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TiO_2 type influences fibronectin adsorption

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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 H_2O_2 solution (TiO_2 cp) and TiO_2 sputtered on Si (TiO_2 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 ^{125}I, ^{125}I-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 TiO_2 cp surface than for the TiO_2 sp. As expected from the surface free energy values, the work of adhesion of FN is higher for the TiO_2 cp substrate, the more hydrophilic one, and lower for the TiO_2 sp substrate, the more hydrophobic one.

The adsorption isotherms were evaluated by two different techniques: radiolabelling of FN (^{125}I-FN) and XPS. TiO_2 cp adsorbs more FN than the TiO_2 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 ^{125}I-FN also suggests that FN adsorbs as a multilayer for FN concentrations in solution higher than 100 μg/mL.

1. Introduction

FN is a product of most mesenchemyal 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 cells [2] to the biomaterial surface through its central-binding domain RGD sequence. Indeed, this protein plays a key role in the biointergration of implants. Therefore the study of FN conformation adsorbed on biomaterials is of high relevance.

FN (MW ~500 KD) is a dimmer 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 H_2O_2 solution (TiO_2 cp) and TiO_2 sputtered on Si (TiO_2 sp). These surfaces were characterized elsewhere [7].

Adsorption isotherms, exchangeability of bound FN by free FN, and work of adhesion of FN to titanium

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oxide surfaces were assessed by wettability studies, X-ray photoelectron spectroscopy (XPS), and by radiolabelling of FN with ^{125}I, ^{125}I-FN.

2. Materials and methods

2.1. TiO_2 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 HNO_3 (v/v)), for 30 s. The substrates were pre-incubated in H_2O_2 10 mM for 48 h to obtain a regular titanium oxide film.

TiO_2 sputtered substrates were prepared using an automated, load locked ion beam deposition system (Nordiko N3000), described elsewhere [8].

The TiO_2 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-O_2 beam coming from the assist gun (15.02% O_2 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 ($5 \times 5\ \mu m^2$) 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, TiO_2 sputtered films were cleaned twice in acetone and immersed in a "piranha" solution (7 parts of 98% H_2SO_4 and 3 parts of 30% H_2O_2 (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.

2.2. 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.

2.3. X-ray photoelectron spectroscopy, XPS

XPS measurements were carried out on a VG Scientific ESCALAB 200A (UK) spectrometer using MgKα (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).

2.4. 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:

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

2.5. Protein quantification by radioactivity

Quantification of FN adsorbed on the TiO_2 surface was performed using ^{125}I labelled FN.

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

The contribution of free ^{125}I 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 ^{125}I ion equivalent to that present as ^{125}I 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 ^{125}I labelled FN occurred on TiO_2 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 ^{125}I-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 μl ^{125}I-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:

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

where the counts measure the radioactivity of the samples, $|\mathrm{FN}|_{\mathrm{solution}}$ 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 μg/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 μg/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.

3. Results

3.1. XPS

Wide scans were performed on TiO_2 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 TiO_2 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

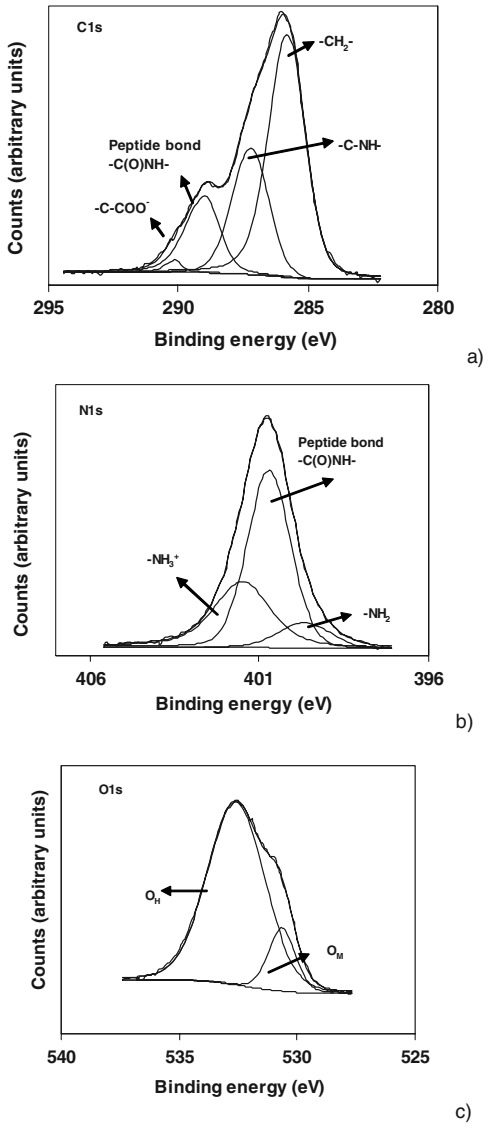

Figure 1 Representative deconvolution of XPS envelope for (a) C1s, (b) N1s and (c) O1s peak (after 60 min of FN 200 μg/ml adsorption at TiO_2 sp surface) at 0°.

to carbon atoms in different environments, i.e., saturated hydrocarbon groups (─CH_3, ─CH_2─) 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 eV [13]. Fig. 1(a)) shows an envelope for C1s deconvolution peak after FN adsorption ($|\mathrm{FN}| = 200\,\mu$g/mL), being the ─CH_2─ 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 ─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 [13, 14] (Fig. 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 adsorption [7], 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 Fig. 2.

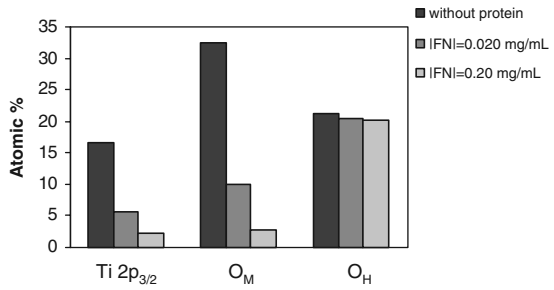

Figure 2 Atomic % with |FN| in solution after 60 min of adsorption on a TiO_2 cp surface.

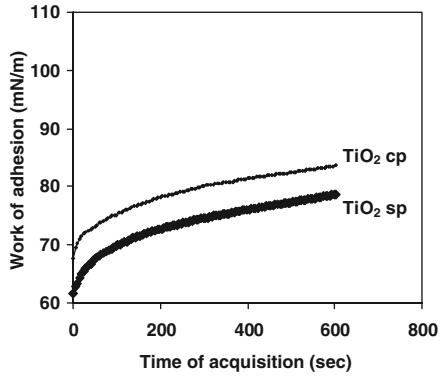

Figure 3 Work of adhesion between TiO_2 substrates and FN (|FN| = 200 μg/mL).

Upon adsorption the Ti% and the O_M % decreases, indicating that a film of protein is covering the titanium oxide (Fig. 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].

3.2. 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 Equation 1. The values of the contact angles of several solutions, given in Table I, were obtained by extrapolation of time-dependent experimental results to $t = 0$ s for PBS [7] 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 TiO_2 surfaces is dependent of FN concentration (Table I), since they are statistically different. When a FN solution was assayed it is observed a higher affinity of the solution to the TiO_2 cp surface. When a plasma solution was assayed it is observed a higher affinity of the solution to the TiO_2 sp surface.

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 I.

The time dependence of the work of adhesion between FN and TiO_2 surfaces was evaluated through the Young–Dupré equation, from the correspondent values of γ_{LV} and θ. Fig. 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 Fig. 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 (Fig. 4).

The work of adhesion between plasma and TiO_2 cp is opposite to the one found for the FN solutions (Fig. 4).

TABLE I Contact angle values of different solutions at TiO_2 surface ($n = 10$)

Solution	γ_{LV} (mN/m)	θ, degrees	
		TiO_2 sputtered	TiO_2 cp (after H_2O_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

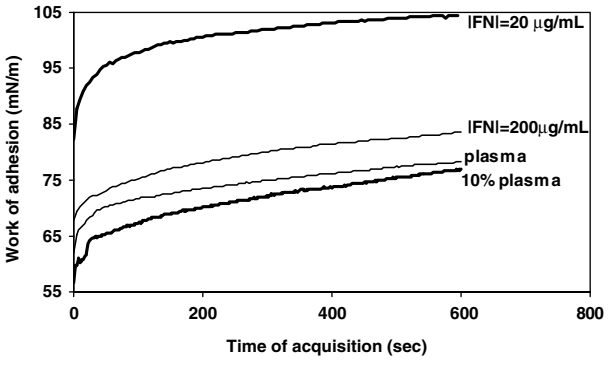

Figure 4 Work of adhesion between TiO_2 cp and protein solutions.

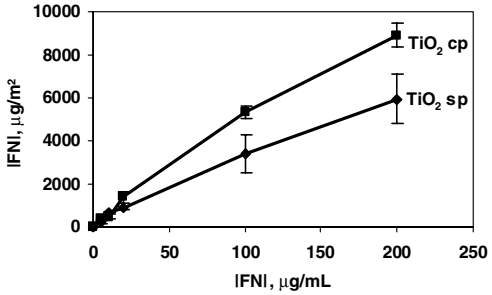

Figure 5 Adsorption isotherm of FN on TiO_2.

3.3. 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 μg/mL as determined by the bicinchoninic acid (BCA) assay. The amount of FN adsorbed in all experiments to TiO_2 cp was 2870 ± 1.95 μg/m^2.

Adsorption isotherms were performed using FN concentrations between 0 and 200 μg/ml, on both TiO_2 surfaces, and Fig. 5 shows average curves.

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 Fig. 6 it can be seen that for the FN concentrations studied, 60–70% of FN molecules are eluted from TiO_2 surface sp and only 10–55% of adsorbed protein was desorbed from TiO_2 cp surface.

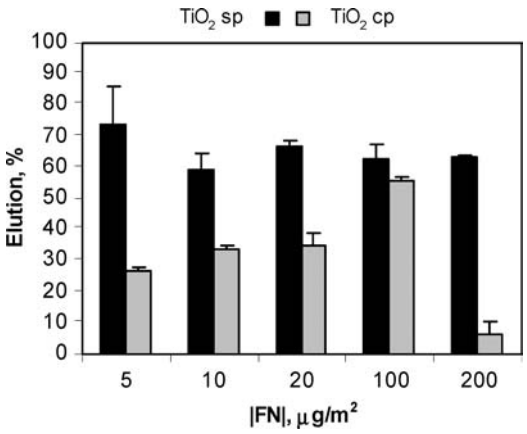

Figure 6 Elution of FN molecules by other FN molecules from TiO_2 sp and from TiO_2 cp as a function of FN concentration in solution. The elution time was 24 h.

4. 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 TiO_2 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 (Fig. 4). The opposite correlation was found for the plasma and TiO_2 cp substrate (Fig. 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 TiO_2 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 μg/m^2 and 41000 μg/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 μg/mL.

The FN molecules adsorbed on TiO_2 sp seem to be more easily exchanged (Fig. 6) than those adsorbed on TiO_2 cp after 24 h, since 60–70% of FN is eluted from TiO_2 sp and only 10–55% of adsorbed protein was desorbed from TiO_2 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 TiO_2 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 [17]. Tamada, Y. *et al* [24] referred that the minimum amount of FN required for fibroblast cell adhesion was 500 μg/m^2. After elution, and for FN concentration $\geq$20 μg/mL for TiO_2 cp, and $\geq$100 μg/mL for TiO_2 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 [17]. In the present study, FN is more weakly held (higher elutabily shown in Fig. 6, and lower work of adhesion shown in Fig. 3) at the TiO_2 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.

5. Conclusions

It has been demonstrated that when a FN solution was assayed it is observed a higher affinity of the solution to the TiO_2 cp surface. In contrast, when a plasma solution was assayed a higher affinity of the solution to the TiO_2 sp surface occurred. TiO_2 cp adsorbs also more FN than the TiO_2 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 TiO_2 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 ^{125}I-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 TiO_2 surface for the binding of FN and thus for the formation of a protein layer.

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