Interrogating protonated/deuterated fibronectin fragment layers adsorbed to titania by neutron reflectivity and their concomitant control over cell adhesion

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The fibronectin fragment, 9th–10th-type III domains (FIII9–10), mediates cell attachment and spreading and is commonly investigated as a bioadhesive interface for implant materials such as titania (TiO₂). How the extent of the cell attachment–spreading response is related to the nature of the adsorbed protein layer is largely unknown. Here, the layer thickness and surface fraction of two FIII9–10 mutants (both protonated and deuterated) adsorbed to TiO₂ were determined over concentrations used in cell adhesion assays. Unexpectedly, the isotopic forms had different adsorption behaviours. At solution concentrations of 10 mg l⁻¹, the surface fraction of the less conformationally stable mutant (FIII⁹¹⁰) was 42% for the deuterated form and 19% for the protonated form (fitted to the same monolayer thickness). Similarly, the surface fraction of the more stable mutant (FIII⁹¹⁰–H₂P) was 34% and 18% for the deuterated and protonated forms, respectively. All proteins showed a transition from monolayer to bilayer between 30 and 100 mg l⁻¹, with the protein longitudinal orientation moving away from the plane of the TiO₂ surface at high concentrations. Baby hamster kidney cells adherent to TiO₂ surfaces coated with the proteins (100 mg l⁻¹) showed a strong spreading response, irrespective of protein conformational stability. After surface washing, FIII⁹¹⁰ and FIII⁹¹⁰–H₂P bilayer surface fractions were 30/25% and 42/39% for the lower/upper layers, respectively, implying that the cell spreading response requires only a partial protein surface fraction. Thus, we can use neutron reflectivity to inform the coating process for generating bioadhesive TiO₂ surfaces.

1. Introduction

Extracellular matrix (ECM) proteins such as fibronectin (FN), laminin and collagen can be used to create biomimetic surfaces for cell adhesion and tissue engineering. By immobilizing biocompatible ECM proteins on a biomaterial surface, the interaction between it and the host cells can be influenced [1]. The large size of ECM proteins, however, can make it difficult to control orientation, and the binding site can be hidden when incorporated onto biomaterial surfaces leading to partial or complete loss of bimolecular activity [2]. Use of the RGD tripeptide (arginine–aspartic acid–glycine) has, therefore, become popular, given its relative amenability for polymer conjugation generating biomimetic scaffolds for tissue engineering and regenerative medicine [3]. Although RGD interacts with several integrin receptors, its use is limited because it cannot encapsulate the complexities of the binding environment [4]. This has prompted the revisiting of protein fragments based on ECM proteins. One example is the FN type III 9th and 10th domain pair (FIII⁹–¹⁰), which displays the RGD binding motif...
(FIII10) and the PHSRN synergy site (FIII9) and elicits a full integrin α5β1 binding response [5].

The conformational stability of native human FIII9–10 is relatively poor, with the FIII9 domain being especially unstable. However, mouse FIII9–10 has increased conformational stability compared with human and an 83% sequence identity over FIII9. Using this, stable-mutated human FIII9–10 domain pairs were developed by incorporating substitutions-identity over FIII9. Using this, stable-mutated human FIII9–10 is relatively poor, with the FIII9 domain being especially unstable. There is one other Pro–Pro pair in mouse FIII9–10 which we theorized could be a source of stability and so further substituted His in FIII9 for Pro-1377, terming this variant FIII910–H2P. Here, we assessed the stability of FIII910–H2P in the bulk and at the solid/liquid interface, because one potential application is to support cell adhesion and growth. In particular, determination of the conformation of the adsorbed FIII910–H2P is of importance, because the spatial relationship between RGD and PHSRN is critical for optimum integrin binding [8].

This is particularly relevant to generating biomimetic surfaces, because when proteins are adsorbed non-specifically to a surface this is generally associated with a change in conformation [9], leading to unfolding, ‘relaxation’ and partial desorption [10]. The ligand conformation may be compromised by immobilized proteins denaturing or adsorbing in the incorrect orientation. In order to have well-defined substrates with reproducible results, these adsorption changes should be characterized or controlled. The functional importance of adsorption-induced conformational change can be illustrated by a report of non-specific adsorption of FN to a gold substrate. Adsorption of FN alone resulted in the exposure of the type III domains from the surface, thus allowing the RGD and PHSRN regions to interact with cells. However, when FN was co-adsorbed with human serum albumin a rearrangement occurred such that the type I domains were exposed from the surface, essentially obscuring the cell binding domains [11].

Biomedical devices are commonly constructed from titanium because they have good fatigue strength, corrosion resistance and biocompatibility [12,13]. The surface layer of a titanium implant in contact with tissue will be composed of TiO2, which will influence its biocompatibility. Several studies have explored the effects of pre-treating the implant surface with integrin binding peptides to enhance biocompatibility. For example, RGD peptides grafted onto titanium rods supported increased bone thickness in rats [14]. In vivo osseointegration at grafted titanium implants in rats was improved threefold when using FII7–10 coatings instead of RGD coatings at the implant surface, attributed to the preferential binding of FII7–10 to integrin α5β1 [15].

The functional importance of the structure adopted by FN on surface adsorption and subsequent cellular response(s) has been noted in several studies. Eisenberg and co-workers have measured the function of RGD and PHSRN by the use of gold- or titanium-treated glass coverslips coated with phosphonate ligands, to covalently immobilize variants of FIII ninth and 10th domain fragments which appear to cooperatively enhance cell adhesion and spreading [16]. The surface properties of the support may have an influence on the behaviour of the proteins adsorbed to them. Toworfe et al. determined that surface hydrophobicity of the synthetic support altered the density of adsorbed whole-length fluorescent-labelled FN [17]. The change in density was estimated from surface roughness revealed in the atomic force microscopic (AFM) images. This could effectively alter the orientations of the protein which could reduce the area available to interact with the cell ligands. Surface wettability of the support influences the organization of both soluble and adsorbed FN [18]. Garcia et al. reviewed the adsorption of full-length FN onto varied treated polystyrene surfaces and the subsequent effect on integrin binding of myoblast C2C12 cells [19]. The production of variants containing differences surrounding the RGD motif allowed an aspect of the structural component on surface association to be examined.

Despite the evidence then that the cell-adhesion response to an ECM-like surface is dependent both on the binding motif and adsorption-induced changes, full biophysical characterization of the latter is difficult. Imaging techniques such as AFM have yielded insight regarding large conformational changes to large modular proteins such as FN, but provide only indirect measurement of the orientation, thickness and adsorbed fraction of a protein layer at the solid/liquid interface. Here, we have determined these parameters using neutron reflectivity (NR), whose resolution is further able to distinguish between one/more adsorbed protein layers. The confidence in modelling (multiple) adsorbed protein layers from NR data is greater when more than one contrasting dataset is acquired under the same conditions. To achieve this, we adsorbed both protonated and deuterated protein and measured the NR against D2O and H2O backgrounds, respectively. By relating the nature of the adsorbed wild-type and mutant FIII9–10 layers to the respective cell adhesion response, the importance of adsorption-induced events can be understood in the context of ECM-like coatings to biomaterials-relevant (Ti) surfaces. Characterization of cell adhesive protein surfaces to this level of detail has to date been lacking, but is necessary for understanding the cell—ligand interface in vivo and for the directed engineering of cell substrates.

2. Experimental

2.1. Protein expression and characterization

Primers were designed to mutate the FII910 cDNA to substitute His-1377 for Pro (FII910–H2P), see electronic supplementary material, figure S1. Expression of the wild-type and mutant FII9–10 proteins has been described in (FII9–10) [18], (FII910) [5] and (FII910–CC) [6]. A protein purity of more than 95% was determined by size exclusion chromatography.

Circular dichroism (CD) spectra were recorded in the far UV (180–260 nm) for the proteins diluted with water to 1 mg ml−1 in a 0.01 cm cell, with scan speed 50 nm min−1, 3 s time constant, bandwidth of 0.5 nm and six repeats (ChirascanTM spectrometer, Applied Photophysics, Surrey, UK). Mean residual ellipticity (deg cm2 dmol−1) was calculated from the measured ellipticity (millidegrees) for a mean residue weight of 110 MW. ‘Solid-state’ CD spectra were acquired in the far UV for the proteins adsorbed to silica beads in a rotating cell holder. Full experimental details are provided in the electronic supplementary material.

For equilibrium chemical denaturation, proteins (approx. 0.01 mg ml−1) were incubated for more than 2 h in 0 to approximately 8 M guanidine hydrochloride (GdmCl) in
10 mM NaH2PO4, 50 mM NaCl pH 6 (‘phosphate buffer’). Fluorescence spectra were recorded in a 1 cm path length cell with λex 290 nm and λem 350 ± 2 nm (Cary Eclipse spectrofluorometer, Agilent Technologies, Berkshire, UK). Calculation of the conformational stability of the protein as described by ΔG_H2O is described in the electronic supplementary material.

We determined the isoelectric point of the proteins by isoelectric focusing (IEF) using Immobiline™ DryStrips (pH 6–9). Full experimental details are provided in the electronic supplementary material.

2.2. Cell attachment and imaging

Cell attachment assays were carried out according to methods previously described in [5], with some variations. Nuclon® 96-well plates were coated with doubling dilutions of the proteins from stock 100 mg l⁻¹ overnight at 4°C. Excess protein was washed off with phosphate-buffered saline (PBS), pH 7.4, and uncoated plastic blocked with 5% bovine serum albumin in PBS. Ten thousand baby hamster kidney (BHK) cells were inoculated into each well for 1 h at 37°C, 5% CO2. Adherent cells were washed gently with PBS and fixed with 100 µl of 4% glutaraldehyde/4% formaldehyde. Adhesion was determined by staining with 0.1% crystal violet for 30 min and solubilized with 200 µl methanol. Absorbance was read at 570 nm, and the data normalized by the largest reading, expressed as the means ± standard error of eight replicates from three independent experiments.

For immunocytochemistry, 12 mm diameter glass coverslips were thin film coated with titanium by Helia Photonics Ltd, West Lothian, Scotland, generating TiO₂-plated glass coverslips. The TiO₂ surface was coated with protein at a bulk concentration fitted upon spontaneous oxidation of the titanium layer. The TiO₂ surface was coated with protein at a bulk concentration 100 mg l⁻¹ and washed. BHK cells were seeded gently with PBS and fixed with 100 µl of 4% glutaraldehyde/4% formaldehyde. Adhesion was determined by staining with 0.1% crystal violet for 30 min and solubilized with 200 µl methanol. Absorbance was read at 570 nm, and the data normalized by the largest reading, expressed as the means ± standard error of eight replicates from three independent experiments.

2.3. Neutron reflectometry and data fitting

NR measurements were made at the Australian Nuclear Science and Technology Organisation (ANSTO) using the ‘Platypus’ neutron reflectometer. The wavelength of the neutrons used ranged from 2.5 to 17.0 Å. Deuterated proteins, referred to as d-FIII9‘10, and d-FIII9‘10-D2P, were expressed and purified at the National Deuteration Facility, ANSTO, and lyophilized (see electronic supplementary material). Freshly polished silicon (100) substrates of 10 cm diameter and 1 cm thick were thin film coated with TiO₂ by Helia Photonics Ltd. Immediately before use, the substrates were cleaned in Decon 90 and thoroughly rinsed with deionized water and dried before clamping into a Teflon cell (void volume approx. 3 ml) into which protein was introduced for 30 min before data acquisition; the concentration being increased in a stepwise manner (10, 30, 100 and 200 mg l⁻¹ in phosphate buffer), followed by a final wash step with five volumes of buffer (made in H₂O or D₂O as appropriate). Each reflectivity profile was measured at incident angles of 0.5°, 1.8° and 5.0° at approximately 25°C, and the data reduced by dividing the raw data by direct beam spectrums collected with the same slit openings and then combined to yield a single reflectivity profile. The neutron beam illuminated an area of 40 x 60 mm² on the planar interface. Blank buffer measurements were performed for substrate characterization. A flat background, determined by extrapolation to high values of momentum transfer Q (Q = (4π sin θ)/λ, where θ is the glancing angle of incidence and λ the wavelength) was fitted to the datasets.

Theoretical scattering length density (SLD) values of the proteins were calculated as previously described [20], assuming an H/D exchange of approximately 85% consistent with levels found in the literature [21]; yielding r_calculated values for the protonated (in D₂O) and deuterated (in H₂O) forms of the proteins of 3.014 x 10⁻⁶ and 5.121 x 10⁻⁶ Å⁻², respectively. For each protein concentration series, datasets were analysed simultaneously using the ‘global fit’ within MOTOFIT [22], assuming that the interface was composed of a series of parallel layers and using the parameters fitted to the respective bare substrate in water contrast. The model parameters fitted to the data were SLD, layer thickness and interfacial roughness (constrained between 2 and 10 Å for proteins). The SLD for silicon was constrained to 2.07 x 10⁻⁵ Å⁻² because concentrated solutions of the (protonated) proteins were diluted into D₂O buffers, the SLD of D₂O was allowed to fluctuate from 6.0 to 6.3 x 10⁻⁶ Å⁻²; similarly, the SLD of H₂O was maintained at −0.56 to −0.55 x 10⁻⁶ Å⁻² taking into account the reconstitution of the lyophilized, deuterated proteins into H₂O. Calculation of the surface fraction (α) of the protein layer followed

\[ P_{\text{fitted}} = (1 - \alpha)P_{\text{D}_2\text{O}} + \alpha P_{\text{calculated}} \]

where \( P_{\text{fitted}} \) is the SLD made up from contributions of the protein and subphase in D₂O or H₂O as appropriate. The final data fits were then analysed via a Monte Carlo resampling method [23,24]. In this procedure, 1000 new datasets, based upon the original with Gaussian noise based upon the counting statistic added, are individually fitted. The output from these fits are.histogrammed for each parameters with the midpoint taken as the parameter value and the 95% confidence level determined from the distribution.

3. Results

3.1. Physical characterization of the mutants in solution

Proteins were obtained at modest yields following expression in E. coli and purification by affinity chromatography. The M₉ obtained from the SDS–PAGE corroborated the calculated MW of the proteins. The CD spectra (electronic supplementary material, figure S2A) showed that the proteins all displayed a similar, characteristically β-sheet spectra, consistent with the known structure and previously reported CD spectra [25]. Therefore, the introduction of the mutations to FIII9‘10 did not alter its solution structure.

For both mutant proteins, a two-step unfolding curve was observed with the initial step representing unfolding of the ninth domain and the second step corresponding to the 10th domain, consistent with the wild-type FIII9‘10 [26] (electronic supplementary material, figure S3). The values obtained from linear regression analysis of the slope and y-axis intercept of the corresponding unfolding domain are shown in table 1. The Leu-1408 to Pro mutation within the FIII9‘10 domain
Table 1. Equilibrium unfolding parameters for the first (ninth FIII domain) and second (10th FIII domain) denaturation step of FIII9–10, FIII9’10 and FIII9’10–H2P (cf. electronic supplementary material, figure S3).

<table>
<thead>
<tr>
<th>FIII domain</th>
<th>[GdmCl] (M)</th>
<th>m (kcal mol⁻¹ M⁻¹)</th>
<th>ΔG_H2O (kcal mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIII9</td>
<td>2.15</td>
<td>3.41</td>
<td>7.33</td>
</tr>
<tr>
<td>FIII9’–H2P</td>
<td>2.85</td>
<td>3.23</td>
<td>9.20</td>
</tr>
<tr>
<td>wild-type FIII9’</td>
<td>1.1</td>
<td>4.3</td>
<td>4.9</td>
</tr>
<tr>
<td>FIII10 (of FIII9’10)</td>
<td>5.36</td>
<td>1.86</td>
<td>10.00</td>
</tr>
<tr>
<td>FIII10 (of FIII9’10–H2P)</td>
<td>5.41</td>
<td>2.04</td>
<td>11.03</td>
</tr>
<tr>
<td>wild-type FIII10’</td>
<td>4.5</td>
<td>2.8</td>
<td>12.6</td>
</tr>
</tbody>
</table>

aData from [7].

bSee electronic supplementary material for calculation of ΔG_H2O.

pair is also able to confer a significant increase in structural stability to the native FIII9 domain, consistent with earlier studies [7]. The conformational stability of the double-mutated FIII9 domain (H-1377/P, L-1408/P) in the FIII9’10–H2P protein was increased further, approaching even that of FIII10 as measured by ΔG_H2O on account of denaturation occurring only at higher [GdmCl] and a steeper slope (m). FIII9’10–H2P is therefore the most stable human FIII9–10 mutant reported to date and warranted further interrogation of functional activity and surface adsorption characteristics.

3.2. Functional characterization of the mutants

The BHK cells attached to the plastic wells coated with FIII9–10, FIII9’10 or FIII9’10–H2P from concentrations as low as 1 mg l⁻¹ (electronic supplementary material, figure S4). Comparison of the cell attachment curves showed that the mutants had an equivalent functional activity compared with the wild-type at low coating concentrations (less than or equal to 10 µg ml⁻¹) but greater activity at concentrations greater than or equal to 50 mg l⁻¹, where the wild-type supported approximately 75% of the maximal cell attachment observed for the two mutants; this difference being statistically significant (p < 0.05), calculated by one-way ANOVA assuming Gaussian distributions. The greater cell attachment on substrates coated with the more conformationally stable mutants is in agreement with previous data [7]. Cell attachment to uncoated surfaces was very limited, and the absorbance value obtained was subtracted from protein-coated surfaces to compensate for any unspecific binding of the cells. It is important to note that the range of concentrations chosen for the BHK cell attachment studies (0.1–100 mg l⁻¹) and NR studies (10–200 mg l⁻¹ and wash) overlap, purposefully, to facilitate direct interpretation between these two quantitative datasets.

BHK cell attachment to TiO₂-coated glass coverslips lacking any additional protein coating was only marginal (figure 1a,b); just a few small, rounded cells were observed in which only the nucleus could be visualized, the majority lacking clear focal adhesions and organized actin stress fibres. Cells attached to TiO₂ surfaces coated with 100 mg l⁻¹ FIII9–10, FIII9’10 or FIII9’10–H2P showed dramatically greater cell attachment and spreading (figure 1c–h). The cells were mostly large and well spread with clearly visible actin stress fibres and focal adhesions at the periphery (contrasting against the fewer small, rounded cells). The immunofluorescence images only suggested slight differences between cell behaviour on FIII9–10, FIII9’10- or FIII9’10–H2P-coated surfaces: a greater cell density was evident on FIII9’10 and FIII9’10–H2P, with the latter supporting some large focal adhesions but (at higher magnification) with shortened stress fibres. However, these remain interpretations and only give a suggestion as to the behaviour of the cells on the different surfaces.

3.3. Protein adsorption to TiO₂ surfaces

Four titania (TiO₂)-coated silicon substrates were used in total for these experiments, one for each protein: FIII9’10, d-FIII9’10, FIII9’10–H2P and d-FIII9’10–H2P (d- refers to the deuterated protein). We did not study the wild-type FIII9–10 by NR, because our previous data for FIII9–10 adsorption on silicon oxide (SiO₂) surfaces showed a rather featureless reflectivity profile; a simple model wherein FIII9–10 adsorbed side-on as a monolayer was sufficient to fit the data for concentrations up to 400 mg l⁻¹ [27]. A global fit was made within MOTOFIT, wherein the substrate parameters were maintained for all datasets acquired for the same protein concentration series. The protonated and deuterated forms of the same mutant protein were expected to adsorb to the surface with the same layer thicknesses on the basis of solid-state CD [28] data (see below); the best fit for this parameter was therefore constrained leaving only the change in surface fraction between the two forms to be determined. It was found that the four substrates were very similar as regards the thickness, SLD and roughness of the TiO₂ layer: 251.4–253.6 Å, 2.13 ± 0.03 × 10⁻⁶ Å⁻² and approximately 2–6 Å, respectively (electronic supplementary material, table S1). The difference in the SLD of the TiO₂ layers was likely due to small variations in deposition. The global error of fit (χ²) was 5.87 and 7.23 for the FIII9’10 and FIII9’10–H2P forms, respectively, which was considered very reasonable for the simultaneous fit of these datasets.

Solid-state CD spectra acquired for FIII9’10 and FIII9’10–H2P adsorbed to silica beads showed that both mutants retained their native β-sheet structure. This is in contrast to FIII9–10 which partially lost secondary structure on adsorption, as measured by smaller positive ellipticities at 203 and 226 nm (electronic supplementary material, figure S2B). For FN domains, the positive CD band centred around 226 nm (electronic supplementary material, figure S2B). For FN domains, the positive CD band centred around 226 nm (electronic supplementary material, figure S2B).
aligning horizontally with the substrate surface. At 30 mg l\(^{-1}\), the domain pair was considered to have tilted upwards (‘tilt’ here referring to the domain pair as a whole, rather than with reference to a change in the interdomain angle) away from the plane of the surface presumably on account of increased crowding between neighbouring molecules.

During the fitting process, while maintaining layer thicknesses between the protonated and deuterated proteins and then comparing the fitted SLDs, it was surprising to find clear differences in the surface fraction of the two isotopic forms. For bulk concentrations of 10 and 30 mg l\(^{-1}\), there was an upper limit of approximately 19% and 27% surface fraction for the FIII\(_9\)10 monolayer and 41.5% and 42%, respectively, for the d-FIII\(_9\)10 monolayer. The differences observed in the surface fraction between the protonated and deuterated forms were seen consistently throughout these studies and will be discussed below.

The reflectivity profiles at concentrations of 100–200 mg l\(^{-1}\) for FIII\(_9\)10 could only be fitted to a bilayer model (table 2), where the thickness of the two layers was dependent on bulk concentration. The upward tilt of the domain pair directly adsorbed to the TiO\(_2\) surface (the ‘lower’ layer), already observed for the monolayer at 30 mg l\(^{-1}\), caused a further layer thickness increase to approximately 53 Å at 100 mg l\(^{-1}\). The increased tilt was again accompanied by an increase in crowding at the surface. A second ‘upper’ layer was also fitted with a side-orientation. At 200 mg l\(^{-1}\), the surface fraction of the protonated FIII\(_9\)10 reached a maximum at 31% and 35% for lower and upper layers, respectively. The increase in crowding at the upper layer was accompanied by a tilt away from the plane of the surface. However, because the thickness of the lower layer decreased, this implied some form of compression of the lower layer, or tilt back towards the plane of the surface. For deuterated FIII\(_9\)10 fitted to the same layer thicknesses, surface fraction behaviour was distinguished by a dense lower and sparse upper layer at 100 mg l\(^{-1}\), and a maximal density for both layers (51–53%) at 200 mg l\(^{-1}\).

On washing of the surface the apparent compression of the lower layer, seen with increasing solution concentration from 100 to 200 mg l\(^{-1}\), was reversed. Because this was accompanied by a decrease in surface fraction of the upper layer, this compression was most likely to be due to a change in the interaction between the layers and neighbouring molecules therein (rather than as a result of a ‘compressive force’). While the deuterated FIII\(_9\)10 had the greater adsorbed mass per unit area at high bulk concentrations, the same was not true following the wash step. Almost all the upper layer desorbed for the deuterated form, while only a small fraction desorbed for the protonated form, such that the sum of the surface fractions was almost the same (54% and 55%, respectively, table 2). This differential behaviour between the protonated and deuterated FIII\(_9\)10 layers can also be clearly seen when plotted as the histogrammed output for the data fits analysed via the Monte Carlo resampling method (electronic supplementary material, figure S6). Note, for non-covalent adsorption, a diffusion gradient exists at the surface such that the model SLD profiles should be asymmetric in shape; however, while the position of individual protein molecules will fluctuate in equilibrium systems, on the timescale of the NR experiments only the average position of the proteins is probed and can be modelled accordingly.

The reflectivity profiles for FIII\(_9\)10–H2P were fitted to a monolayer at solution concentrations of 10 and 30 mg l\(^{-1}\).
and a bilayer a 100 and 200 mg l⁻¹ and after the wash (table 3); this behaviour matching that of FIII9–10. The thickness of the FIII9–10–H2P layer in direct contact with the TiO2 surface was greater than the equivalent FIII9–10 layer over all concentrations except after the wash (cf. h for layer 1 in tables 2 and 3). One would therefore infer that FIII9–10–H2P adsorbed to the surface with an increased upwards tilt of the domain pair away from the surface. This behaviour has previously been observed by our group when comparing the wild-type FIII9–10 with the more stable FIII9 mutant [27], and is likely to be due to a decreased mobility around the domain interface. At 100–200 mg l⁻¹, the approximately 62 Å thickness of the adsorbed lower FIII9–10–H2P layer implied a near perpendicular orientation from the surface (given that the axial length is approx. 70 Å as described above). Thus, the apparent compression seen for FIII9–10 at 200 mg l⁻¹ was not observed for FIII9–10–H2P and is likely to reflect its hindered domain–domain mobility on account of greater conformational stability.

As for FIII9–10, the deuterated FIII9–10–H2P displayed a different adsorption behaviour to the protonated FIII9–10–H2P, with greater monolayer surface fraction at low bulk concentrations. The behaviour as the bilayer was also distinct, but in this case, the deuterated form had a lower surface fraction at high bulk concentrations up to the wash step at which point the surface fraction for the lower layer was similar (42% versus 39% for protonated and deuterated forms, respectively). Also similar to FIII9–10, the deuterated FIII9–10–H2P desorbed from the upper layer to a greater extent than the protonated FIII9–10–H2P (table 3). The histogrammed outputs for the corresponding data fits clearly show these distinct trends for the two isotopic forms during the wash step (electronic supplementary material, figure S7). When comparing the Monte Carlo generated histograms, it is interesting to note that the protein fraction of the lower and upper layers for either FIII9–10 or FIII9–10–H2P are very similar either at 200 mg l⁻¹ or after the wash (electronic supplementary material, figures S6 and S7). This is not the case for the corresponding deuterated protein layers where the upper layer is largely washed off. For upper and lower layers to have similar protein fractions either before or after the wash step would suggest a near optimal packing arrangement of (protonated) molecules which consequently undergo minimal desorption.

4. Discussion

The introduction of a second Pro–Pro pair by mutation of His-1377 in the FIII9 cDNA yielded a significant increase in the conformational stability of the resultant double-mutated FIII9 domain. The two Pro–Pro pairs in the mouse FIII9 domain (cf. none in wild-type human FIII9) would be expected to severely restrict the local flexibility of the protein backbone because of the narrow range of dihedral angles allowed by the Pro residue. Because a stepwise increase in ΔG_{H2O} is seen with each additional Pro–Pro pair (table 1), the relatively high conformational stability of FIII9–10–H2P can be directly attributed to these two mutations (H-1377/P, L-1408/P).

It was important to verify that FIII9–10–H2P retained functional activity, measured as cell attachment and spreading, because His-1377 is present in the human FIII9 PHSRN synergy site, which increases the binding affinity for integrin α5β1. It has been shown that the key amino acid in the PHSRN sequence is the Arg-1379, although for optimum efficacy all five residues are required [32]. BHK cells attached equally to plasticware coated with the three proteins from bulk concentrations less than or equal to 20 mg l⁻¹. At concentrations greater than or equal to 50 mg l⁻¹, the FIII9–10 and FIII9–10–H2P mutants supported approximately 33% greater cell adhesion than FIII9–10. This is consistent with previous data showing that FIII9–10 had improved functional activity over FIII9–10⁻¹. This study therefore provides a further example of how a single residue substitution away from the RGD
Table 2. Fitted layer parameters for one- and two-layer models of protonated and deuterated FIII9–10 with surface fractions derived from the SLD values in electronic supplementary material, table S2. The numbers in parentheses are the error in the final figure of the surface fraction.

<table>
<thead>
<tr>
<th>protein conc. (mg l⁻¹)</th>
<th>h₁, layer 1$^\circ$ (Å)</th>
<th>h₂, layer 2$^\circ$ (Å)</th>
<th>FIII9–10 surface fraction</th>
<th>d-FIII9–10 surface fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>layer 1</td>
<td>layer 2</td>
<td>layer 1</td>
<td>layer 2</td>
</tr>
<tr>
<td>10</td>
<td>—</td>
<td>36</td>
<td>0.19 (1)</td>
<td>—</td>
</tr>
<tr>
<td>30</td>
<td>46</td>
<td>—</td>
<td>0.27 (5)</td>
<td>—</td>
</tr>
<tr>
<td>100</td>
<td>53</td>
<td>36</td>
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<td>0.22 (3)</td>
</tr>
<tr>
<td>200</td>
<td>42</td>
<td>41.7</td>
<td>0.31 (1)</td>
<td>0.35 (1)</td>
</tr>
<tr>
<td>wash</td>
<td>53</td>
<td>36</td>
<td>0.30 (1)</td>
<td>0.25 (1)</td>
</tr>
</tbody>
</table>

$^\circ$, deuterated protein; wash, exchange of bulk protein solution (200 mg ml⁻¹) for buffer; h, layer thickness; $^\circ$, layer 1 is in direct contact with the substrate and layer 2 is the second, outer layer. χ² for all fits was 5.87; 95% CIs for all fitted parameters are shown in electronic supplementary material, table S2 (see electronic supplementary material). Data for the protonated and deuterated proteins was acquired in D₂O and H₂O phosphate buffers, respectively.

Table 3. Fitted layer parameters for one- and two-layer models of protonated and deuterated FIII9–10–H₂P with surface fractions derived from the SLD values in electronic supplementary material, table S3. The numbers in brackets are the error in the final figure of the surface fraction. Notations as described in table 2. χ² for all fits was 7.23; 95% CIs for all fitted parameters are shown in electronic supplementary material, table S3.

<table>
<thead>
<tr>
<th>protein conc. (mg l⁻¹)</th>
<th>h₁, layer 1$^\circ$ (Å)</th>
<th>h₂, layer 2$^\circ$ (Å)</th>
<th>FIII9–10–H₂P surface fraction</th>
<th>d-FIII9–10–H₂P surface fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>layer 1</td>
<td>layer 2</td>
<td>layer 1</td>
<td>layer 2</td>
</tr>
<tr>
<td>10</td>
<td>43</td>
<td>—</td>
<td>0.18 (1)</td>
<td>—</td>
</tr>
<tr>
<td>30</td>
<td>60</td>
<td>—</td>
<td>0.26 (1)</td>
<td>—</td>
</tr>
<tr>
<td>100</td>
<td>62</td>
<td>36</td>
<td>0.6 (2)</td>
<td>0.48 (2)</td>
</tr>
<tr>
<td>200</td>
<td>62</td>
<td>36</td>
<td>0.53 (2)</td>
<td>0.48 (1)</td>
</tr>
<tr>
<td>wash</td>
<td>52</td>
<td>40</td>
<td>0.42 (1)</td>
<td>0.39 (1)</td>
</tr>
</tbody>
</table>

d-, deuterated protein; wash, exchange of bulk protein solution (200 mg ml⁻¹) for buffer; h, layer thickness; $^\circ$, layer 1 is in direct contact with the substrate and layer 2 is the second, outer layer. χ² for all fits was 7.23; 95% CIs for all fitted parameters are shown in electronic supplementary material, table S3.

Attachment and spreading of BHK cells on TiO₂ was clearly dependent on presentation of the FIII9–10 proteins, in agreement with previous work [15]. In these experiments, the TiO₂ surface was coated with protein from a bulk concentration of 100 mg l⁻¹, within the upper range of concentrations studied by NR. As mentioned above, it is not possible to accurately quantify changes in cell behaviour and relate these to differences in the adsorbed layer thickness and protein surface fraction. However, we can relate the overall cell spreading response to the NR-determined surface fractions following the wash step (as used in the cell coating procedure); these were 30/25% and 42/39% for the lower/upper layers of FIII9–10 and FIII9–10–H₂P, respectively, implying that the cell spreading response requires a partial protein surface fraction.

Adsorption of proteins to TiO₂ surfaces has been shown to be mostly irreversible and can occur spontaneously from solution [33]. Full-length FN has been shown to adsorb to TiO₂ at a maximum concentration following only 30 min incubation time, irrespective of surface hydrophobicity [13]. In our NR experiments, the protein solution was incubated with the surface for 30 min and so it was assumed that free versus adsorbed protein would be at equilibrium upon measurement. The surface fraction of all of the proteins studied was found to be concentration-dependent, with the presence of a second upper layer at higher concentrations. As for adsorption of FIII9–10 to the negatively charged SiO₂ surface [27], adsorption of FIII9–10 and FIII9–10–H₂P to the negatively charged TiO₂ surface at approximately pH 6 [34] was likely to be driven by electrostatic interactions with the arginine cluster of FIII9 (Arg-1358, 1360, 1369, 1371, 1374, 1379—which can be readily visualized and compared against the relatively weak cationic face of FIII10 [27]).

Monolayer and bilayer thicknesses for both mutants generally increased with increasing bulk concentrations, with the tilt of the more stable FIII9–10–H₂P implying a near perpendicular orientation relative to the surface at the highest concentrations studied. This is consistent with NR data for another highly conformationally constrained FIII9–10 mutant, which was similarly tilted upwards away from the surface [27]. We have previously explained the increased upward tilt of FIII9–10 proteins relative to the surface (SiO₂) with increasing concentration with reference to the surface pressure area isotherm of a surfactant at the air/liquid interface [27]. Comparing the protonated mutants, the increased surface fraction seen for FIII9–10–H₂P over FIII9–10 fits the same surface pressure hypothesis, wherein the less conformationally stable (more mobile) FIII9–10 generates a greater barrier to the adsorption of further FIII9–10 molecules. Interestingly, the lower FIII9–10 (but not FIII9–10–H₂P) layer underwent apparent compression at 200 mg l⁻¹ bulk concentration (table 2). The likely explanation for this is

binding site can increase functional activity via a long range conformational change.

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the greater domain–domain mobility of FIII9’10, facilitating a greater change in the intermolecular packing between layers and neighbours as surface fraction increases. The apparent compression of an adsorbed, lower protein layer to a TiO₂ surface is reflected in another study [32], which describes mixed protein bilayers whose properties are dependent on the sequence of injection of the two protein systems. Similarly, reorientation of proteins such as lysozyme, antibodies and FN at the surface with a change in bulk concentration has also been observed [11,35,36].

We can now begin to interpret how surface adsorption affects cell behaviour, aided by the overlap in concentrations used in our biophysical and cell experiments. First, the smaller fraction of cells attached to FIII9–10 at higher coating concentrations can be linked to adsorption-induced partial denaturation. Denaturation will perturb the spatial relationship between the RGD motif on the FIII10 domain and the synergy site on FIII9. Because this spatial relationship switches between high and low integrin binding activity [8], maximal cell attachment was only observed on surfaces coated with the more conformationally rigid FIII9’10 and FIII9’10–H₂P, because these mutants did not undergo adsorption-induced denaturation.

Having established that adsorbed FIII9’10 and FIII9’10–H₂P retain a near native structure, the NR data for concentrations equivalent to those used in the cell studies unambiguously showed that only partial surface fractions were required to support cell attachment and cell spreading. Coating concentrations of 100 mg l⁻¹ supported maximal cell adhesion and pronounced cell spreading which equated to surface fractions of 30/22% and 60/48% for the lower/upper layers of FIII9’10 and FIII9’10–H₂P, respectively. Lower surface fractions were adequate to support submaximal cell attachment, for example, 10 mg l⁻¹ bulk concentrations equated to approximately 20% surface fraction of either mutant and a cell attachment of approximately 80%. Below a bulk concentration of 10 mg l⁻¹, direct correlation between NR and cell attachment data can no longer be made here, but the cell attachment data strongly imply that BHK cells can attach to surfaces with protein surface fractions less than 20%.

It is reasonable to assume that the cell surface integrin receptors can only interact with the upper adsorbed protein layer where a bilayer arrangement exists, because the lower layer cannot be fully solvent exposed. Because there was no evidence of denaturation of adsorbed FIII9’10 and FIII9’10–H₂P by NR or CD (i.e. it is not the case that the adsorbed lower layer is denatured), it would appear unnecessary for there to exist a bilayer structure in order to engage adherent cells. However, the nature of non-specific adsorption means that there is no control of bilayer or monolayer other than through a change in bulk concentration, e.g. bulk concentrations less than or equal to 30 mg l⁻¹ resulted in a monolayer at the surface. What would be required in terms of coating concentration to generate functional surfaces for a specific in vivo tissue/cell engineering goal is outside the scope of this work, but prompts exploration by more specialized, biomedical research teams.

The emergence of differences in the surface fraction between the protonated and deuterated forms of the same protein were somewhat surprising, and the authors are not aware of similar reports in the literature. If the initial adsorption event of a protein to a charged surface is driven by electrostatic interactions (as assumed to be the case here), then it would be expected that the protein pi and pH/ionic strength of the buffer are critical. Given that the buffer recipe was the same and account was made for pD/pH in preparation, a subtle change in pi may provide an explanation. IEF was therefore employed but the resolution of the IEF strips was insufficient to discern the very small differences that may have been present (electronic supplementary material, table S4). An alternative explanation as regards conformational effects owing to deuteration was unlikely, because the far UV CD spectrum of d-FIII9’10 had the same profile as for FIII9’10 (data not shown). This finding is further supported by a data mining report suggesting that in general deuterated proteins are structurally indistinct from their protonated counterparts (a bar select few) [30]. However, the same study also suggested that exchange into a deuterated solvent may lead to slight ‘stiffening’ as regards the mechanical properties of the protein. This is corroborated by a study of ribonuclease A [37], which has an increase in melting temperature when buffer exchanged into deuterated solvent, and is thought to represent a more compact structure in D₂O. By extension, if the FIII9–10 mutants were indeed ‘stiffer’ or more compact in D₂O then one would anticipate a greater surface fraction for the protonated form (which was studied in D₂O) with reference to the surface pressure area isotherm analogy, above. However, this effect would be offset by inherent structural stabilities and may explain the complexity in interpreting surface fraction changes between the two mutants and their isotopic forms.

5. Conclusion

We have characterized in detail the nature of adsorption of a FN fragment at the TiO₂/liquid interface. The adsorption behaviour generally follows a previously described model which is analogous to the surface adsorption isotherm of a surfactant. The BHK cell spreading response was strong on TiO₂ surfaces coated with all FIII9–10 proteins and, so the requirement for solution conformational stability is not evident for the TiO₂ surface (at least at high bulk concentrations). There was no evidence of denaturation of either mutant upon adsorption to TiO₂ from both the solid-state CD data and the layer thicknesses determined by NR, which were comparable to the dimensions of FIII9–10 (as determined by NMR and X-ray crystallography) over all concentrations tested. For maximal cell attachment and cell spreading, it was not necessary for the surface to be saturated with the FN domain pair, indeed, only approximately 20% surface fraction was sufficient for approximately 80% cell attachment. Of particular interest to this study was the unexpected finding that both deuterated mutants have different adsorption behaviour to their protonated counterparts. While this may be on account of the solvent rather than the isotopic form per se, this would require corroboration from other NR studies. In summary, relating the cell response to the characteristics of an adsorbed protein layer is important as a first step to understanding and controlling the integration between cells/tissue and an implant surface.

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