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The strength of the protein-material interaction determines cell fate

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ABSTRACT

Extracellular matrix (ECM) proteins are key mediators of cell/material interactions. The surface density and conformation of these proteins adsorbed on the material surface influence cell adhesion and the cellular response. We have previously shown that subtle variations in surface chemistry lead to drastic changes in the conformation of adsorbed fibronectin (FN). On poly(ethyl acrylate) (PEA), FN unfolds and displays domains for cell adhesion and FN-FN interaction, whereas on poly(methyl acrylate) (PMA) – with only one methyl group less – FN remains globular as it is in solution. The effect of the strength of the protein/material interaction in cell response, and its relation to protein density and conformation, has received limited attention so far. In this work, we used FN-functionalized AFM cantilevers to evaluate, via force spectroscopy, the strength of interaction between fibronectin and the underlying polymer which controls FN conformation (PEA and PMA). We found that the strength of FN/PEA interaction is significantly higher than FN/PMA, which limits the mobility of FN layer on PEA, reduces the ability of cells to mechanically reorganize FN and then leads to enhanced proteolysis and degradation of the surrounding matrix with compromised cell viability. By contrast, both PEA and PMA support cell adhesion when FN density is increased and also in the presence of serum or other serum proteins, including vitronectin (VN) and bovine serum albumin (BSA), which provide a higher degree of mobility to the matrix.

Statement of Significance

The identification of parameters influencing cell response is of paramount importance for the design of biomaterials that will act as synthetic scaffolds for cells to anchor, grow and, eventually, become specialised tissues. Cells interact with materials through an intermediate layer of proteins adsorbed on the material surface. It is known that the density and conformation of these proteins determine cell behaviour. Here we show that the strength of protein/material interactions, which has received very limited attention so far, is key to understand the cellular response to biomaterials. Very strong protein/material interactions reduce the ability of cells to mechanically reorganize proteins at the material interface which results in enhanced matrix degradation, leading ultimately to compromised cell viability.
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1. Introduction

The protein interface is a mediator of cell/material interactions [1–5]. ECM proteins, adsorbed onto the material surfaces after implantation *in vivo* and from the culture media *in vitro*, are recognised by cells through integrins, a family of cell surface receptors that bind to specific adhesive domains of the ECM components [6,7]. This initial interaction leads to integrin clustering and the internal recruitment of cytoplasm proteins, forming focal adhesions and mediating cell adhesion and contractility [8]. It is well known that the physicochemical properties of the material such as chemistry, topography and mechanics play an important role on the adsorption of proteins onto the material surface. These properties influence the protein surface density, conformation and distribution [9–11], directing cell response during the early events of cell attachment and spreading, as well as controlling later events such as proliferation, matrix reorganization and differentiation [1,9,10,12,13].

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Cell interactions with the ECM are highly dynamic *in vivo*; cells receive information from specific cues in the ECM [12], but also, simultaneously, and as consequence, respond to these inputs by remodeling the surrounding matrix and/or secreting new components [12,14,15]. Proteolytic degradation is a mechanism for the removal of excess ECM; these processes are mostly active during development, wound healing and regeneration of tissues, but, when misregulated, can contribute to diseases such as fibrosis, arthritis and cancer invasion [16–18]. The mechanical organization and reorganization of ECM proteins is another important physiological event that happens after the initial cell/protein interaction [12,14,15,19]. *In vivo* cells secrete and reorganize proteins into fibrils to form their own ECM, which provides them with mechanical support and local growth factors delivery [19–21]. One such protein is fibronectin (FN) that plays a key role in cell adhesion and proliferation, controls the availability of growth factors, and so contributes to cell differentiation [22–24]. FN is synthesized by various anchorage-dependent cells, which then assemble it into a fibrillar network through an integrin dependent mechanism. FN assembly is the initial step which orchestrates the assembly of further ECM proteins such as collagen [25]. There have been several attempts to recapitulate the physiological organization of FN *in vitro*, including the addition of reducing or oxidizing agents [26], using of denaturing or ionic compounds [27] and the use of peptidic FN fragments [28]. Our group identified a specific chemistry, poly(ethyl acrylate) (PEA), which induces the spontaneous organization of FN into physiological-like networks in the absence of cells; the so-called material-driven FN fibrillogenesis [29]. This extended conformation of the FN molecules on PEA provides better availability of cell and growth factors binding regions and leads to enhanced cell adhesion and differentiation on PEA [22–24,29,30]. However, although there are many studies investigating the influence of protein conformation on cell response [22,29], only few have focused on the importance of the strength of the protein-material interaction [31–33].

There are evidences that the biocompatibility of materials can be linked to the ability of cells to remodel surface associated proteins, presumably as an attempt to form their own matrix [12]. It has been shown that cells reorganize more effectively FN molecules that are loosely adsorbed, generally on hydrophilic surfaces [12]. However, the reorganization of the proteins adsorbed on surfaces is usually investigated in the presence of other serum proteins in the culture media [11,12,20,34]. These serum proteins may actively participate in the remodeling of FN previously adsorbed on surfaces [35,36], displace/block the existing FN coating (the so called Vroman effect) [37,38], or can contribute to FN reorganization through physical processes e.g. macromolecular crowding [39].

In this work we investigate the role of the strength of interaction between FN and polymer surfaces with similar physicochemical properties on which FN is adsorbed in different conformations. We have used two polymers with similar chemistry (only one methyl difference in the side chain) and physical properties: PEA and poly(methyl acrylate) (PMA). PEA unfolds FN whereas PMA maintains the native globular conformation of FN in solution [29]. We correlate protein conformation with the strength of the protein/material interaction to reveal the importance of this parameter on cell fate.

2. Material and methods

2.1. Preparation of films

Polymer sheets were obtained by radical polymerization of a solution of the corresponding methyl (MA) and ethyl (EA) acrylate (Sigma-Aldrich), using 0.2 wt% benzoin (98% pure, Scharlau) as a photoinitiator. After polymerization, low molecular-mass substances were extracted from the material by drying *in vacuo* at 60 °C. Each of the synthesized polymers were dissolved in toluene at a concentration of 2 wt% and then spin coated onto glass coverslips at 2000 rpm for 30 s to prepare thin polymer films. Samples were dried *in vacuo* at 60 °C before further characterization. Untreated glass coverslips were employed as a control.

2.2. Protein adsorption

Fibronectin, from human plasma (Sigma), was adsorbed on the different substrates by immersing the material sheets in a 5 or 20 μg/ml FN solution in PBS for 10 min or 1 h. Vitronectin (Sigma) and fibrinogen (Sigma) were adsorbed from protein solutions with the same molar concentration as the fibronectin solution (3.4 and 15.45 μg/ml respectively). Poly(L-lysine) (Sigma) substrates were prepared by adsorption of the protein on glass substrates, from a 40 μg/ml PLL solution for 1 h. After adsorption, samples were rinsed in PBS to eliminate the non-adsorbed protein.

2.3. FN quantification by BCA assay

The surface density of FN adsorbed onto the different substrates was quantified by measuring the amount of non-adsorbed protein using the Bicinchoninic Acid Protein Assay (Thermo Scientific). The kit was used following specification of the manufacturer and the absorbance was measured in a spectrometer at 550 nm. Protein density was normalized respect to the protein density adsorbed at 20 μg/ml in [40]. The experiment was performed in triplicate.

2.4. Atomic force microscopy

Atomic force microscopy (AFM) was employed to analyse the distribution and conformation of FN on the substrates. AFM imaging experiments were performed using a Multimode AFM equipped with NanoScope IIIa controller from Veeco (Manchester, UK) operating in tapping mode in air. The Nanoscope 5.30r2 software version was used. Si-cantilevers from Veeco (Manchester, UK) were used with force constant of 2.8 N/m and resonance frequency of 75 kHz. The phase signal was set to zero at a frequency 5–10% lower than the resonance. Drive amplitude was 600 mV and the amplitude setpoint (A_{sp}) was 1.8 V. The ratio between the amplitude setpoint and the free amplitude A_{sp}/A_0 was kept equal to 0.8. FN-coated samples (20 μg/ml, 10 min) were dried with nitrogen for 2–3 min and then analyzed in the AFM; height, phase and amplitude magnitudes were recorded simultaneously for each image.

Force spectroscopy measurements were performed in order to determine the strength of the FN-material interaction using a JPK NanoWizard® 3 BioScience AFM in force mapping mode. Si-nitride PEGylated cantilevers (COOH terminated) from Novascan with nominal force constant of 0.06 N/m were functionalized with FN according to the manufacturer's instructions. Briefly, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC; Thermo Scientific) was used as a carboxyl and amine-reactive zero-length crosslinker to bind FN to the COOH functionalized tip. Free reactive groups were then blocked with 1 mM Glycine in DPBS. The FN-material interaction was tested for every material in ≥50 different points at a relative setpoint of 0.4 V (≈500–600 pN), constant speed of 0.5 μm/s, z-length of 5 μm and extension delay of 1 s. Calibration of the cantilever sensitivity was performed in the same conditions as the experiments (wet conditions) using a flat, rigid surface and the value was used to correct the force-height curves for the deflection of the cantilever. The thermal noise method (which is implemented in the JPK Nanowizard Control

software v.4) was used to measure the actual spring constant of the cantilever. The strength of the protein-material interaction was calculated from the retract segments of the force-distance curves using the JPK Data Processing software (version spm-4.3.42), and quantified in terms of adhesion force (maximum adhesion force measured by the minimum value of the curve) and work of adhesion (area enclosed by the force curve and the x axis). In addition, the rupture length (distance at which the force reaches zero) was measured as an indication of protein unfolding/stretching.

2.5. Cell culture

MC3T3-E1 cells were obtained from the RIKEN CELL BANK (Japan). Prior to seeding on FN-coated substrates, cells were maintained in DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin, and passaged twice a week using standard techniques. Sample disks (12 mm diameter) were placed in a 24-well tissue culture plate then coated with FN at 5 or 20 μg/ml for 10 min and 1 h. $2 \cdot 10^4$ cells were then placed onto each substrate (cell density: 10,000 cells/cm^2) and maintained at 37 °C in a humidified atmosphere under 5% CO_2 for different time points: 30 min, 2 h and 48 h, in the presence or absence of FBS or BSA. MC3T3 cells were also cultured on VN or Fibrinogen (FG) – coated PEA for 2 h in serum free conditions. Each experiment was performed in triplicate.

2.6. Immunofluorescence

The ability of cells to reorganize the adsorbed FN layer (i.e. early matrix) was monitored after 30 min, 1 h or 2 h of culture. Cells were washed in Dulbecco's phosphate buffered saline (DPBS, Invitrogen) and fixed in 10% formalin solution (Sigma) at 4 °C for 30 min. Samples were rinsed with DPBS and permeabilised at room temperature for 5 min. In order to reduce the background signal, the samples were then incubated in 1% BSA/DPBS at room temperature for 30 min. Samples were incubated with primary antibody against FN (polyclonal rabbit; 1:400, Sigma) or cellular FN (mouse monoclonal, Sigma), then in 1% BSA/DPBS at room temperature for 1 h, rinsed in 0.5% Tween 20/DPBS three times for 5 min each and incubated with the anti-rabbit Cy3-conjugated secondary antibody (1:200 in 1% BSA/DPBS, Invitrogen) at room temperature for 1 h. Simultaneously, BODIPY FL phallacidin was added for the duration of this incubation (1:40 in 1% BSA/PBS; Invitrogen). Finally, samples were washed in 0.5% Tween 20/DPBS three times before mounted in Vectashield containing DAPI (Vector Laboratories, Peterborough, UK). A Nikon fluorescence microscope was used for cellular imaging.

2.7. Cell-mediated protein remodeling

Tapping mode AFM in air was employed to assess, at the nanoscale, the cell morphology and the protein distribution onto the substrate after cell culture. To do this, cells were washed in Dulbecco's phosphate buffered saline (DPBS, Invitrogen) after 30 min of culture and fixed in Formalin solution 10% (Sigma) at 4 °C for 30 min. Samples were then washed with DPBS and gently dried by exposing the sample to a nitrogen flow for 2–3 min prior to AFM analysis.

Protein degradation was analysed by measuring the intensity of the fluorescence on the surfaces coated with labelled FN. FITC-labelled FN (Sigma) was adsorbed on the different surfaces at 20 μg/ml and the extracted fluorescence intensity was measured before and after 2 h incubation with MC3T3 cells. The extraction was performed with 0.2 M NaOH for 2 h, as previously described [41].

To further investigate the process of FN remodeling, MC3T3 cells were also incubated in the presence of protease inhibitors (SIGMA*FAST*™ Protease Inhibitor Cocktail Tablets, Sigma) at different concentrations (dilutions 1:1, 1:5 and 1:100) for 2 h.

2.8. Live/dead viability assay

MC3T3-E1 cells were incubated for 2 h on FN-coated substrates in serum-free conditions. Cell viability was measured by a live/dead assay (Invitrogen) according to manufacturer recommendations (15 min incubation in 2 μM calcein AM and 4 μM Ethidium homodimer-1). Viable green fluorescent cells and dead red fluorescent cells were visualized and quantified under a fluorescence microscope to assess viability as the percentage of living cells.

2.9. Time-lapse cell imaging

Phase contrast images were acquired every 5 min for 2 h using an inverted microscope (Zeiss) with a 20× dry objective. During the observation, the samples were maintained at 37 °C and supplied with a 95% air and 5% CO2 humidified gas mixture. After 2 h incubation, FN reorganisation was investigated via immunofluorescence for FN (see Section 2.6)

2.10. Statistics

Results are shown as an average ± standard deviation and were analysed by one-way ANOVA. If treatment level differences were determined to be significant, pair-wise comparisons were performed using Tukey post hoc test.

3. Results

3.1. FN adsorption

In this study we used PEA and PMA, two polymers with similar chemistry – i.e. only differ in one methyl group in the lateral chain, as shown by their molecular structure and XPS analysis – providing two material surfaces with similar wettability and mechanical properties (Fig. S1) [29,30]. Polymeric films of these materials, prepared by spin coating, display smooth (Rrms < 1 nm) and homogenous surfaces. FN distribution after adsorption on material surfaces was assessed by AFM. Fig. 1a shows AFM images of FN adsorbed on the different substrates from a solution of 20 μg/ml for 10 min. On PEA, FN is organized into networks, whereas it adopts a globular form on both PMA and the control glass. FN density after adsorption is similar on the three substrates and it increases along with the concentration of the protein solution and the adsorption time (Fig. 1b). Although the surface coverage after 1 h of protein adsorption is limited to ~90%, this corresponds to an equilibrium state, thus it does not interfere with the biological activity of the surface. We note that there are no statistical differences between the density of FN on glass, PMA and PEA regardless of the concentration of the solution and adsorption time.

The strength of the protein/material interaction was characterized by force spectroscopy using FN-functionalized AFM tips. Fig. 2a shows representative force-distance curves obtained for each material during the retraction of the tip from the surface. The parameters Adhesion force, Work of adhesion and Rupture length, were calculated from the average of ≥50 force-curves (Fig. 2c). The adhesion force and work required to detach the functionalized FN tip from the substrate were higher on PEA compared to PMA, whereas the lowest values were found on glass, revealing higher strength of protein-material interaction on PEA. The rupture length was similar on PMA and PEA, whilst lower on glass. The

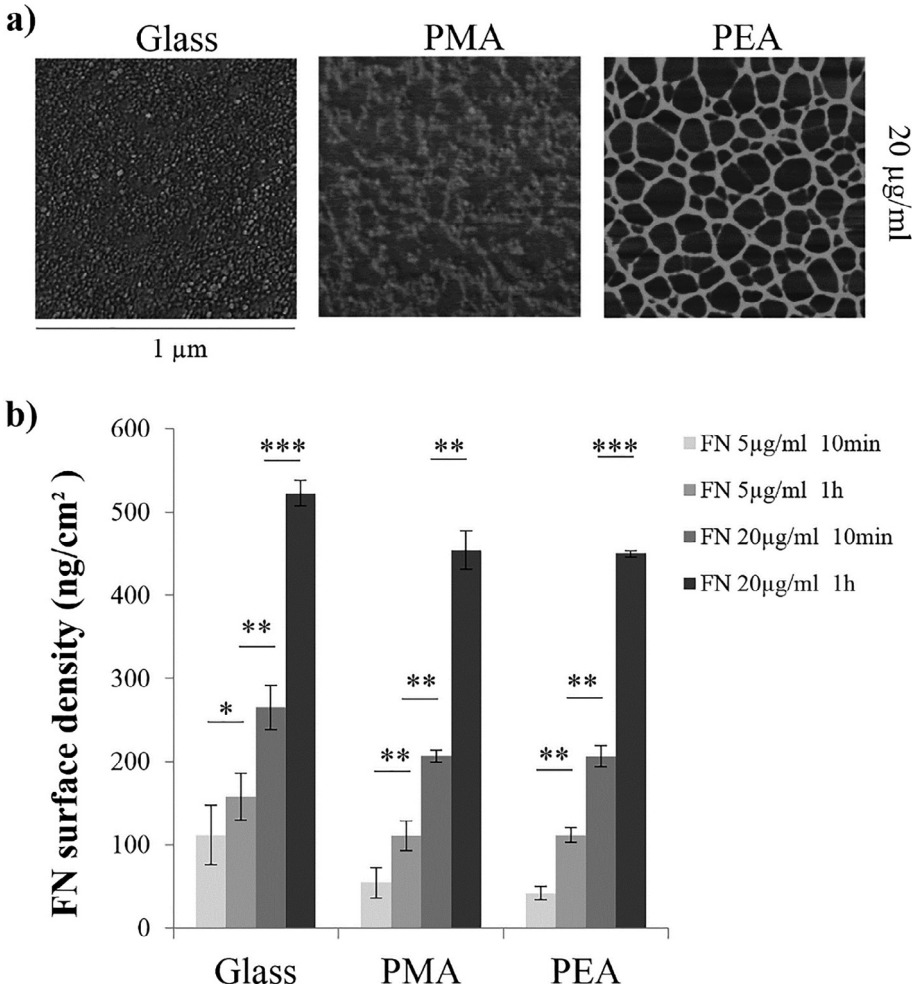


Fig. 1. FN adsorption on PMA and PEA surfaces and control glass. a) Distribution of FN adsorbed on the different substrates from a 20 μg/ml solution as observed by the phase magnitude in tapping mode AFM. b) Quantification by BCA assay of FN adsorbed on these surfaces from solutions of different concentrations (5 and 20 μg/ml) for 10 min and 1 h. Statistically significant differences (as determined by *t*-test) are indicated with *P < 0.05, **P < 0.01, ***P < 0.001.

interaction of non-functionalized tips with the surface was also characterized (see Fig. S2).

3.2. Cells degrade adsorbed FN on PEA in absence of serum

Cellular reorganization of the adsorbed FN was assessed by immunofluorescence on FN-coated substrates in serum-free conditions after 2 h (Fig. 3). On the control glass and PMA, cells presented spread morphology on a homogeneously stained FN layer. However, a brighter area was consistently observed in the protein layer around cells adhered on PEA. We hypothesize that this phenomenon is related to the degradation (remodeling) around the cell of the FN fibrils assembled on PEA, leading to a higher fraction of fluorophores exposed after staining, what leads to a brighter area on the FN layer. The second row in Fig. 3 shows merged images after staining for FN and cell nuclei (ethidium homodimer), seeking to visualize the bright FN area around cells (cell-mediated FN remodeling) and simultaneously identify non-viable cells. Interestingly, most cells surrounded by a brighter FN area were positively stained for ethidium homodimer (see also Videos and Fig. S3 in Supplementary data).

This brighter FN staining observed around cells growing on PEA suggested remodeling of the adsorbed FN layer. In fact, staining for cell-secreted FN did not show this trace of this effect (Supp. Fig. S3). To further investigate this phenomenon, we used AFM to evaluate at the nanoscale the topology of the FN layer assembled on PEA. Fig. 4 shows cell morphology and FN distribution on PEA after 30 min of culture. A change in the FN patterns was clearly observed around the cells, in accordance with the immunostained images of FN (Fig. 3). The distribution of the protein layer is better observed at higher magnification in Fig. 4b. The characteristically dense, interconnected FN network was only observed far away from cells (compare to PEA in Fig. 1a), whereas fibronectin molecules were hardly interconnected in areas closer to cells. The less interconnected FN fibrils shown in Fig. 4b, nearby cells, corresponded to the brighter areas in Fig. 3 (PEA, immunostained FN) and suggest cell mediated degradation of FN fibrils on PEA.

The degradation of the FN matrix was assessed by coating PEA and PMA surfaces with FITC-labelled FN and analyzing the intensity of fluorescence remaining on the samples before and after cell incubation for 2 h (Fig. 4c). Similar fluorescence signal was found on both substrates before cell seeding, which is expected as the amount of FN adsorbed on PEA and PMA is similar (Fig. 1b). However, the intensity decreases significantly with cells on PEA, which suggests a decrease in the amount of protein on the surface of PEA as cells degrade the adsorbed FN layer (Fig. 4c).

The dynamics of matrix remodeling on FN-coated PEA was evaluated after different cell incubation times. Immunostained

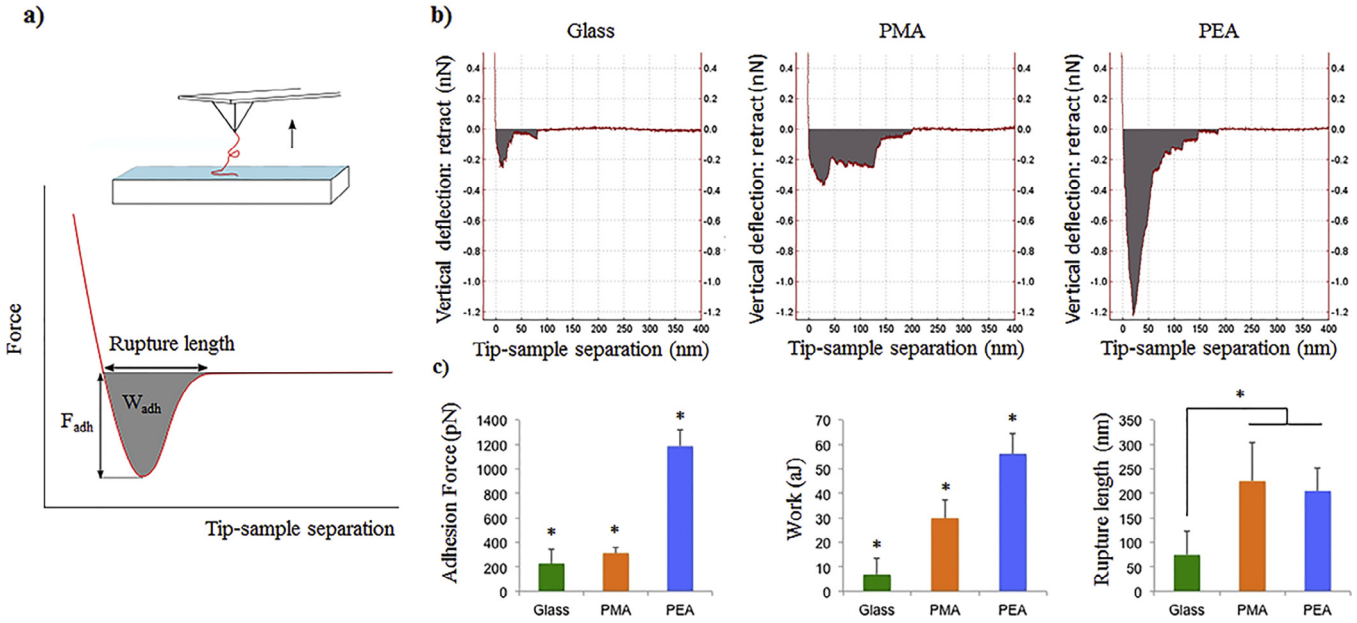


Fig. 2. Strength of FN-material interaction via force spectroscopy. a) A FN-functionalised AFM cantilever is approached to the surface and force curves are obtained during the retraction of the cantilever after 1 s of contact with the surface. The strength of the protein-material interaction is analyzed by quantifying the adhesion force, adhesion work and rupture length. b) Force-distance curves obtained during the retraction of the FN-functionalized cantilevers from the different surfaces. c) Quantification of the adhesion force, work of adhesion and rupture length. Statistically significant differences (as determined by ANOVA) are indicated with *P < 0.01.

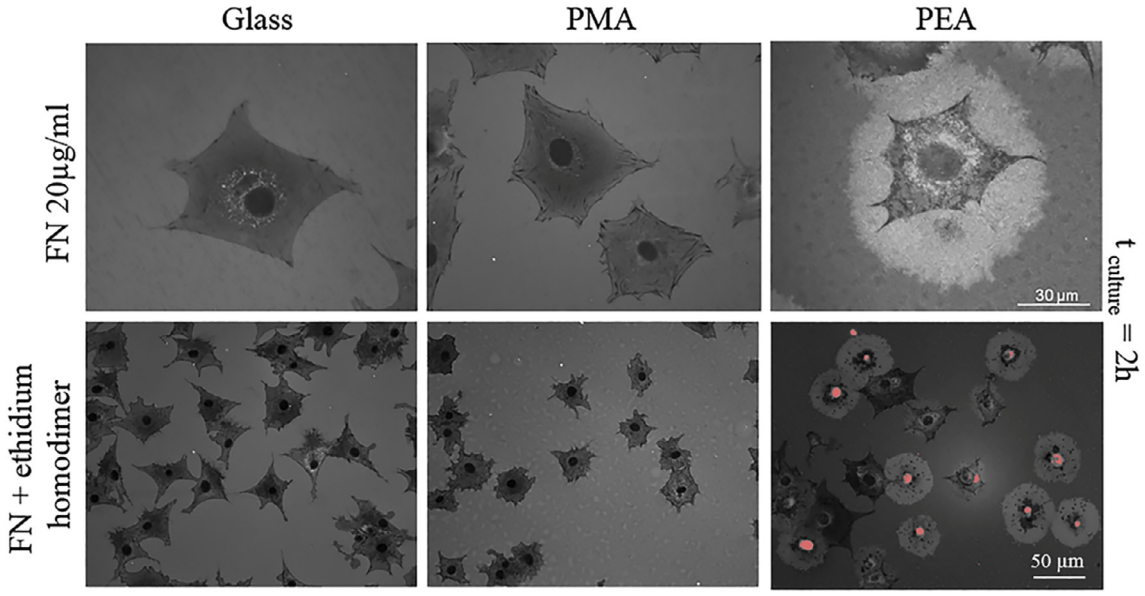
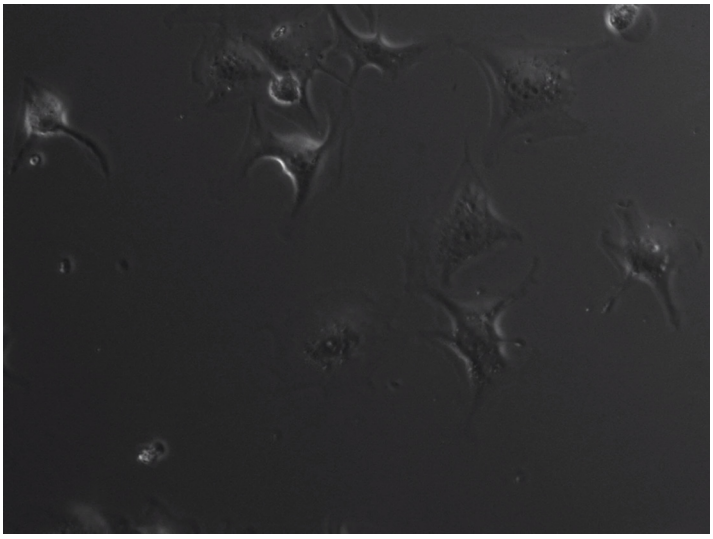


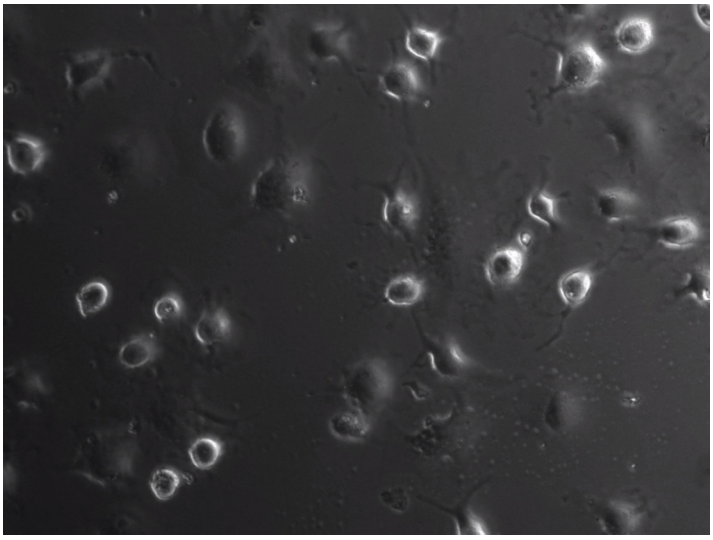
Fig. 3. Cell-mediated FN remodeling on the different substrates after 2 h in serum-free conditions. An imprint (observed as a brighter area of the FN staining) is left by the cells on the protein layer on PEA but not on PMA and glass. The second row shows that dead cells (after staining with ethidium homodimer) correspond to the cells where the cell imprint is observed.

images for FN, in Fig. 5, reveal that cells modified the surrounding FN layer very quickly, at very short culture times (5 min). The area of the protein layer influenced (degraded) by the cells increased with the incubation time. In tandem, after initial spreading (15 min), cell morphology evolves, with cells decreasing in size and being more rounded over time. After 2 h of cell adhesion, signs of FN degradation leading to brighter areas around cells are observed for the majority of the cells growing on PEA.

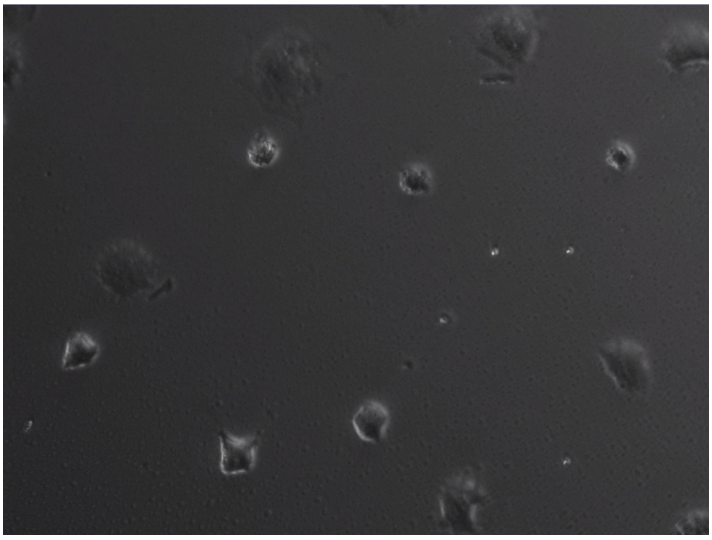
To better follow this process, 2 h time-lapse experiments were performed on the three different substrates. Movies 1, 2 and 3 clearly show the cell response after adhesion to each material (see Supplementary Video): upon seeding, cells attached to the adsorbed FN layer and spread on the substrate. On PMA and control glass, cells remained spread and with some mobility for the entire incubation time. However, on PEA, cells started shrinking after approximately 30 min and remained static, degrading the protein layer.



Video 1.



Video 2.



Video 3.

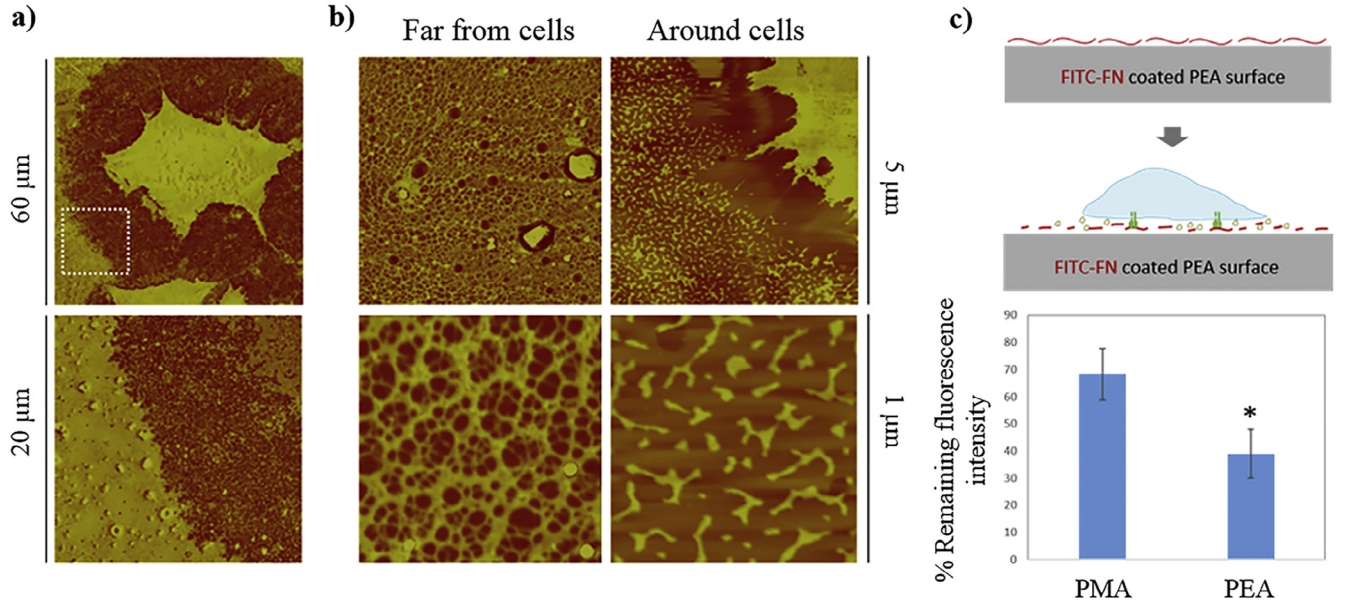


Fig. 4. Cell-mediated FN matrix degradation on PEA surfaces coated with FN at 20 μg/ml for 1 h. a) Cell-mediated FN degradation at the nanoscale. AFM phase images show different protein patterns in dependence of the proximity (far vs around) to cells. FN distributions in areas around cells and far from cells are showed at higher magnification in b). c) Remaining fluorescence intensity of FITC-labelled FN-coated PMA and PEA surfaces after incubation of MC3T3 cells for 2 h. The fluorescence intensity decreases significantly on PEA, consistent with higher levels of cellular proteolytic activity on this material.

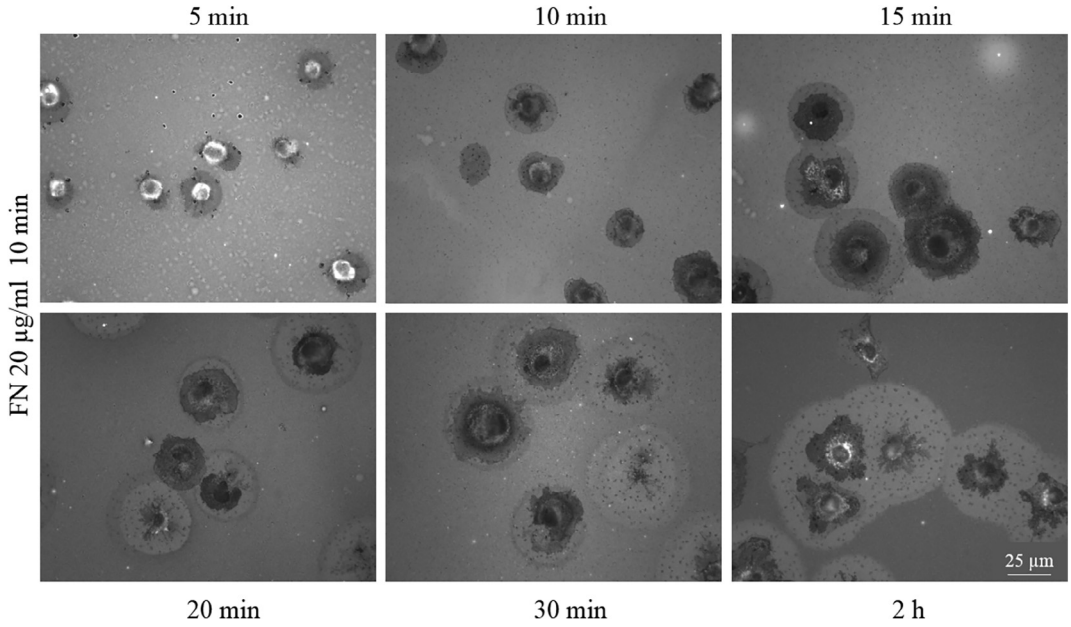


Fig. 5. Protein matrix degradation during increasing culture time on FN-coated PEA substrates. Cells start degrading the FN layer from short culture times. Scale bar: 25 μm.

3.3. The presence of proteins in the culture medium prevents cells to degrade FN on PEA

The ability of cells to remodel FN on PEA was evaluated varying the concentration of the adsorbing solution and the adsorption time. Cells remodeled FN on PEA regardless of the culture time as well as the concentration of FN and adsorption time (Fig. 6). However, when a higher amount of FN was adsorbed on PEA, cells presented a more spread morphology with less evidences of degradation of the FN layer (compare *b,e* to *a,d* in Fig. 6). When cells were cultured on PEA with FBS containing medium, they showed a well spread morphology with no signs of degradation of the FN layer (Fig. 6c and f, and Fig. 7).

As serum contains adhesive proteins and growth factors, we wondered if FN degradation on PEA could also be prevented by adding BSA, a non-adhesive protein, to the culture medium (Fig. 7). Cells spread well on the three substrates with no signs of degradation observed on the protein layer around cells growing on PEA. Moreover, the addition of BSA fostered a strong FN reorganisation on glass surfaces, where FN is loosely adsorbed (Fig. 7).

Additionally, we investigated if this stronger interaction between FN and PEA leading to protein remodeling at the cell/material interface and cell death occurs for other adhesive proteins. To do so, MC3T3 were seeded on PEA coated with two other proteins, VN and FG. We found that viability was also compromised on coated PEA but not on Glass (see Fig. S7).

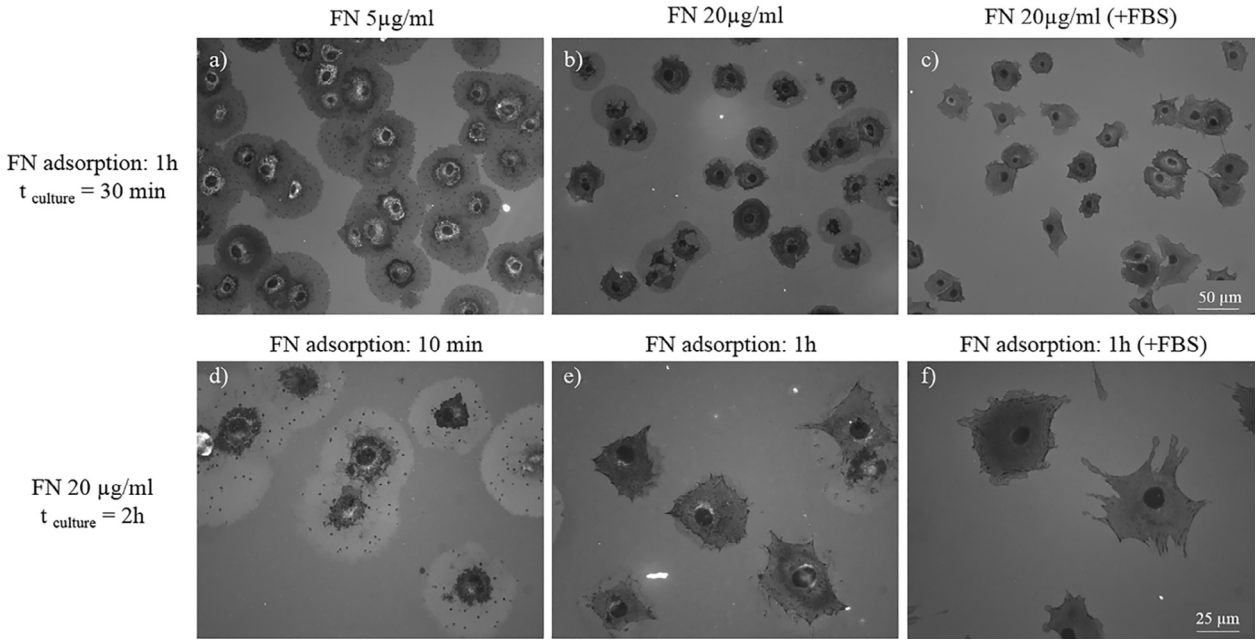


Fig. 6. Cell-mediated FN reorganization on FN-coated PEA after 30 min and 2 h incubation. FN degradation is observed regardless of the culture time (a-b, d-e), FN concentration (a-b) and adsorption time (d-e). However, less evidences of degradation are observed with higher FN concentration, which results in better spread cells. In the presence of serum (c,f) no signs of FN degradation are observed and cells present a spread morphology. Top scale bar: 50 µm, bottom scale bar: 25 µm.

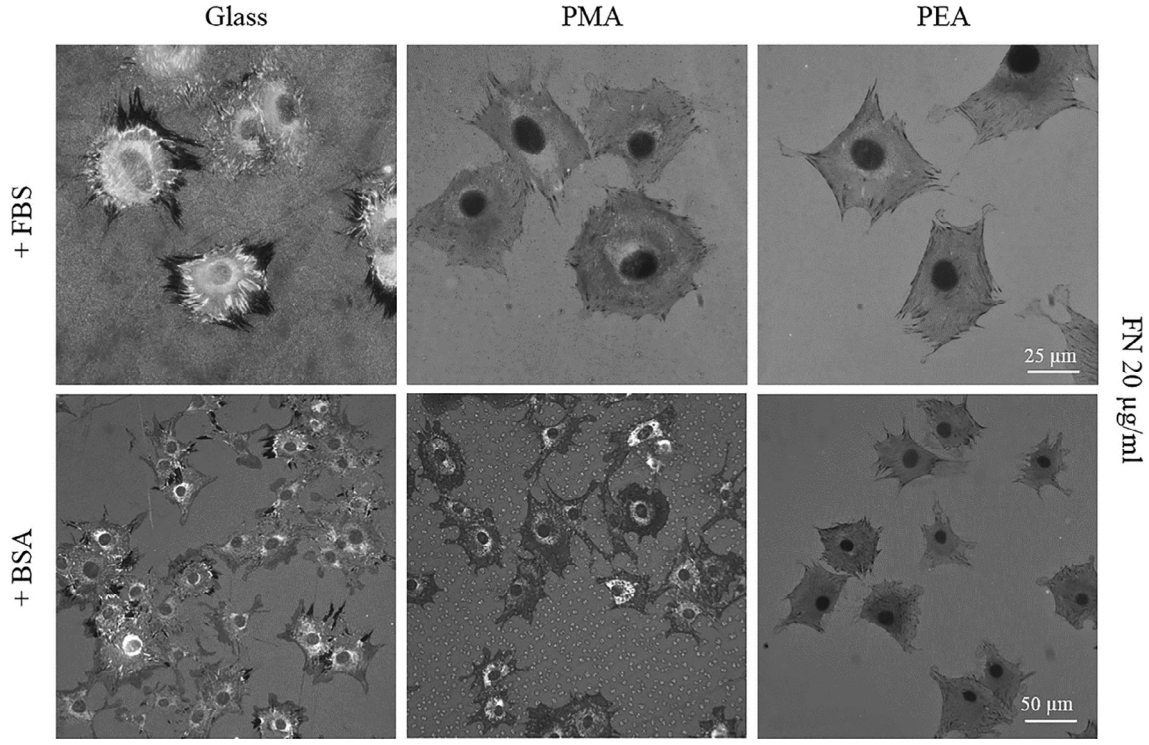


Fig. 7. Cell-mediated FN reorganization in the presence of FBS or BSA on the different substrates after 2 h incubation. Substrates were coated with FN at 20 µg/ml for 10 min before cell seeding. Cells remain well spread and are able to reorganise the underlying protein layer in the presence of FBS (top) or BSA (bottom) proteins. When these proteins are present, the degradation of FN observed on PEA is prevented. Scale bar: 50 µm.

Finally, we investigated whether the partial inhibition of the degradation process of the FN matrix could prevent cells to enter in apoptosis on PEA. To do so, cells were incubated in the presence of protease inhibitors at different concentrations and the cell-mediated FN remodeling process was assessed. In Fig. 8 we can observed that, whereas a very low concentration of protease inhibitors does not have any effect in the cellular behavior, a high concentration of the inhibitors leads to cell death on both glass and PEA substrates. However, cell viability was enhanced on PEA when the burst of proteolytic activity was partially inhibited.

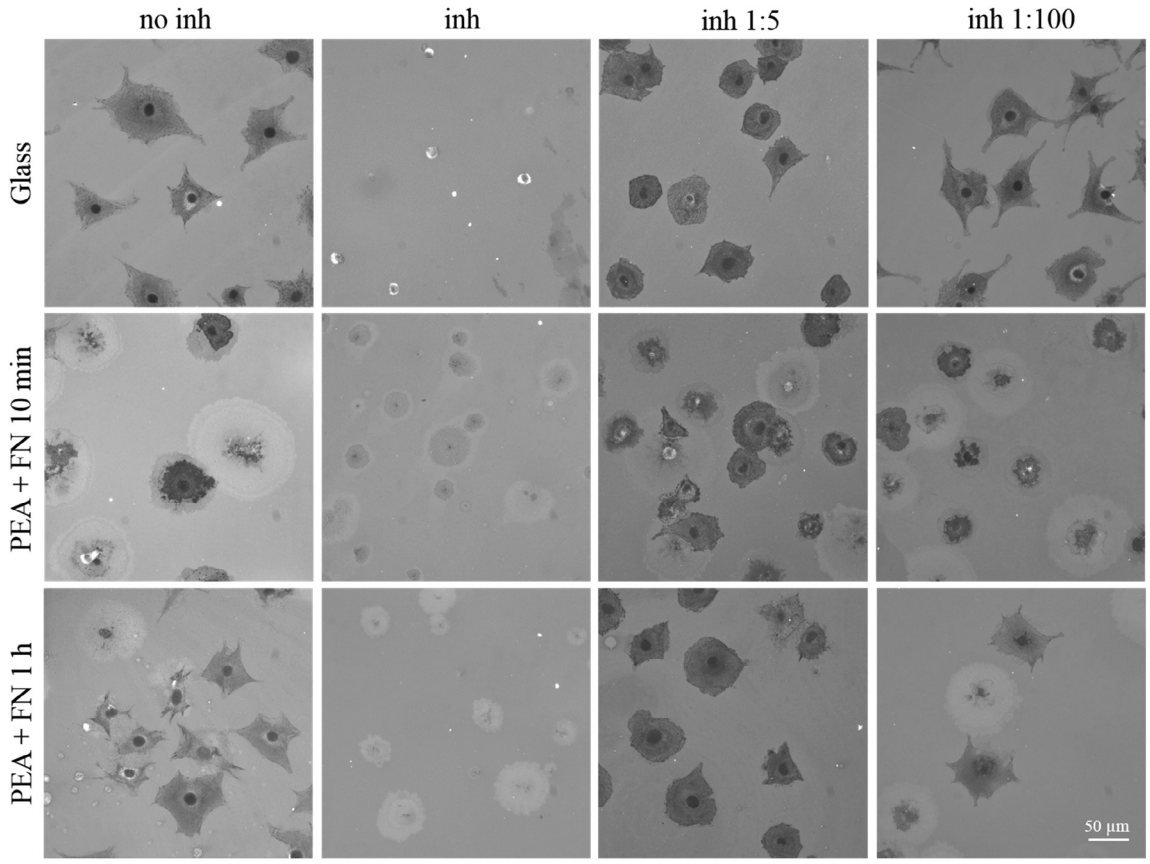


Fig. 8. Cell-mediated degradation of the FN matrix in the presence of protease inhibitors at different concentrations for 2 h incubation. Substrates were coated with FN at 20 μg/ml before cell seeding. The presence of protease inhibitors, at a suitable concentration, reduces the burst of FN degradation and enhances cell viability on PEA substrates. Scale bar: 50 μm.

4. Discussion

Remodeling of the ECM is a highly dynamic process, involving proteolytic degradation, protein secretion and mechanical reorganization of proteins [42]. Reorganization and degradation of the ECM are necessary for morphogenesis and other cell functions such as migration and differentiation [43]. An unbalanced turnover of matrix remodeling has been associated with cell apoptosis [43–46] and diseases such as fibrosis, arthritis, reduced angiogenesis and cancer invasion [17,47].

After initial adhesion, cells mechanically rearrange adsorbed proteins on material surfaces in a fibril-like pattern, presumably seeking to mimic the ECM organization *in vivo* [36,42]. Thus, since cells need to reorganize adsorbed FN for their normal function, it is suggested that materials that adsorb proteins loosely may facilitate cells to mechanically rearrange the protein layer at the material surface and result in better biocompatibility [34]. Notwithstanding this, few studies recognize the importance of the strength of the interaction between proteins and material surfaces when investigating cell response, and even fewer have attempted measuring this property quantitatively [31,33].

Cells secrete globular FN that is afterwards reorganized into fibrillar matrices through an integrin mediated process. This process involves FN unfolding and organization of the ECM that provides mechanical support and specific cues that direct their behaviour [42]. Several routes have been investigated to recapitulate cell-mediated organization of FN [26–28]. We showed that PEA unfolds FN and induces assembly into fibrillar networks. The phenomenon is very specific of PEA. For example, on PMA (similar chemistry with only one less carbon in the lateral chain), FN remains globular after adsorption (Fig. 1). This different FN organisation on both substrates is driven by the increased mobility of the longer lateral chain on PEA, which increases the mobility on the polymer surface [30,49] and leads to the unfolding of the FN molecule. FN assembly on PEA provides more efficient presentation of integrin and growth factor binding domains, leading to an enhanced cell adhesion and differentiation *in vitro*, [24,29,30], along with bone regeneration and vascularization *in vivo* [22,23]. Here, we have used this family of polymers, PEA and PMA, that despite of their similar chemistry, mechanical properties and nanotopography (Fig. S1) [30], induce a very different FN conformation, to investigate the importance of the strength of the protein/material interactions in cell/material interactions. First, using FN-functionalized AFM cantilevers (Fig. 2a), we found that FN adsorbed strongly on PEA compared to PMA (and control glass) (Fig. 2), which is at least partly dependent on the unfolded conformation of FN on PEA [29]. This stronger interaction between FN and PEA is consistent with previous data that revealed lower mobility of FN on PEA compared to PMA [48].

Differences observed on the strength of the protein-material interaction have previously been suggested to influence the ability of cells to reorganize the underlying protein layer at the cell/material interface [31]. Lin et al. investigated initial cell adhesion and FN remodeling on self-assembled monolayers (SAMs) with different surface chemistry and charge, finding that the ability of cells to reorganize the matrix changed as a consequence of the initial strength between FN and the surface on which it is adsorbed [31]. Our results show that the high interaction strength between FN and PEA prevents cells to reorganize FN and then triggers

degradation of the adsorbed FN. We hypothesize that it is the strong proteolytic cascade on PEA which leads to apoptosis. Alternatively, cells on PMA (and glass), where FN was more loosely bound (Fig. 2), reorganize FN with lower secretion of proteases [49]. Indeed, AFM showed breaking of the FN network assembled on PEA into unconnected protein patches around cells (Fig. 4), resulting in a brighter FN staining observed at the microscale when stained with an antibody (Fig. 3). The loss of fluorescence of adsorbed FITC-labelled FN confirmed proteolysis (Fig. 4), in accordance with previous results from our group [11]. The degradation of the FN matrix around cells on PEA corresponds to non-endogenous FN, which is confirmed by using FITC-FN coated surfaces, where the matrix degradation is still observed (Fig. S5), and labelling of the cellular FN, where the degradation of the FN matrix is no longer observed (Fig. S4). Additionally, we found that this cellular behavior is not exclusive of FN on PEA, but that there is a similar response when PEA is coated with other ECM proteins such as VN or FG (see Fig. S7).

As a consequence, since FN adsorbs strongly on PEA, it is suggested that cells, unable to reorganize FN, respond with a proteolytic burst in an attempt to remodel the provisional matrix at the cell/material interface, which eventually determines cell fate and compromised cell viability (Fig. 3). Although cellular apoptosis leads to matrix degradation [50], there are evidences that high levels of protease activity may also induce apoptosis [51,52]. In fact, several works have shown that the inhibition of matrix metalloproteinases (MMPs), the main enzymes involved in ECM degradation, via tissue inhibitors of MMPs (TIMPs) may prevent cellular apoptosis [45,53,54]. In our previous work we found that PEA promotes higher expression of MMPs than PMA and glass, suggesting triggering of a stronger proteolytic activity on this material in the absence of FBS [49]. Interestingly, we observed here that the presence of protease inhibitors reduces the proteolytic burst on PEA and enhances cell viability (Fig. 8).

No signs of matrix degradation on FN-coated PEA are observed if other ECM proteins are added to the culture media (i.e. serum proteins) (Fig. 7). In this case, cells are not only well spread on FN-coated PEA, but FN assembled on PEA also promotes cell differentiation *in vitro* [24,29,31] and tissue repair *in vivo* [22,23], compared to e.g. globular FN on PMA [22–24,29,30]. Here it is important to note that the inability of reorganizing FN on PEA is overcome by the high activity of the protein on this material, i.e. both integrin- and growth factor- binding regions highly available on FN assembled on PEA. However, other proteins are needed that increase the mobility of FN at the cell/material interface and so prevent strong proteolytic cascades (Figs. 6, 7 and Refs. [35,36]).

The presence of other serum proteins, such as vitronectin [35,36], helps in the cellular assembly of FN fibrils and provide cells with higher ability to reorganize their surrounding protein matrix. We have previously shown that when vitronectin is co-adsorbed with FN on PEA, a protein network with higher mobility is assembled on PEA [35]. This alleviates the proteolytic burst in C2C12 myoblasts [55] and in fibroblasts [35], suggesting that this behaviour is more universal and not limited to the cell type used in this study. A similar effect on FN reorganization on PEA is also noted in the presence of other proteins, such as when BSA is added to the culture media instead of FBS (Fig. 7). Protein reorganization is enhanced when high concentrations of macromolecules, such as proteins, are present in the cell microenvironment, due to a reduction of the excluded volume [39]. This crowding effect may explain the enhanced FN reorganization on PEA observed in the presence of BSA or FBS, leading to a reduction in the degradation of the protein layer and high cell viability and adhesion on both PEA (Fig. 7). All these findings together suggest that a degree of mobility in the adsorbed ECM layer, at least at the nanoscale, is necessary for cells to be able to reorganize their own provisional matrix at biomaterials interface [11,12,14], and thus provide themselves with a functional environment.

5. Conclusions

We show that the ability of cells to reorganise adsorbed matrix proteins at the material interface, at least at the nanoscale level, is crucial for the development of normal cellular functions, to the extent that high strength of protein/material interaction prevents reorganization and so may compromise cell viability. FN is assembled into fibrillar networks on PEA, which are strongly attached to the substrate. These fibrils are unable to be reorganized by cells resulting in a proteolysis burst. This is in contrast reverted by increasing the mobility of FN at the material interface, by e.g. increasing the protein surface density or adding other proteins to the culture medium. The strength of interaction between proteins and materials determine cell fate and it is an important material to be quantified to understand cell response to synthetic materials.

6. Author contribution

CGG, MC, JBB and GA performed the experiments. MC, JBB, GA revised the manuscript. CGG and MSS conceived the research and wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.actbio.2018.07.016.

Research data for this article

All the original data related to this manuscript are within the depository of the University of Glasgow with https://doi.org/10.5525/gla.researchdata.638.

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