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Fibronectin adsorption studied using neutron reflectometry and complementary techniques

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Abstract. In implantology it is known that fibronectin affects cell-substrate adhesion, consequently, the structure and composition of the initially adsorbed fibronectin layer to a large extent determines the biological response to a biomaterial implanted into the body. In this study we have used neutron reflectometry and quartz-crystal microbalance with dissipation to investigate the amount of fibronectin adsorbed, the layer density, thickness and structure of films adsorbed to polished silicon oxide surfaces. We have cultured MG63 osteoblast-like cells on surfaces coated and uncoated with fibronectin and monitored the cellular response to these surfaces. The results show that at fibronectin concentrations in the range 0.01 to 0.1 mg/ml a single highly hydrated layer of fibronectin approximately 40–50 Å in thickness adsorbs to a polished silicon oxide surface and is likely to correspond to one diffuse monolayer of fibronectin arranged side-on. Cells cultured on this fibronectin layer have dramatically different morphology and growth to those grown on bare surfaces. Using a model silicon oxide surface has enabled us to study the substrate/protein interface, together with the impact of a fibronectin layer on the cellular response using consistent experimental conditions across a unique set of experimental techniques.

PACS. 61.05.fj Neutron reflectometry – 68.08.-p Liquid-solid interfaces – 68.43.Mn Adsorption kinetics

1 Introduction

The acceptance of implants into the body relies on cells being able to bind to the surface of the implant. Immediately after implantation into a patient, a proteinaceous layer will coat the implant and play a key role in the subsequent cell adhesion. The interactions at the implant surface give rise to osteoinduction by proliferation and differentiation of cells, revascularisation and eventual gap closure. Sometimes a strong bond is not formed between implant and local tissue. In these cases connective tissue can form at the interface resulting in a fibrous tissue capsule that prevents osteointegration and ultimately can cause implant failure. Understanding the nature of the initial protein layer and the cellular response to such a layer will help provide explanation as to why some implant materials perform better than others. Understanding these interfacial events at the molecular and cellular levels will allow us to optimize the integration of implants through the prediction of the local-tissue response to biomaterials.

Fibronectin is a well-characterized extracellular matrix cell-binding protein located in body fluid and connective tissue. Plasma fibronectin is one of the principle plasma proteins that adsorb rapidly onto a surface following implantation. Along with vitronectin, fibrinogen, collagens, laminin, osteopontin and other trace proteins, it mediates the specific interaction of cells with surfaces via cell integrin receptors [1]. Consequently the structure and composition of the adsorbed plasma fibronectin layer is a major factor in determining the biological response to a biomaterial. Fibronectin consists of two similar polypeptide chains each 600 Å in length and 25 Å in diameter, connected by flexible disulfide bonds. The adsorption of fibronectin (Fn) to various substrates has been studied in the past using scanning electron microscopy (SEM), X-ray photoelectron spectroscopy (XPS) and atomic force microscopy (AFM) [2–4]. Most previous studies report the effect that adsorption has on the cell growth and proliferation on different substrates such as hydroxyapatite ceramics and titatium [5–7]. For example, Galtayries *et al.* [3] used XPS to study Fn adsorption onto steel at various concentrations. They found that Fn adsorption reached a maximum at 60 μg/ml, above which increasing the concentration did not increase the Fn adsorption [3]. They measured the Fn layer thickness to be 63 Å and this was interpreted as one layer thickness. However, to use XPS,

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the fibronectin layer is dried onto the substrate, therefore the experiment was not performed close to *in vivo* conditions. Bergkvist and coworkers studied Fn adsorbed onto mica, silica, and methylated silica surfaces using liquid and ambient AFM methods. They revealed that surface physical properties influence the molecular structure of fibronectin on adsorption [8]. Their study indicates different Fn orientations on different surfaces, but the protein density and thickness were not measured.

In our work the interfacial adsorption of fibronectin has been examined as a function of protein concentration on a well-characterized model silicon oxide surface using neutron reflectometry (NR) and quartz-crystal microbalance with dissipation (QCM-D) techniques. To our knowledge, the adsorption of Fn has never been studied using neutron reflectometry previously, and we believe this technique in conjunction with QCM-D provides invaluable information in understanding the amount, density and structural orientation of the adsorbed fibronectin layer. In addition, using the same conditions we have monitored the cellular response to the bare model surface and a Fn-coated surface as a function of time over 14 days. Using the model silicon oxide surface has enabled us to study the adsorption of a fibronectin layer, and its impact on the cellular response using consistent experimental conditions across a unique set of experimental techniques.

2 Materials and methods

2.1 Sample preparation

Commercially available human plasma fibronectin (Sigma, UK) was dissolved in 0.1 M phosphate buffered saline (PBS) at *p*H 7.4 at a range of concentrations between 0.01 and 0.1 mg/ml and adsorbed onto polished ⟨111⟩ Si wafers with roughness average 6–10 Å (Crystran, UK). For all measurements the protein solutions were freshly prepared less than one hour in advance. For neutron reflectivity and cell response studies the Si substrate was incubated with protein solution for two hours at 37 °C. The QCM-D study was performed at room temperature using quartz single crystals coated with a silicon oxide layer (Q-Sense, Sweden).

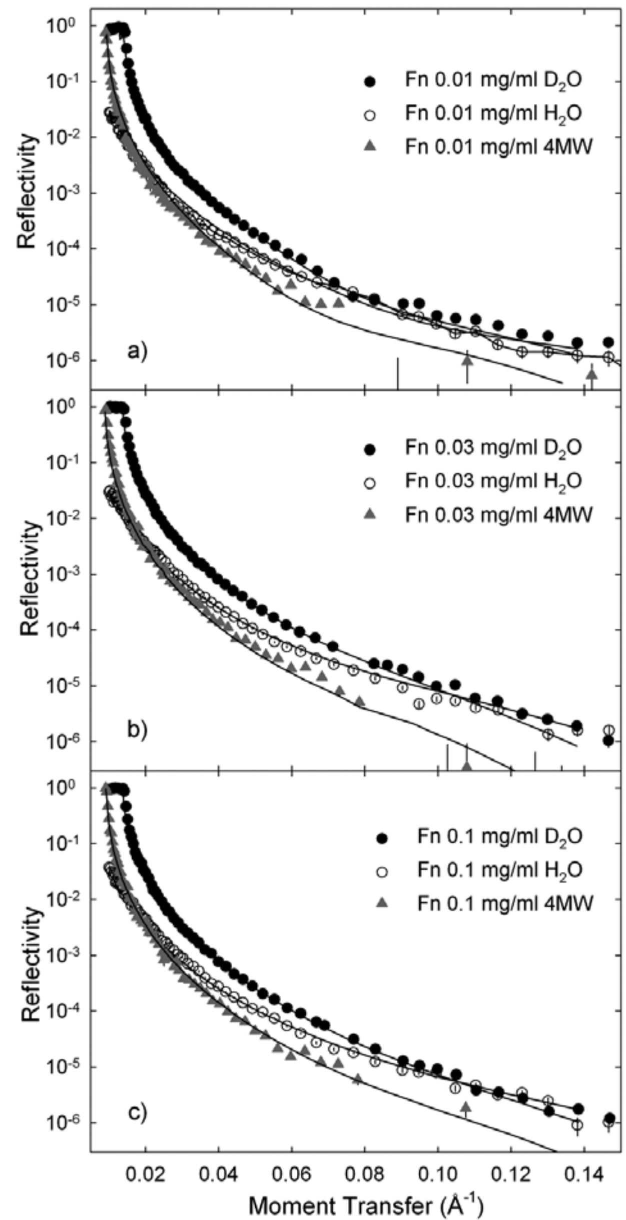

Fig. 1. Neutron reflectivity curves (symbols) and fits (lines) for fibronectin adsorbed onto SiO_2 at three concentrations a) 0.01, b) 0.03, and c) 0.1 mg/ml.

2.2 Neutron reflectivity

Neutron reflectometry is a well-established technique in the physical sciences for the study of surfaces and interfaces at the nanometer length scale, and is increasingly being recognized for its potential to study processes on this length scale in biological systems [9,10]. By measuring the reflected over the incoming intensity of a well-collimated neutron beam striking an interface, as a function of the incident angle and wavelength, the reflectivity curve is calculated.

We have used NR on the D17 instrument [11] at the Institut Laue Langevin, Grenoble to measure the protein layer density and thickness and to derive the orientation of Fn on SiO_2 at three different concentrations: 0.01, 0.03 and 0.1 mg/ml. Time of flight mode with three detector angle setup positions allowed a Q-range of 0.002 Å^{-1} to 0.4 Å^{-1} to be explored. The fluids were held in a Teflon trough clamped to the Si block to create the solid/liquid interface. The SiO_2 substrates were first cleaned using a UV/ozone treatment [12]. This treatment produces extremely hydrophilic surfaces (readily wetted by water) leaving a small negative charge on the silicon oxide surface (10–20%). The samples were maintained at 37 ± 1 °C by water circulating from a temperature-controlled water bath. Each sample was first aligned using a laser beam

Table 1. Parameters used for neutron reflectivity fits given in Figure 1. The data are interpreted with the presence of a monolayer that partially covers the surface.

Fibronectin concentration (mg/ml)	Layer thickness (Å)	SLD_{layer} (Å^2)	Solvent percentage ($\%_{vol}$)	Mass per unit area (mg/m^2)
0.01	39(1)	2.7(1)	78(2)	1.1(1)
0.03	50(5)	2.2(4)	95(2)	1.5(1)
0.1	41(2)	2.6(2)	92(2)	1.1(1)

following the same path as the neutron beam. This alignment was then optimized using the neutron beam. The SiO_2 surfaces were then characterized prior to protein adsorption with three standard contrast-matched water solutions: D_2O, H_2O and a 66–34% (v/v) D_2O-H_2O mixture labeled four-matched water (denoted 4MW), since it corresponds to a scattering length density of 4×10^{-6} Å^{-2}. These three water solutions were used to make up the PBS buffer in order to vary the scattering length density (SLD) for the measurements in the presence of protein. The reflectivity data were modelled using the optical matrix method [13], where the solid substrate and the liquid solution are considered having infinite thickness with fixed scattering length density. The interfacial region is described as discrete layers of uniform scattering material, each with a particular composition and corresponding SLD, thickness, and roughness with the adjoining layer. The parameters of the model were refined until the best least-squares fit to the data was achieved within the constraints imposed by the use of water contrasts.

2.3 Quartz crystal microbalance with dissipation (QCM-D)

QCM-D is a technique used to measure very small changes in the mass adsorbed (nanogram sensitivity) onto a surface by detecting the change in resonant frequency of a quartz crystal. We have used the Q-Sense E4 QCM-D to study the kinetics of Fn adsorption as a function of fibronectin concentration at 0.01, 0.03 and 0.1 mg/ml at room temperature.

The adsorbed surface mass density, Γ, was calculated from the Sauerbrey model where the change in the fundamental resonant frequency of the quartz crystal due to deposition of uniform films on the crystal surface should be linearly related to the increase in the mass by

$$\Gamma = \frac{\Delta f}{nC}\,, \qquad (1)$$

where Γ is the adsorbed surface mass density, Δf is the change in frequency, n is the overtone number and C is mass sensitivity constant ($5.72\,\mathrm{m^2\,Hz/mg}$ at $f_0 = 5\,\mathrm{MHz}$).

2.4 Cell culture

MG63 osteoblast-like cells at passage 8 were subcultured in modified Eagle's medium (MEM) supplemented with 10% fetal bovine serum, 100 units/ml of penicillin and 100 μg/ml of streptomycin, at a seeding density of 0.8×10^6 on sterile Si disks either uncoated or coated with fibronectin at two concentrations 0.01 and 0.1 mg/ml. The cultures were maintained at $37 \pm 1\,^\circ$C in a humidified atmosphere containing 5% CO_2 and 95% air. The cells were cultured for up to 14 days with the medium changed every other day. After 1, 7, and 14 days the cells were harvested and used for either cell morphology studies under the SEM or cell counting.

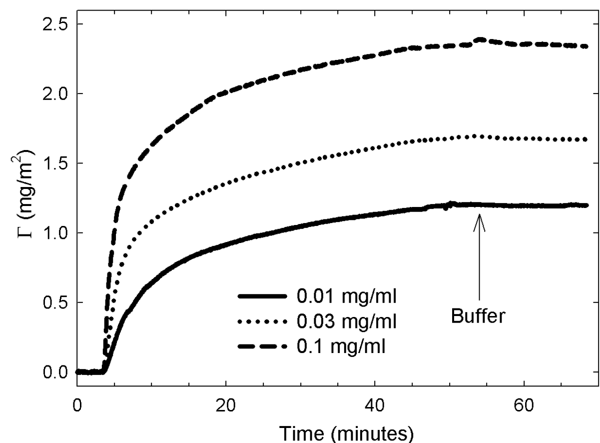

Fig. 2. Mass in mg/m^2 of adsorbed fibronectin as a function of time for three Fn concentrations: 0.01, 0.03 and 0.1 mg/ml. Γ has been calculated from equation (1).

3 Results and discussion

For the neutron reflectometry experiments, solvent deuteration was used to prepare three contrast-matched Fn solutions for data analysis. Figure 1 shows the neutron reflectivity curves for three Fn concentrations a) 0.01 b) 0.03 and c) 0.1 mg/ml and three solvents D_2O, H_2O and 4MW (four-matched water: 66–34% (v/v) D_2O-H_2O). For the D_2O and 4MW datasets the protein SLD value was calculated for 80% H-D exchange —this value was used as the starting value for refinement during the least-squares fitting procedure. Before introduction of fibronectin solution, the bare Si blocks were first characterized using neutron reflection in order to model the oxide layer (SiO_2). This layer was found to have a thickness of 11.9 ± 0.6 Å and a roughness of 8.0 ± 0.3 Å. The calculated specular reflectivities were obtained using the optical matrix model, and were fitted to the observed data by least-squares methods using the computer program drydoc [14]. The parameters used in the fits are summarized in Table 1. The scattering length density of fibronectin was calculated to be 1.94×10^{-6} Å^{-2} using its known amino acid sequence [15]. A single monolayer of fibronectin with thickness between 40 Å and 50 Å was found to be an adequate model for describing the adsorbed layer for all three Fn concentrations. Across all three Fn concentrations, a low volume fraction Fn layer with between 78% to 95% solvent inclusion was

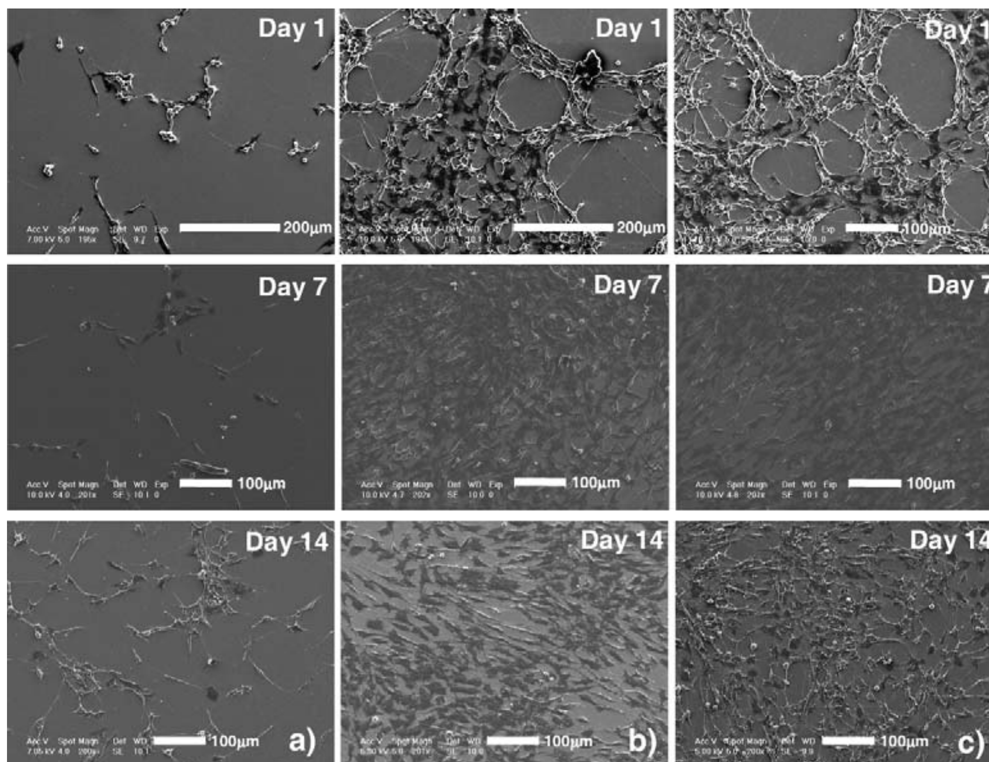

Fig. 3. SEM images of MG63 cell morphology after 1, 7 and 14 days incubation on a) bare silica substrate, b) Fn preadsorbed at 0.01 mg/ml, and c) Fn preadsorbed at 0.1 mg/ml.

observed. NR fit parameters reveal the Fn layer is approximately the same thickness at all three concentrations (within errors), however the percentage solvent appears to increase by approximately 10–15% as a function of concentration from $\sim$ 80% at 0.01 mg/ml to $\sim$ 90–95% at 0.03 and 0.1 mg/ml. It is generally agreed that after adsorption protein molecules undergo conformational relaxation resulting in structural rearrangements on a characteristic time scale (longer for globular proteins). These rearrangements are more pronounced for lower rates of adsorption or for adsorption from solutions of lower protein concentration [16]. As we have observed in our NR results study, this may well result in a more densely packed adsorbed layer from a lower protein concentration solution.

QCM-D was used to measure the kinetics of fibronectin adsorption on SiO_2. Figure 2 shows the QCM-D data plotting the mass per unit area in mg/m^2 of Fn adsorbed as a function of time calculated using (1) for three Fn concentrations. The Fn solution was introduced at 5 minutes, then after a further 50 minutes when the adsorption curve was seen to flatten, PBS was flushed through the sample cell to remove any loosely bound protein. It can be seen that after 20 minutes the majority of protein adsorption is complete, and after a further 30 minutes the adsorption process is complete. Using the Sauerbrey equation, the mass density of absorbed Fn is seen to increase as a function of time and concentration reaching maxima of $1.2 \pm 0.1\,mg/m^2$, $1.7 \pm 0.1\,mg/m^2$, and $2.3 \pm 0.2\,mg/m^2$ for the three concentrations 0.01, 0.03 and 0.1 mg/ml respectively. These values are in good agreement with the parameters obtained modeling the neutron reflectivity data (see Tab. 1) for the lower concentrations, however there appears to be a discrepancy between the values at 0.1 mg/ml. Although the Sauerbrey model provides an adequate description of the Fn adsorption at the lower concentrations, it assumes that the Fn layer is rigid (*i.e.* no dissipation) whereas the NR results indicate a very diffuse 5% protein layer at 0.1 mg/ml which may not behave in line with this model. Further modeling of QCM-D data which includes dissipative effects caused by a diffuse layer may be necessary in order to more accurately describe the Fn bound to the SiO_2 surface at 0.1 mg/ml.

The culture of MG63 cells onto sterile SiO_2 surfaces coated with Fn was used to investigate the cellular response to the characterized Fn layer. Figure 3 gives the SEM images of cell morphology at day 1, 7 and 14, with a) bare silicon oxide substrate, b) Fn preadsorbed at 0.01 mg/ml, and c) Fn preadsorbed at 0.1 mg/ml. At all three time points a large difference in cell coverage can be seen between the bare silicon oxide surface and those coated with fibronectin. Additionally at each time point the cell morphology appears different on the bare silicon oxide as compared to the Fn-coated disks. The cells in a) are isolated and globular in shape. In b) and c) they are more stretched out and appear to be interconnected —this is likely to be extracellular matrix produced by the cells. As a function of time, the SEM images reveal that the cell density increases at all concentrations.

One-way analysis of variance (ANOVA) statistical analysis of counted cells was used to compare the cell den-

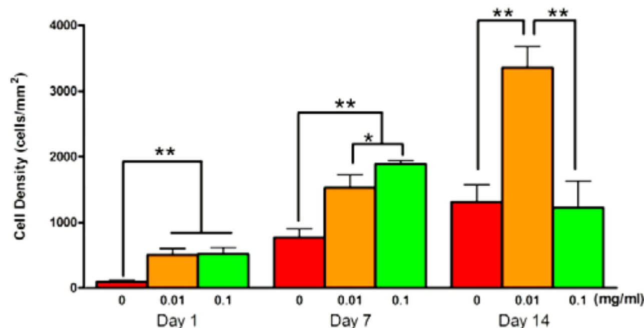

Fig. 4. Time evolution of MG63 cell density culture on Si disks coated with Fn at concentrations 0, 0.01 and 0.1 mg/ml. * indicates a statistical significance of $p < 0.05$, ** indicates $p < 0.01$.

sity at 1, 7 and 14 days with Fn preadsorbed at 0, 0.01 and 0.1 mg/ml. Figure 4 shows the cell density in cells per mm^2 as a function of surface and time. At day 1, the cell density is seen to increase very significantly between the bare and coated surfaces, however there is no difference between the surfaces coated with Fn at 0.01 and 0.1 mg/ml. At day 7, there is still a very significant increase in cell density when going from the bare Si to Fn-coated substrates, and there is also a significant difference in the two Fn-coated surfaces. By day 14 there is still a very significant difference in cell density between 0 and 0.01 mg/ml. The low value for cell density at day 14 and 0.1 mg/ml is most likely due to the cells becoming confluent and detaching from the Si surface, therefore it is difficult to interpret these results. ANOVA analysis has revealed that the cell density increases significantly as a function of Fn concentration and time on all three substrates. At all time points, the most dramatic increase appears to be between the bare SiO_2 substrate and Fn coated substrates —in good agreement with the SEM results.

4 Conclusions

We have shown that neutron reflectometry is a powerful tool for investigating the solid/liquid interface during fibronectin adsorption events. Neutron reflectivity data analysis has shown that a fibronectin layer of approximately 40–50 Å in thickness adsorbs to a polished silicon oxide surface at three concentrations 0.01, 0.03 and 0.1 mg/ml. This indicates that Fn has the same orientation at each concentration, and is likely to correspond to one diffuse monolayer of Fn arranged side-on. Neutron reflectometry also reveals that there is a high percentage of solvent within the fibronectin layer, and this solvent percentage increases slightly with concentration from 80% at 0.01 mg/ml to around 90–95% at 0.03 and 0.1 mg/ml. The QCM-D measurements agree generally with the low protein amount within the layer seen with NR. The cell culture study has revealed a dramatic difference in cellular response between the bare silica, and Fn coated silica substrates indicating that the presence of fibronectin is a major factor in cell response to the SiO_2 surface, while the Fn concentration affects the cell response much less in the range of 0.01 mg/ml to 0.1 mg/ml explored in this study. This highlights the importance of fibronectin in accelerating cell adhesion and growth even at very low concentrations. Since the NR results indicate the same orientation of Fn at 0.01 and 0.1 mg/ml concentrations and a similar surface mass density of Fn, it is perhaps not surprising that the cellular response to these two surfaces is similar. The use of a model silicon oxide surface to study Fn adsorption has given our investigation the advantage that we can directly relate the cellular response at the surface to the adsorbed Fn layer orientation, density, and amount, characterized using NR and QCM-D. This will have added importance in our future studies of the effect of substrate material, for example comparing ordered and disordered hydroxyapatite substrates as candidate biomaterials for implant coating. Our results provide new insights into fibronectin adsorption phenomena which may influence areas of biomedical research and clinical practice such as biomaterial design, tissue engineering and accelerated wound healing.

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