *Journal of Biomedical Materials Research Part A* 2003;64A(1):20-37.
6. Horbett TA. The role of adsorbed proteins in animal cell adhesion. *Colloids and Surfaces B: Biointerfaces* 1994;2(1-3):225-240.
7. Huang HH, Ho CT, Lee TH, Lee TL, Liao KK, Chen FL. Effect of surface roughness of ground titanium on initial cell adhesion. *Biomolecular Engineering* 2004;21(3-5):93-97.
8. Vitte J, Benoliel AM, Pierres A, Bongrand P. Is there a predictable relationship between surface physical-chemical properties and cell behaviour at the interface? *European Cell Materials* 2004;n° 7.
9. Tsai WB, Grunkemeier JM, McFarland CD, Horbett TA. Platelet adhesion to polystyrene-based surfaces preadsorbed with plasmas selectively depleted in

- fibrinogen, fibronectin, vitronectin, or von Willebrand's factor. *Journal of Biomedical Materials Research* 2002;60(3):348-359.
10. Grainger DW, Pavon-Djavid G, Migonney V, Josefowicz M. Assessment of fibronectin conformation adsorbed to polytetrafluoroethylene surfaces from serum protein mixtures and correlation to support of cell attachment in culture. *Journal of Biomaterials Science-Polymer Edition* 2003;14(9):973-988.
 11. Goncalves IC, Martins MCL, Barbosa MA, Ratner BD. Protein adsorption on 18-alkyl chains immobilized on hydroxyl-terminated self-assembled monolayers. *Biomaterials* 2005;26(18):3891-3899.
 12. Culp LA, Sukenik CN. Cell type-specific modulation of fibronectin adhesion functions on chemically-derivatized self-assembled monolayers. *Journal of Biomaterials Science-Polymer Edition* 1998;9(11):1161-1176.
 13. Baugh L, Vogel V. Structural changes of fibronectin adsorbed to model surfaces probed by fluorescence resonance energy transfer. *Journal of Biomedical Materials Research Part A* 2004;69A(3):525-534.
 14. McClary KB, Ugarova T, Grainger DW. Modulating fibroblast adhesion, spreading, and proliferation using self-assembled monolayer films of alkylthiolates on gold. *Journal of Biomedical Materials Research* 2000;50(3):428-439.
 15. Gehanno V, Freitas PP, Ferreira J, Almeida B, Sousa JB, Kling A, Soares JC, Silva MF. Ion beam deposition of Mn-Ir spin valves. *IEEE Trans Magn* 1999;35:4361-4367.
 16. Iodine-125. Little Chalfont, Buckinghamshire, U.K.: Amersham International; 1993. 64 p.
 17. Lima J, Sousa SR, Ferreira A, Barbosa MA. Interactions between calcium, phosphate, and albumin on the surface of titanium. *J Biomed Mater Res* 2001;55(1):45-53.
 18. Sousa SR, Moradas-Ferreira P, Barbosa M. TiO₂ type influences fibronectin adsorption. *Journal of Materials Science: Materials in Medicine* 2005;16(12):1173-1178.
 19. Hederos M, Konradsson P, Liedberg B. Synthesis and self-assembly of galactose-terminated alkanethiols and their ability to resist proteins. *Langmuir* 2005;21(7):2971-2980.
 20. McFarland CD, Mayer S, Scotchford C, Dalton BA, Steele JG, Downes S. Attachment of cultured human bone cells to novel polymers. *Journal of Biomedical Materials Research* 1999;44(1):1-11.

21. McFarland CD, Thomas CH, DeFilippis C, Steele JG, Healy KE. Protein adsorption and cell attachment to patterned surfaces. *Journal of Biomedical Materials Research* 2000;49(2):200-210.
22. Yang YZ, Cavin R, Ong JL. Protein adsorption on titanium surfaces and their effect on osteoblast attachment. *Journal of Biomedical Materials Research Part A* 2003;67A(1):344-349.
23. MacDonald DE, Rapuano BE, Deo N, Stranick M, Somasundaran P, Boskey AL. Thermal and chemical modification of titanium-aluminum-vanadium implant materials: effects on surface properties, glycoprotein adsorption, and MG63 cell attachment. *Biomaterials* 2004;25(16):3135-3146.
24. Steele JG, Johnson G, McFarland C, Dalton BA, Gengenbach TR, Chatelier RC, Underwood PA, Griesser HJ. Roles of Serum Vitronectin and Fibronectin in Initial Attachment of Human Vein Endothelial-Cells and Dermal Fibroblasts on Oxygen-Containing and Nitrogen-Containing Surfaces Made by Radiofrequency Plasmas. *Journal of Biomaterials Science-Polymer Edition* 1994;6(6):511-532.
25. Keselowsky BG, Collard DM, Garcia AJ. Surface chemistry modulates fibronectin conformation and directs integrin binding and specificity to control cell adhesion. *Journal of Biomedical Materials Research Part A* 2003;66A(2):247-259.
26. Jenney CR, Anderson JM. Adsorbed serum proteins responsible for surface dependent human macrophage behavior. *Journal of Biomedical Materials Research* 1999;49(4):435-447.
27. Webb K, Hlady V, Tresco PA. Relationships among cell attachment, spreading, cytoskeletal organization, and migration rate for anchorage-dependent cells on model surfaces. *Journal of Biomedical Materials Research* 2000;49(3):362-368.
28. Amaral IF, Lamghari M, Sousa SR, Sampaio P, Barbosa MA. Rat bone marrow stromal cell osteogenic differentiation and fibronectin adsorption on chitosan membranes: The effect of the degree of acetylation. *Journal of Biomedical Materials Research Part A* 2005;75A(2):387-397.
29. Scotchford CA, Gilmore CP, Cooper E, Leggett GJ, Downes S. Protein adsorption and human osteoblast-like cell attachment and growth on alkylthiol on gold self-assembled monolayers. *Journal of Biomedical Materials Research* 2002;59(1):84-99.

30. Scotchford CA, Ball M, Winkelmann M, Voros J, Csucs C, Brunette DM, Danuser G, Textor M. Chemically patterned, metal-oxide-based surfaces produced by photolithographic techniques for studying protein- and cell-interactions. II: Protein adsorption and early cell interactions. *Biomaterials* 2003;24(7):1147-1158.
31. Pettit DK, Horbett TA, Hoffman AS. Influence of the substrate binding characteristics of fibronectin on corneal epithelial cell outgrowth. *J Biomed Mater Res.* 1992;26(Oct 10):1259-1275.
32. Steele JG, Johnson G, Underwood PA. Role of Serum Vitronectin and Fibronectin in Adhesion of Fibroblasts Following Seeding onto Tissue-Culture Polystyrene. *Journal of Biomedical Materials Research* 1992;26(7):861-884.

Concluding remarks and Future work

1. Concluding Remarks

The initial attachment of cells to the implant surface is a key event regarding the success of the tissue response to implants. This adhesion is related to the ability of cells to interact with the protein layer previously adsorbed on the implant surface, and this interaction is dependent on the protein characteristics. Indeed, serum and plasma proteins such as albumin and IgG decrease or inhibit cell attachment, while fibronectin and vitronectin can facilitate the adhesion of cells.¹⁻⁴ It is believed that the efficiency of cell adhesion and subsequent growth is dependent on the balance between adhesion-promoting versus adhesion-inhibiting proteins that competitively adsorb to a surface. Surface characteristics, such as chemistry, surface charge and topography of the material surface, have a relevant effect on protein adsorption, playing a critical role in governing biocompatibility.^{2,5-13}

Titanium has properties such as physiological stability, corrosion resistance, mechanical properties and favorable osteointegration, that make it one of the most used biomaterials for bone and cartilage replacement.¹⁴⁻¹⁷ Abundant information on protein adsorption onto Ti is available in the literature.⁹⁻²² Still, there are no generally accepted views on the importance of the protein film, or specific proteins, on biological reaction to Ti.¹⁸

This thesis aims to disclose the main features that govern the protein adsorption of two key proteins – albumin and fibronectin - on titanium oxide surfaces with different nanometric scale topography. The experimental approach involved human serum albumin, HSA, and human plasma fibronectin, FN, both from single protein or complex protein solutions. The influence of

competitive preadsorption of the above proteins on titanium surfaces on the osteoblast adhesion was also addressed.

The work started by preparing titanium oxide surfaces chemically similar to those used in clinical practice, with different topographies at the nanometric scale. Thus, two surfaces, TiO₂ sputtered on Si (TiO₂ sp) and TiO₂ formed on commercially pure titanium after H₂O₂ immersion (TiO₂ cp), were employed.

Chapter II describes the several surface treatments of commercially pure Ti that were employed: exposure to air (48 h), immersion in H₂O₂ 10 mM and in H₂O₂ 100 mM during two different time periods: 24 h and 48 h. The treatment selected was the immersion in H₂O₂ 10 mM for 48 h, since it led to a thicker oxide film composed by TiO₂, without sub-oxides, such as TiO and Ti₂O₃. The influence of the nature of titanium oxide and of its hydrated state, as well as of the surface roughness on biocompatibility, has become an important area of research,¹⁹⁻²² and it has been shown that the *in vivo* performance of the titanium²³ is dependent on the method of preparation of titanium oxide.²⁴⁻²⁶

Using XPS it was revealed that the Ti/O_M ratio acquires values near the theoretical ratio for a stoichiometric oxide (1/2). As expected, the values of Ti/O_H are higher near the interface for the TiO₂ cp substrate, due to the presence of a hydrated film. The data obtained in the present work is in agreement with the report²¹ showing that Ti/O ratio decreases when titanium surfaces are immersed in solutions of up to 10 mM H₂O₂ (**Chapter II**). In addition, the TiO₂ film was thicker in 10 mM H₂O₂ than in 100 mM H₂O₂,²² which corroborates our choice of the peroxide concentration. A thicker oxide is advantageous because prevents release of Ti(IV) ions.

The two TiO₂ surfaces showed similar chemical composition but distinct roughnesses at the nanometric scale, assessed by AFM roughness analysis. As compared to the TiO₂ cp, TiO₂ sputtered had mean R_a and R_q values 14 times smaller. AFM roughness analysis (**Chapter II and IV**) also revealed that both TiO₂ surfaces exhibited R_q and R_{max} lower values in air than in water. In addition, depending on the medium (air or water) different topographies were found for the TiO₂ surfaces. The height and phase images showed topographic features, such as globules, characteristic of titanium oxide with different morphologies in air and in water that may result from combined effects. A possible explanation may be that different interaction between

the tip and the sample are found in liquid and in water. Also, after hydration, TiO_2 is expected to swell, becoming gel-like with a layer of hydration instead of a dehydrated oxide.

Wenzel was one of the first investigators to propose a model relating contact angles and surface roughness.²⁷ Roughening a substrate usually decreases its wettability,²⁸ which is in agreement with the results obtained. Moreover, the increased surface energy observed in H_2O_2 treated surfaces was also indicative of higher wettability.²² Both substrates were predominantly dispersive. However, after the H_2O_2 treatment the polar component of the surface energy of the Ti substrates increased, due to the presence of ionic species. Considering that polarity is a quantitative indicator of hydrophilicity, we can say that the TiO_2 cp substrate was the most hydrophilic one.

Aiming at identifying the main features that govern protein adsorption, the above TiO_2 surfaces were evaluated regarding their ability to adsorb albumin from single protein solutions, plasma and diluted plasma (**Chapter II**). The adsorption kinetics and isotherms were obtained using two different techniques, namely radiolabelling of albumin with ^{125}I (^{125}I -HSA) and XPS. Additionally, the work of adhesion between protein solutions and TiO_2 substrates was determined through wettability studies. Finally, exchangeability tests were performed using FN and HSA.

It was observed that the quantity of albumin adsorbed was higher on the more hydrophobic surface (TiO_2 sp), which is in agreement with the view defended by many authors,²⁹⁻³² according to whom hydrophobic surfaces generally adsorb more albumin than hydrophilic surfaces. However, the adherent molecules were more strongly attached to the TiO_2 cp, as indicated by the work of adhesion and by the desorption studies. This result was unexpected if we consider the work of adhesion for both substrates, i.e. HSA are less tightly bound at hydrophobic surfaces than to hydrophilic ones. Globular proteins, such as HSA, tend to minimize exposure of their hydrophobic groups to the aqueous environment. However, as a result of geometrical restrictions, the protein exterior is often partly hydrophobic.³³ According to Norde³³ the binding strength seems to be at a maximum if both polar and non-polar interactions are involved, as it is the case of TiO_2 cp substrate. Besides, Kurrat et al³⁴ and Martins et al³⁵ suggested that the main mode of interaction of HSA onto hydroxylated metals was via hydrogen bonds. Although hydrogen bonds are individually weak, the probability of breaking many hydrogen bonds simultaneously is low.

Comparing the theoretical amount for monolayer formation with the data obtained by ^{125}I -HSA, it may be suggested that albumin adsorbs as a multilayer. Although proteins often adsorb as monolayers, multilayer adsorption is not uncommon, mainly for high concentration solutions.^{33,36}

Subsequently, the surfaces were evaluated regarding their ability to adsorb fibronectin from single protein solutions, plasma and diluted plasma (**Chapter III and IV**). Adsorption isotherms and the work of adhesion were assessed by wettability studies, XPS and by radiolabelling of FN with ^{125}I (^{125}I -FN). FN adsorption as a function of time was followed by XPS, ^{125}I -FN, and ellipsometry. Exchangeability tests were performed using FN and HSA. In addition, fibronectin morphological properties induced by its adsorption onto the TiO_2 surfaces with AFM, were investigated by imaging and roughness analysis in air and in water.

FN showed a different affinity to the TiO_2 surfaces, compared to albumin. Higher adsorbed amounts of FN were found on the TiO_2 cp surfaces, conversely to what was observed with HSA, as shown by the radiolabelling data. Besides, FN molecules appeared to be more strongly attached to the former surface, as indicated by the work of adhesion and by the exchangeability studies, following the trend observed for albumin. The wettability results also showed that the work of adhesion of FN to TiO_2 cp decreased when FN concentration increased. The adsorption of proteins is mediated by several polypeptide domains. At low concentrations, adsorbed proteins display a conformation that leads to exposure of more binding sites. It is known^{37,38} that the driving forces for adsorption of “soft” proteins, like FN and HSA, are related to structural rearrangements in the molecules that enable them to overcome the unfavourable conditions offered by an electrostatically repelling surface. At higher protein concentration the adsorption layer becomes more compressed and molecules with different degrees of unfolding will coexist at the interface,³⁹ being more difficult or even impossible for some molecules to overcome the unfavourable conditions for adsorption. This fact can explain the highest work of adhesion obtained for the lowest FN concentration observed. Desorption results also showed that the elution seems to be more dependent on the nature of the surface than on the time of adsorption or on the type of protein in the eluting solution.

Until now AFM imaging has been limited to adsorbed protein on relatively smooth model surfaces, such as mica, graphite, or self assembled monolayers (SAMs)⁴⁰⁻⁴⁶ on which the

proteins can be easily distinguished from the background. The inherent roughness of biomaterial surfaces may be unable to visualize adsorbed proteins in topographic images. However, Holland N. B. et al.⁴⁵ showed that the phase imaging signal of tapping mode AFM provides information on differences in surface properties, and was used to distinguish individual protein molecules from the underlying surface. AFM studies (**Chapter IV**) allowed the visualization of FN on TiO₂ sp both in air and in liquid. AFM phase imaging successfully showed FN adsorbed on the rougher TiO₂ surfaces, TiO₂ cp. In addition, the presence of FN on both substrates was also confirmed by roughness parameters, namely R_q and R_{max} . The overall results suggested that FN spontaneously adsorbs as self-assembled molecules at TiO₂ sp surfaces as a function of time. The aggregate structure is an intermediate feature shared by some protein fibrillar assemblies at interfaces, which is believed to promote cell adhesion, and cytoskeleton organization.⁴⁷⁻⁴⁹

Comparing the theoretical amount for closely packed monolayers⁵⁰ with the data obtained by ¹²⁵I-FN and by ellipsometry, it is concluded that the amount of FN adsorbed is insufficient for monolayer formation, which is consistent with the AFM results.

Finally, **Chapter V** describes the effect of competitive preadsorption of HSA and FN on both TiO₂ substrates from binary protein mixtures and from 10 % plasma on MC3T3-E1 osteoblasts adhesion and morphology. The competitive adsorption of proteins was evaluated by measuring the amount and the effectiveness of binding with radiolabelled proteins, ¹²⁵I-FN and ¹²⁵I-HSA.

HSA and FN adsorbed amounts decrease significantly when HSA/FN solutions are used in comparison to those found for single protein solutions. Albumin competitively adsorbed from binary solutions depends on the TiO₂ substrate, higher amounts adsorbing at the TiO₂ sp surfaces. Similarly, FN competitively adsorbed from binary solutions also depends on the TiO₂ type, higher amounts of FN adsorbing at TiO₂ cp substrates.

The amounts of adsorbed HSA are significantly reduced when other serum or plasma proteins are present. Other authors found that the adsorption of serum FN to different surfaces decreases significantly or is completely blocked by other serum components (e.g., albumin or vitronectin) at serum concentrations greater than 1%. Therefore, small amounts are expected to adsorb onto surfaces at serum concentrations (5-30 %). The lower levels of FN adsorption relative to HSA are expected since plasma contains a 200-fold excess of albumin compared

with FN, yet its competitive effectiveness for adsorption is similar to that of albumin.⁵¹ Although the density of FN adsorbed onto TiO₂ substrates was low, it may be sufficient to support focal contact formation, which is in agreement with the findings of McFarland² and Keselowsky¹² who showed that this process is dependent on the conformation of the adsorbed FN.

As far as the ability of a TiO₂ type substrate to support MC3T3-E1 adhesion is concerned, after 4 h it seems to correlate well with the substrate affinity to HSA and FN. As previously referred, TiO₂ cp adsorbs less albumin and more FN than TiO₂ sp, thus explaining a higher number of adherent cells, as observed for all the pre-adsorbing conditions. After 24 h of incubation TiO₂ substrates still harbors fewer cells for substrates pre-adsorbed with HSA, HSA/FN=200 and 10 % plasma. Although surface wettability might have contributed to higher cell affinity toward TiO₂ cp, it has to be taken into account that for osteoblasts, cell attachment and spreading vary with surface chemistry rather than with wettability.⁵²⁻⁵⁴ The two TiO₂ differ in surface roughness, the TiO₂ cp being ~14 times rougher than TiO₂ sp. It is unlikely that the differences observed for cell behavior between the two substrates can be attributed solely to morphological differences. While surface roughness of TiO₂ cp is higher than that for TiO₂ sp, the number of cells after 24h and not at 4 h of incubation is similar for substrates without pre-adsorbed protein. Similarly, cell growth differs significantly for a given TiO₂ substrate preadsorbed with proteins, yet the surface roughness is equivalent.

Our results indicated that the ability of both substrates to support cell adhesion was negatively affected by the preadsorption of HSA, HSA/FN=200, and 10% plasma, when compared to the positive effect of FN and HSA/FN=1 pre-adsorption. For the two latter preadsorption conditions, albumin is found in a larger proportion relatively to FN. Both the high albumin concentration and relatively small molecular mass, kinetically favor more rapid mass transport to surfaces compared to larger and less concentrated proteins like FN. The presence of small amounts of co-adsorbed albumin apparently increases adsorbed FN cell binding domain availability, resulting in a more biological active FN molecular conformation, an effect called "albumin rescuing".^{4,51,55} At equal protein ratio, HSA/FN=1, FN was able to compensate the negative effect of HSA with exception of TiO₂ sp after 4 h of MC3T3-E1 incubation. For the short incubation time a combined effect is probably occurring: the substrate capacity to adsorb more HSA than FN, and the higher albumin diffusivity lead to inhibition of cell adhesion. With time the elution of HSA seems to be more difficult than elution of FN, suggesting that HSA may undergo

conformational changes at the surface while FN would not. Although the type and the amount of protein adsorbed on a substrate will influence cell spreading and growth, the biological activity of the adsorbed proteins may have a more significant impact on cellular interactions. While some studies suggest that tight protein binding may be conducive to cell attachment and growth,⁵⁶ other studies provide evidence that tight protein binding may not be desirable,⁵⁷ since tight binding of a protein to a surface may indicate that conformation or orientation changes (especially denaturation) have taken place during or after protein adsorption.

The preadsorption of serum or plasma components is observed to decrease cell adhesion which is in accordance with other studies.^{51,58}

The overall results reported in this thesis provided evidence that HSA and FN adsorption depended on the TiO₂ type surface, in what concerns affinity, amount and strength of adhesion. When both proteins are present in solution, they competed for TiO₂ surface sites. For HSA/FN 1:1 ratio, the amounts of FN adsorbed are higher than those observed for HSA and thus enhanced levels of MC3T3-E1 adhesion are observed. The presence of small amounts of co-adsorbed albumin seems to improve the exposure of FN in a more integrin-recognized conformation, effect that is known as “albumin rescuing”.

Elutability results suggested that FN adsorbs to TiO₂ in a favorable conformation and does not suffer subsequent conformational changes, thus allowing exchange with other FN molecules in solution.

2. Future work

The work carried out and discussed in the present thesis focused on two protein adsorption on titanium oxide surfaces. Further analysis of protein behavior regarding biomaterials surfaces is still required. An interesting work would be the study of adsorption of other adhesive proteins on TiO₂ such as vitronectin, laminin, and osteopontin. This requires additional work using radiolabelling proteins to understand the role of adsorbed proteins on osteoblasts cell adhesion, cytoskeleton organization, proliferation and osteogenic differentiation.

The protein competition was carried out with HSA and FN but, the study could be performed using the referred glycoproteins.

The conformation of adsorbed proteins is a key factor for the biocompatibility of surfaces. This is due to the fact that substrate-induced changes in conformation usually lead to a decrease in functional activity, and consequently to a decrease in cell adhesion. Therefore, the data generated by studies involving protein conformation at titanium oxides surfaces is a relevant contribution. Immunofluorescence, and ellipsometry or SPR associated with monoclonal primary antibodies can be very useful to assess the HSA and FN conformation, both from single protein solutions as from binary protein solutions or plasma. The results reported in the final part of this thesis (**Chapter V**), provided evidence that small amounts of co-adsorbed albumin improve the exposure of FN in a more integrin-recognized conformation, an effect called “albumin rescuing”. Also, the exchangeability results suggested that FN competitively adsorbs to TiO₂ in a favorable conformation and does not suffer subsequent conformational changes allowing exchange with other FN molecules in solution. Although both evidences are in agreement, there is a need to gain knowledge on the molecular basis of this protein/surface interaction behavior.

A number of biochemical mechanisms are influenced by electrostatic interactions, especially those that involve proteins. Protein adsorption, formation of protein-protein interactions, folding and stability of proteins, denaturation of proteins at high and low pH values, are some examples. Electrostatic interactions are one of the most relevant phenomena involved in the process of protein adsorption, as it was referred in **Chapter I**. The radiolabelling of HSA and FN, zeta potential analyses and XPS can be used to assess the amount of each protein, as a function of pH or charge. Fluorescence studies can be performed to assess the possible conformational changes experienced by proteins, under pH variation.

REFERENCES:

1. McFarland CD, Mayer S, Scotchford C, Dalton BA, Steele JG, Downes S. Attachment of cultured human bone cells to novel polymers. *Journal of Biomedical Materials Research* 1999;44(1):1-11.
2. McFarland CD, Thomas CH, DeFilippis C, Steele JG, Healy KE. Protein adsorption and cell attachment to patterned surfaces. *Journal of Biomedical Materials Research* 2000;49(2):200-210.
3. Yang YZ, Cavin R, Ong JL. Protein adsorption on titanium surfaces and their effect on osteoblast attachment. *Journal of Biomedical Materials Research Part A* 2003;67A(1):344-349.
4. Koenig AL, Gambillara V, Grainger DW. Correlating fibronectin adsorption with endothelial cell adhesion and signaling on polymer substrates. *Journal of Biomedical Materials Research Part A* 2003;64A(1):20-37.
5. Castner DG, Ratner BD. Biomedical surface science: Foundations to frontiers. *Surface Science* 2002;500(1-3):28-60.
6. Ostuni E, Chapman RG, Liang MN, Meluleni G, Pier G, Ingber DE, Whitesides GM. Self-Assembled Monolayers That Resist the Adsorption of Proteins and the Adhesion of Bacterial and Mammalian Cells. *Langmuir* 2001;17(20):6336-6343.
7. Horbett TA. The role of adsorbed proteins in tissue response to biomaterials. In: Ratner BD, Hoffman AS, Schoen FJ, Lemons JE, editors. *Biomaterials Science: An Introduction to Materials in Medicine*. San Diego, CA, USA: Elsevier Academic Press; 2004. p 237-247.
8. Horbett TA. Proteins: structure, properties and adsorption to surfaces. In: Ratner BD, Hoffman AS, Schoen FJ, Lemons JL, editors. *Biomaterials Science - An Introduction to Materials in Medicine*. San Diego, CA, USA: Academic Press Inc.; 1996. p 133-141.
9. MacDonald DE, Rapuano BE, Deo N, Stranick M, Somasundaran P, Boskey AL. Thermal and chemical modification of titanium-aluminum-vanadium implant materials: effects on surface properties, glycoprotein adsorption, and MG63 cell attachment. *Biomaterials* 2004;25(16):3135-3146.

10. Steele JG, Johnson G, McFarland C, Dalton BA, Gengenbach TR, Chatelier RC, Underwood PA, Griesser HJ. Roles of Serum Vitronectin and Fibronectin in Initial Attachment of Human Vein Endothelial-Cells and Dermal Fibroblasts on Oxygen-Containing and Nitrogen-Containing Surfaces Made by Radiofrequency Plasmas. *Journal of Biomaterials Science-Polymer Edition* 1994;6(6):511-532.
11. Tsai WB, Grunkemeier JM, McFarland CD, Horbett TA. Platelet adhesion to polystyrene-based surfaces preadsorbed with plasmas selectively depleted in fibrinogen, fibronectin, vitronectin, or von Willebrand's factor. *Journal of Biomedical Materials Research* 2002;60(3):348-359.
12. Keselowsky BG, Collard DM, Garcia AJ. Surface chemistry modulates fibronectin conformation and directs integrin binding and specificity to control cell adhesion. *Journal of Biomedical Materials Research Part A* 2003;66A(2):247-259.
13. Jenney CR, Anderson JM. Adsorbed serum proteins responsible for surface dependent human macrophage behavior. *Journal of Biomedical Materials Research* 1999;49(4):435-447.
14. Larsson C, Esposito M, Liao H, Thomsen P. The titanium-bone interface *in vivo*. In: Brunette DM, Tengvall P, Textor M, Thomsen P, editors. *Titanium in Medicine: Material Science, Surface Science, Engineering, Biological Responses and Medical Applications*. Berlin: Springer-Verlag; 2001. p 587-648.
15. Windler M, Klabunde R. Titanium for hip and knee prostheses. In: Brunette DM, Tengvall P, Textor M, Thomsen P, editors. *Titanium in Medicine: Material Science, Surface Science, Engineering, Biological Responses and Medical Applications*. Berlin: Springer-Verlag; 2001. p 703-746.
16. Brunski JB. Metals. In: Ratner BR, Hoffman AS, Schoen FJ, Lemons JE, editors. *Biomaterials science: an introduction to materials in medicine*. New York: Elsevier Academic Press, Inc.; 2004. p 37-50.
17. Freese HL, Volas MG, Wood JR. Metallurgy and technological properties of titanium and titanium alloys. In: Brunette DM, Tengvall P, Textor M, Thomsen P, editors. *Titanium in Medicine: Material Science, Surface Science, Engineering, Biological Responses and Medical Applications*: Springer; 2001.

18. Ratner BD. A perspective on titanium biocompatibility. In: Brunette DM, Tengvall P, Textor M, Thomsen P, editors. Titanium in Medicine: Material Science, Surface Science, Engineering, Biological Responses and Medical Applications. Berlin: Springer-Verlag; 2001. p 1-12.
19. Feng B, Weng J, Yang BC, Qu SX, Zhang XD. Characterization of surface oxide films on titanium and adhesion of osteoblast. *Biomaterials* 2003;24(25):4663-70.
20. Morra M, Cassinelli C, Cascardo G, Cahalan P, Cahalan L, Fini M, Giardino R. Surface engineering of titanium by collagen immobilization. Surface characterization and *in vitro* and *in vivo* studies. *Biomaterials* 2003;24(25):4639-54.
21. Tengvall P, Lundstrom I. Physico-chemical considerations of titanium as a biomaterial. *Clinical Materials* 1992;9(2):115-134.
22. Wälivaara B, Lundstrom I, Tengvall P. An in-vitro study of H₂O₂-treated titanium surfaces in contact with blood plasma and a simulated body fluid. *Clin Mater* 1993;12(3):141-48.
23. Ricci JL, Spivak JM, Blumenthal NC, al e. Modulation of bone ingrowth by surface chemistry and roughness. In: Davis JE, editor. The bone biomaterial interface. Toronto: University of Toronto Press; 1991. p 334-349.
24. Oji MO, Wood JV, Downes S. Effects of surface-treated cpTi and Ti6Al4V alloy on the initial attachment of human osteoblast cells. *Journal of Materials Science-Materials in Medicine* 1999;10(12):869-872.
25. Lincks J, Boyan BD, Blanchard CR, Lohmann CH, Liu Y, Cochran DL, Dean DD, Schwartz Z. Response of MG63 osteoblast-like cells to titanium and titanium alloy is dependent on surface roughness and composition. *Biomaterials* 1998;19(23):2219-32.
26. Ong JL, Carnes DL, Cardenas HL, Cavin R. Surface roughness of titanium on bone morphogenetic protein-2 treated osteoblast cells in vitro. *Implant Dent* 1997;6(1):19-24.
27. Busscher HJ, Vanpelt AWJ, Deboer P, Dejong HP, Arends J. The effect of surface roughening of polymers on measured contact angles of liquids. *Colloid Surface* 1984;9(4):319-331.
28. Hitchcock SJ, Carroll NT, Nicholas MG. Some effects of substrate roughness on wettability. *Journal of Materials Science* 1981;16:714-732.

29. Martins MCL, Ratner BD, Barbosa MA. Protein adsorption on mixtures of hydroxyl- and methylterminated alkanethiols self-assembled monolayers. *Journal of Biomedical Materials Research Part A* 2003;67A(1):158-171.
30. Prime KL, Whitesides GM. Self-Assembled Organic Monolayers - Model Systems for Studying Adsorption of Proteins at Surfaces. *Science* 1991;252(5009):1164-1167.
31. Sigal GB, Mrksich M, Whitesides GM. Effect of surface wettability on the adsorption of proteins and detergents. *J. Am. Chem. Soc.* 1998;120(14):3464-3473.
32. Vandulm P, Norde W. The Adsorption of Human-Plasma Albumin on Solid-Surfaces, with Special Attention to the Kinetic Aspects. *J. Colloid Interf. Sci.* 1983;91(1):248-255.
33. Norde W. Adsorption of Proteins from Solution at the Solid-Liquid Interface. *Advances in Colloid and Interface Science* 1986;25(4):267-340.
34. Kurrat R, Prenosil JE, Ramsden JJ. Kinetics of human and bovine serum albumin adsorption at silica-titania surfaces. *J. Colloid Interf. Sci.* 1997;185(1):1-8.
35. Martins MCL. Interaction between Blood Proteins and Surfaces: Self-Assembled Monolayers (SAMs) and Biomedical Polymers [PhD]. Porto: Porto; 2003.
36. Feng L, Andrade JD. Protein adsorption on low temperature isotropic carbon. III. Isotherms, competitiveness, desorption and exchange of human albumin and fibrinogen. *Biomaterials* 1994;5(15):323-333.
37. Serro APVA, Fernandes AC, Saramago BDV, Norde W. Bovine serum albumin adsorption on titania surfaces and its relation to wettability aspects. *Journal of Biomedical Materials Research* 1999;46(3):376-381.
38. Norde W, Anusiem ACI. Adsorption, Desorption and Readsorption of Proteins on Solid-Surfaces. *Colloids and Surfaces* 1992;66(1):73-80.
39. Miller R, Treppo S, Voigt A, Zingg W, Neumann AW. Contact-Angle Kinetics of Human Albumin Solutions at Solid- Surfaces. *Colloids and Surfaces* 1993;69(4):203-208.
40. Lin Y, Wang J, Wan LJ, Fang XH. Study of fibrinogen adsorption on self-assembled monolayers on Au(111) by atomic force microscopy. *Ultramicroscopy* 2005;105(1-4):129-136.
41. Advincula M, Fan XW, Lemons J, Advincula R. Surface modification of surface sol-gel derived titanium oxide films by self-assembled monolayers (SAMS) and non-specific protein adsorption studies. *Colloids and Surfaces B-Biointerfaces* 2005;42(1):29-43.

-
42. Li LY, Chen SF, Zheng J, Ratner BD, Jiang SY. Protein adsorption on oligo(ethylene glycol)-terminated alkanethiolate self-assembled monolayers: The molecular basis for nonfouling behavior. *Journal of Physical Chemistry B* 2005;109(7):2934-2941.
 43. Wang MS, Palmer LB, Schwartz JD, Razatos A. Evaluating protein attraction and adhesion to biomaterials with the atomic force microscope. *Langmuir* 2004;20(18):7753-7759.
 44. Bergkvist M, Carlsson J, Oscarsson S. Surface-dependent conformations of human plasma fibronectin adsorbed to silica, mica, and hydrophobic surfaces, studied with use of Atomic Force Microscopy. *Journal of Biomedical Materials Research Part A* 2003;64A(2):349-356.
 45. Holland NB, Marchant RE. Individual plasma proteins detected on rough biomaterials by phase imaging AFM. *Journal of Biomedical Materials Research* 2000;51(3):307-315.
 46. Jandt KD. Atomic force microscopy of biomaterials surfaces and interfaces. *Surface Science* 2001;491(3):303-332.
 47. Maheshwari G, Brown G, Lauffenburger DA, Wells A, Griffith LG. Cell adhesion and motility depend on nanoscale RGD clustering *Journal of cell science* 2000;113(10):1677-1686.
 48. Meadows PY, Walker GC. Force microscopy studies of fibronectin adsorption and subsequent cellular adhesion to substrates with well-defined surface chemistries. *Langmuir* 2005;21(9):4096-4107.
 49. Pellenc D, Berry H, Gallet O. Adsorption-induced fibronectin aggregation and fibrillogenesis. *Journal of Colloid and Interface Science* 2006;298(1):132-144.
 50. Rouxhet L, Duhoux F, Borecky O, Legras R, Schneider YJ. Adsorption of albumin, collagen, and fibronectin on the surface of poly(hydroxybutyrate-hydroxyvalerate) (PHB/HV) and of poly(epsilon-caprolactone) (PCL) films modified by an alkaline hydrolysis and of poly(ethylene terephthalate) (PET) track-etched membranes. *Journal of Biomaterials Science-Polymer Edition* 1998;9(12):1279-1304.
 51. Grainger DW, Pavon-Djavid G, Migonney V, Josefowicz M. Assessment of fibronectin conformation adsorbed to polytetrafluoroethylene surfaces from serum protein mixtures and correlation to support of cell attachment in culture. *Journal of Biomaterials Science-Polymer Edition* 2003;14(9):973-988.

52. Webb K, Hlady V, Tresco PA. Relationships among cell attachment, spreading, cytoskeletal organization, and migration rate for anchorage-dependent cells on model surfaces. *Journal of Biomedical Materials Research* 2000;49(3):362-368.
53. Amaral IF, Lamghari M, Sousa SR, Sampaio P, Barbosa MA. Rat bone marrow stromal cell osteogenic differentiation and fibronectin adsorption on chitosan membranes: The effect of the degree of acetylation. *Journal of Biomedical Materials Research Part A* 2005;75A(2):387-397.
54. Scotchford CA, Gilmore CP, Cooper E, Leggett GJ, Downes S. Protein adsorption and human osteoblast-like cell attachment and growth on alkylthiol on gold self-assembled monolayers. *Journal of Biomedical Materials Research* 2002;59(1):84-99.
55. Scotchford CA, Ball M, Winkelmann M, Voros J, Csucs C, Brunette DM, Danuser G, Textor M. Chemically patterned, metal-oxide-based surfaces produced by photolithographic techniques for studying protein- and cell-interactions. II: Protein adsorption and early cell interactions. *Biomaterials* 2003;24(7):1147-1158.
56. Pettit DK, Horbett TA, Hoffman AS. Influence of the substrate binding characteristics of fibronectin on corneal epithelial cell outgrowth. *J Biomed Mater Res.* 1992;26(Oct 10):1259-1275.
57. Goncalves IC, Martins MCL, Barbosa MA, Ratner BD. Protein adsorption on 18-alkyl chains immobilized on hydroxyl-terminated self-assembled monolayers. *Biomaterials* 2005;26(18):3891-3899.
58. Steele JG, Johnson G, Underwood PA. Role of Serum Vitronectin and Fibronectin in Adhesion of Fibroblasts Following Seeding onto Tissue-Culture Polystyrene. *Journal of Biomedical Materials Research* 1992;26(7):861-884.