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Vitronectin activity on polymer substrates with controlled –OH density

Georgi Toromanov [a], Cristina González-García [c], George Altankov [a,b], Manuel Salmerón-Sánchez [c,d,e,*]

[a] Institut de Bioenginyeria de Catalunya, Barcelona, Spain
[b] ICREA (Institució Catalana de Recerca i Estudis Avançats), Barcelona, Spain
[c] Center for Biomaterials and Tissue Engineering, Universidad Politécnica de Valencia, 46022 Valencia, Spain
[d] Regenerative Medicine Unit, Centro de Investigación Príncipe Felipe, Autopista del Saler 16, 46013 Valencia, Spain
[e] CIBER de Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), Valencia, Spain

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ABSTRACT

Vitronectin (VN) adsorption on a family of model substrates consisting of copolymers of ethyl acrylate and hydroxyl ethylacrylate in different ratios (to obtain a controlled surface density of –OH groups) was investigated by Atomic Force Microscopy (AFM). It is shown that the fraction of the substrate covered by the protein depends strongly on the amount of hydroxyl groups in the sample and it monotonically decreases as the –OH density increases. Isolated globular-like VN molecules are observed on the surfaces with the higher OH density. As the fraction of hydroxyl groups decreases, aggregates of 3–5 VN molecules are observed on the sample. Overall cell morphology, focal adhesion formation and actin cytoskeleton development are investigated to assess the biological activity of the adsorbed VN on the different surfaces. Dermal fibroblast cells show excellent material interaction on the more hydrophobic samples (OH contents lower than 0.5), which reveals enhanced VN activity on this family of substrates as compared with other extracellular matrix proteins (e.g., fibronectin and fibrinogen).

1. Introduction

Polymers are currently being investigated as synthetic substrates able to trigger the regeneration of a cell population making use of tissue engineering techniques. Cell adhesion is the first step of the regeneration process and plays a fundamental role in subsequent cell differentiation, growth, viability and phenotype expression [1]. Cell adhesion involves different physicochemical phenomena (i.e. ionic and van der Waals interactions) in which several biological molecules participate: Extracellular matrix (ECM) proteins, cell membrane proteins and cytoskeleton proteins that interact to signalling information, promote transcription factors and regulate gene expression. After initial adhesion, cells spread on the substrate to assume a flattened morphology and anchor to the substrate developing the actin cytoskeleton that provides mechanical stability and transmits forces to the cell interior [2,3].

It is now well established that cell adhesion on biomaterials is mediated by a layer of proteins previously adsorbed on the material surface coming from either the physiological fluids in vivo or intentionally deposited in vitro, e.g., from (competitive) adsorption of proteins and serum or even chemical attachment of the protein on the substrate [4–7]. The initial cell–material interaction usually involves the adsorption of proteins such as fibronectin (FN), vitronectin (VN), fibrinogen (FNG), representing the so-called soluble matrix proteins in the biological fluids [6]. Upon longer contact with tissues many other extracellular matrix (ECM) proteins, such as collagens and laminins, will also associate with the surfaces, affecting the cellular interaction. Cells recognize these matrix proteins via a family of trans-membrane proteins – integrins – that provide links between the ECM and the actin cytoskeleton [8]. When integrins are occupied they cluster and develop an aggregate of different proteins, so-called focal adhesions, that anchorage the cell on the substrate. Focal adhesions are supramolecular complexes that contain structural proteins such as vinculin, talin, α-actinin, and signalling molecules, including FAK, Src and paxilin that actually anchorage the cells to the surface and trigger the subsequent cellular response [9,10]. Thus, the initial cell–material interaction is a complex multi-step process consisting of early events, such as adsorption of proteins, followed by cell adhesion and spreading, and late events, related to cell growth, differentiation, matrix deposition and cell functioning. To measure and to quantify some of these parameters comprise the classical approach to characterise the cellular biocompatibility of materials [11].

Vitronectin is an adhesive glycoprotein found in the circulation and different tissues. Its molecular weight varies in the range between 56 and 80 kDa depending on the species. The VN molecule

* Corresponding author. Center for Biomaterials and Tissue Engineering, Universidad Politécnica de Valencia, 46022 Valencia, Spain. Tel.: +34 963877275; fax: +34 963877276.
E-mail address: masalsan@fis.upv.es (M. Salmerón-Sánchez).

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contains binding sites for integrins, collagens, heparin, complement components, plasminogen and plasminogen activator inhibitor [12]. VN has been involved in several physiological and pathological processes including haemostasis, angiogenesis, rheumatoid arthritis, tumour cell invasion and its unique role to bind and maintain plasminogen activator inhibitor in its active conformation to inhibit pericellular proteolysis [13]. Integrin binding to VN is mediated through the RGD motif present in the molecule. Cell adhesion in cultures containing serum is mediated by vitronectin and fibronectin, but adsorption of VN is usually enhanced (up to 40-fold higher), as compared to fibronectin, on different material surfaces that included tissue-culture polystyrene, different polymers and self-assembled monolayers (SAMs) [14–20].

Several studies have investigated VN adsorption on synthetic substrates by making use of different techniques which include immunoblot of desorbed protein from SAMS with different terminating groups [20], adsorption of radiolabelled protein (^{125}I-VN) measuring the radioactivity with a γ-counter [19], surface plasmon resonance [21] and a modified enzyme-linked immunosorbent assays (ELISA) [22]. Scarce work has been done in the recent years to characterise VN adsorption by making use of Atomic Force Microscopy (AFM). AFM images of VN adsorption on mica revealed the initially adsorbed protein molecules extended in a flattened oblate ellipsoidal conformation that appeared more extended on Ti substrates [23]. The effect of pH on VN adsorption on SAMs was investigated by AFM to correlate the orientation and conformation of the adsorbed protein molecules and their bioactivity [24]. AFM has also been used to quantify the strength of interaction between adsorbed VN layers, which was shown to be dependent on the solution ionic strength, ion type and pH [25].

This work investigates VN adsorption on model copolymers in which the surface density of hydroxyl groups can be modulated as an independent parameter. The supramolecular organization, distribution and amount of adsorbed protein on each substrate are obtained by AFM. The biological activity of the adsorbed VN molecules is investigated by means of the initial interaction with human dermal fibroblasts paying particular attention to the overall cell morphology, focal adhesion formation and cytoskeleton development on the different VN-coated model surfaces.

2. Experimental section

2.1. Materials

Copolymer sheets were obtained by polymerization of a solution of both monomers ethyl acrylate, EA, (Aldrich, 99% pure) and hydroxyethyl acrylate, HEA (Aldrich 96% pure), with the desired proportion, using 0.1 wt% of benzoin (Scharlau, 98% pure) as photoinitiator and a 2 wt% of ethyleneglycol dimethacrylate EGDMA (Aldrich, 98% pure) as cross-linking agent. The polymerization was carried out under ultraviolet light at room temperature up to limiting conversion. Five monomer feed compositions were chosen, given by the weight fraction of HEA in the initial mixture of 1, 0.7, 0.5, 0.3, and 0 ($-OH_x$ refers to the sample with fraction x of HEA in the copolymer, x_{OH}). After polymerization, low molecular mass substances were extracted from the material by boiling in ethanol for 24 h and then drying *in vacuo* to constant weight [26,27].

The equilibrium water content (EWC, mass of water absorbed referred to the dry mass of the substrate) and the water contact angle (using a Dataphysics OCA) were measured for the different substrates.

The polymerization procedure gives rise to sheets of 1 mm thickness. Small disks (approximately 10 mm diameter and 1 mm thickness) were cut from the polymerized sheets in order to be used in the protein adsorption and cell adhesion studies. The samples were sterilized with gamma radiation (25 kGy) before the experiments.

2.2. Atomic force microscopy

AFM was performed in a NanoScope III from Digital Instruments (Santa Barbara, CA) operating in the tapping mode; 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 one. Drive amplitude was 200 mV and the amplitude setpoint A_{sp} was 1.4 V. The ratio between the amplitude setpoint and the free amplitude A_{sp}/A_0 was kept equal to 0.7

Vitronectin from human plasma (Sigma, Cat. No. V-8379) was adsorbed on the different substrates by immersing the material sheets in 10 μg/ml physiological solution (NaCl 0.9%) for 10 min. After that, the sample was dried by exposing its surface to a nitrogen flow for a few minutes.

The influence of the concentration of the initial protein solution on the conformation of the adsorbed protein was investigated by immersing the EA sheet in protein solutions of different concentrations: 2, 5, 10, and 20 μg/ml.

2.3. Cell culture

Human Dermal Fibroblasts (PromoCell, Cat. No. C-12302) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 mM Sodium Pyruvate, 2 mM L-Glutamine, and Penicillin–Streptomycin, all of them purchased from Invitrogen.

2.4. Overall cell morphology

To study the overall morphology and vitality of adhering fibroblasts 5×10^4 cells/well were seeded in 24 well tissue-culture plates (Nunc™ Denmark) containing the different substrates or control glass slides at final volume of 1 ml serum free medium. The samples (including the control glass) had been pre-coated with VN (Sigma, V-8379) at concentration 10 μg/ml in PBS. At the end of incubation, the living cells were stained with fluorescein diacetate (FDA) (Sigma) in a final concentration of 0.001% from the stock of 1 mg/ml dissolved in acetone. Under these conditions the vital cells convert FDA in fluorescent analogue via their esterases. The samples were viewed in an inverted fluorescent microscope (Nikon Eclipse E800) and representative pictures were taken at 10× magnification.

2.5. Visualization of actin cytoskeleton

After 2 h of incubation the cells were fixed with 4% paraformaldehyde (5 min), permeabilized with 0.5% Triton X-1000 (5 min) and stained with FITC-Phaloidin (Invitrogen) (dilution 1:100) for 30 min to visualize actin cytoskeleton before washed and mounted with Mowiol. The samples were viewed in an inverted fluorescent microscope (Nikon Eclipse E800) equipped with digital camera and the corresponding software. Representative pictures of the adhered cells were then taken at high magnification as indicated using the green channel of the fluorescent microscope (Nikon Eclipse E800).

2.6. Focal adhesions assembly

To visualize focal adhesions, fixed and permeabilized samples were saturated with 1% albumin in PBS (15 min) and stained for vinculin using monoclonal anti-vinculin antibody (Sigma, Cat. No.

Table 1
Equilibrium water content (EWC: mass of water per unit mass of dry polymer) and water contact angle (WCA) for the different substrates.

x_{OH}	EWC (%)	WCA (°)
0 (pure PEA)	1.7 ± 0.4	89 ± 1
0.3	7.6 ± 0.9	80 ± 2
0.5	18.2 ± 1.7	67 ± 1
0.7	40.6 ± 0.4	55 ± 1
1 (pure PHEA)	134 ± 5	45 ± 2

V9131) dissolved in 1% BSA in PBS for 30 min followed by Cy^3-conjugated Goat Anti-Mouse IgG (H + L) (Jackson ImmunoResearch, Cat. No 115-165-062) as secondary antibody (30 min). The samples were mounted in Mowiol before viewed and photographed on an inverted fluorescent microscope as above.

3. Results and discussion

The material system employed in this work allows one to control the surface density of hydroxy groups without modifying

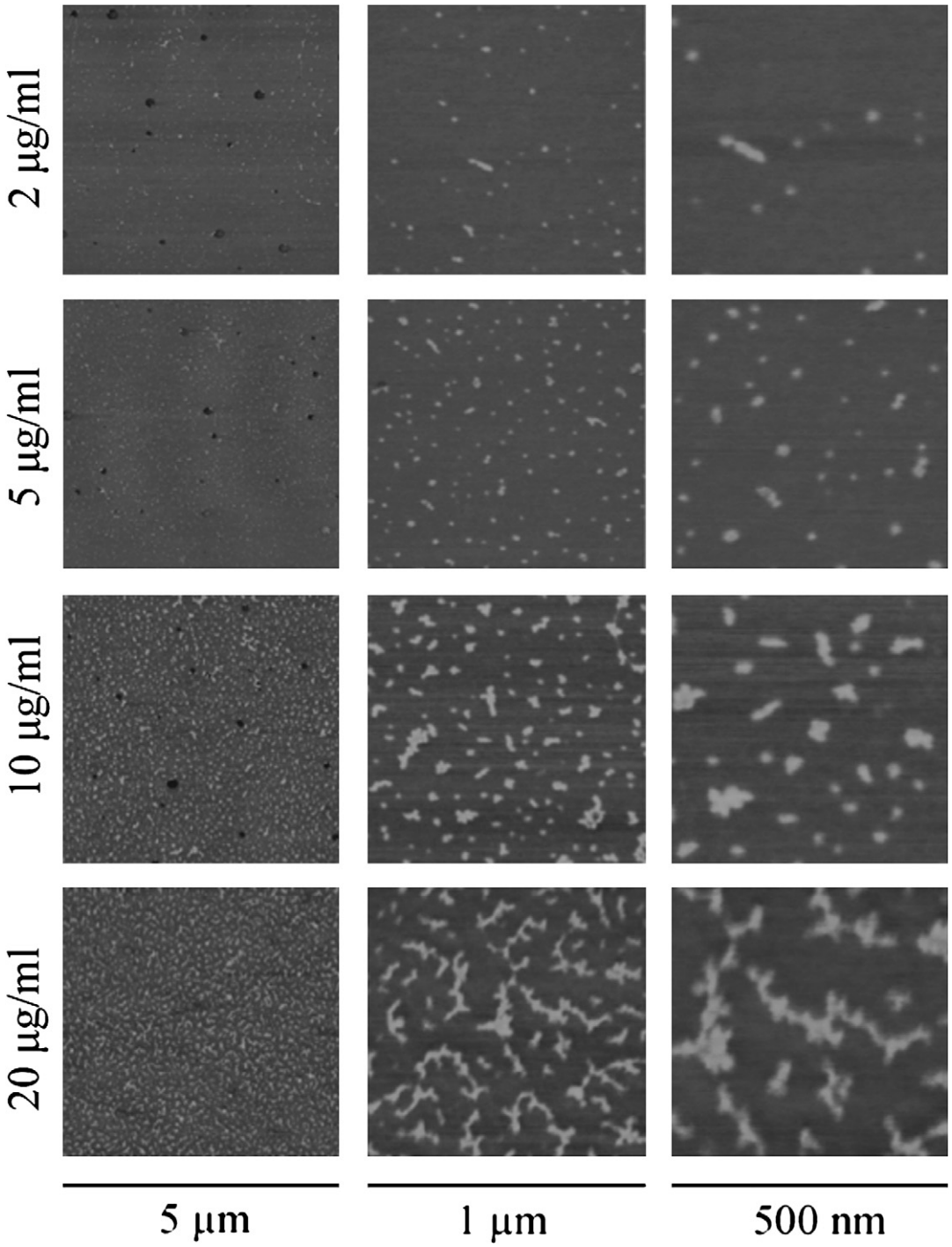

Fig. 1. AFM phase images of VN molecules on PEA ($x_{OH} = 0$) at different magnifications. Each row represents VN distribution after adsorption for 10 min from solutions with a concentration of 2, 5, 10, and 20 μg/mL respectively.

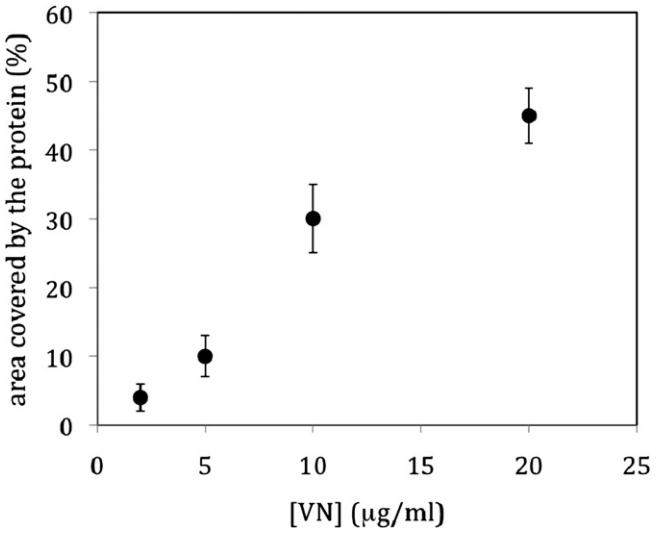

Fig. 2. Fraction of the substrate's surface covered by vitronectin after adsorption on PEA ($x_{OH} = 0$) from solutions of different concentrations [VN]. Error bars represent the standard deviation of four images.

any other functionality in the system. It is based on the copolymerisation of ethyl acrylate and hydroxyethyl acrylate monomers, which have a vinyl backbone chain with the side groups $-COOCH_2CH_3$ and $-COOCH_2CH_2OH$, respectively. Their combination gives rises to a random copolymer [28] in which the concentration of −OH groups determines both the surface energy and the hydrophilicity of the substrate (Table 1). XPS analysis of this family of copolymers revealed that the fraction of hydroxyethyl acrylate units on the material surface was close to the stoichiometric one [29].

Topography of the surfaces examined by AFM prior protein adsorption yielded similar roughness parameters regardless the polymer composition. Besides, we also scanned the surface of the materials after immersion in the physiological solution (i.e. without VN) with no difference when they were compared to the surfaces before adsorption. Roughness parameters obtained for the different surfaces as measured with AFM were $R_a = 18 \pm 4$ nm and $R_{ms} = 22 \pm 4$ nm (R_a is the arithmetic average of the height deviations from the center plane; R_{ms} is defined as the standard deviation of the height values).

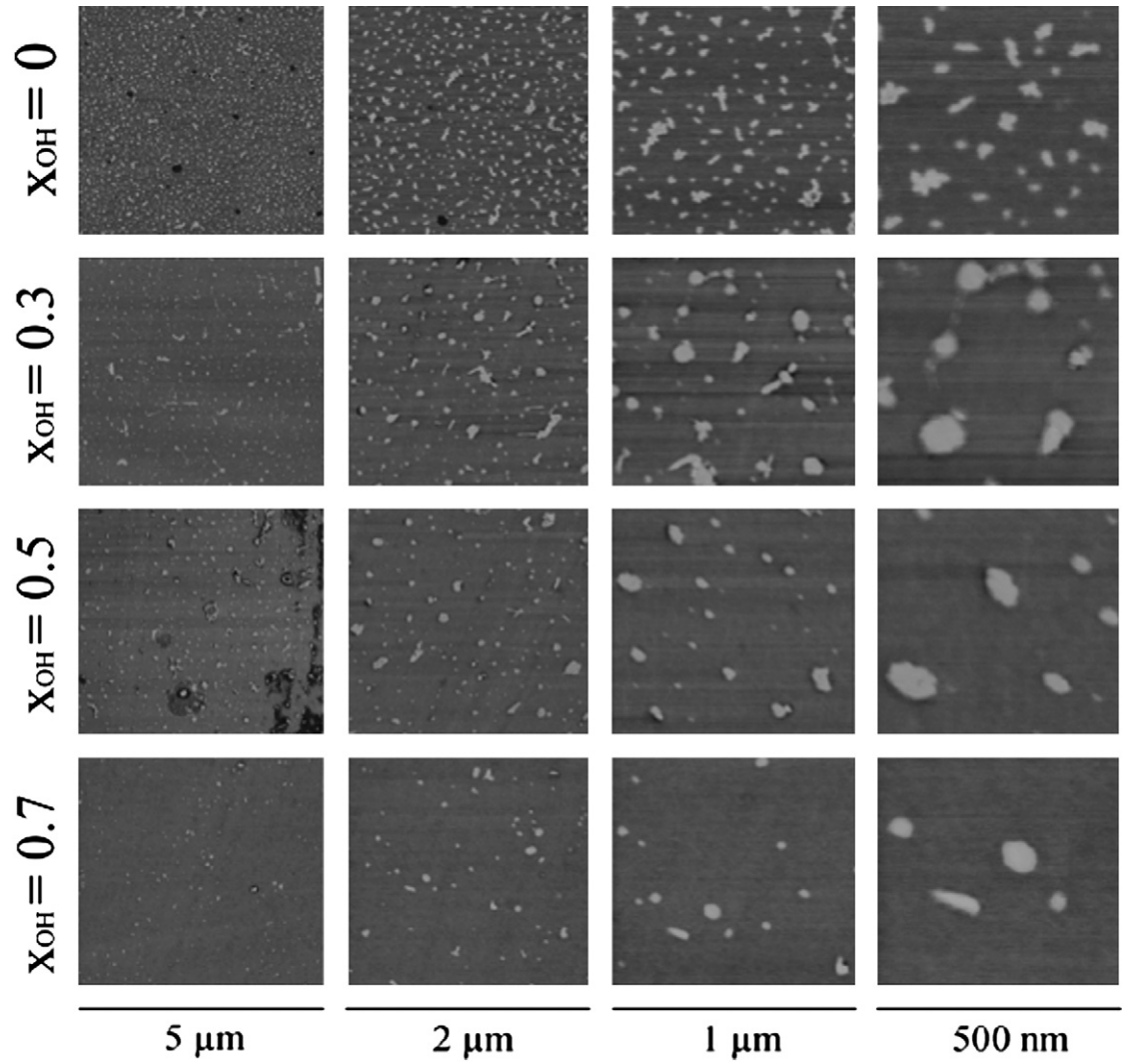

Fig. 3. VN distribution and supramolecular organization as revealed by the phase magnitude in AFM. The protein was adsorbed for 10 min from a solution of concentration 10 μg/ml on substrates with increasing fraction of −OH groups. $x_{OH} = 0$, 0.3, 0.5, 0.7 for each row respectively.

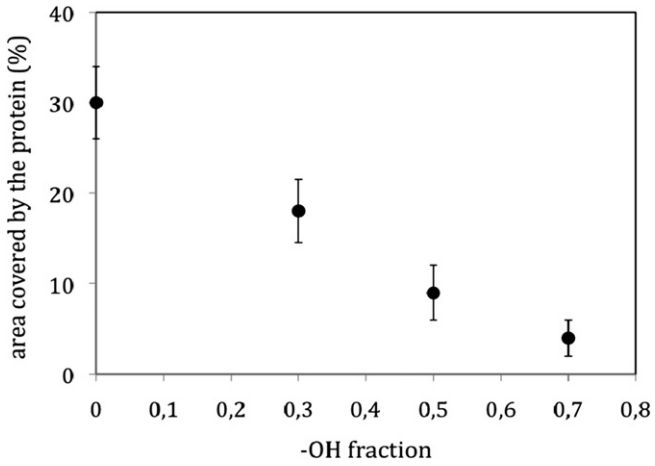

Fig. 4. Fraction of the substrate's surface covered by vitronectin after adsorption on model surfaces with different −OH fractions from a solution of concentrations 10 μg/ml. Error bars represent the standard deviation of four images.

3.1. Protein adsorption by atomic force microscopy

Fig. 1 shows the AFM images of VN adsorbed on the PEA substrate ($x_{OH} = 0$) after 10 min from protein solutions of different concentrations (increasing from top to bottom as indicated in Fig. 1).

VN supramolecular organization and distribution on the surface depend on the concentration of the initial protein solution from which the protein is adsorbed. The lowest concentration of 2 μg/ml, (Fig. 1, top row) results in the deposition of a few isolated globular VN molecules that are almost randomly distributed on the material. For a concentration of 5 μg/ml (Fig. 1) globular protein molecules are still observed, but with a higher density and still homogeneously distributed throughout the surface. VN distribution, after adsorption from a solution of concentration 10 μg/ml, suggests the formation of protein (nano)aggregates of approx. 3–5 molecules that cover the surface of the material homogeneously. When the concentration of the VN solution increases (up to 20 μg/ml) the surface of the material is covered by aligned aggregates of VN which tend to interconnect each other but not completely (Fig. 1). The amount of adsorbed VN can be qualitatively obtained by calculating the fraction of the area covered by the protein from AFM images (Fig. 2). It is observed that the amount of VN increases monotonically as the concentration of the solution does, though saturation of the surface is almost achieved when adsorbing from 20 μg/ml solution.

Surface density of −OH groups influences the amount of VN adsorbed on the different substrates. Fig. 3 shows protein supramolecular organization and distribution after adsorption on the different copolymers – with different −OH fractions – from a 10 μg/ml protein solution, which is the concentration afterwards employed when coating these substrate for cell adhesion studies. Globular-like features are observed whose number decreases as the

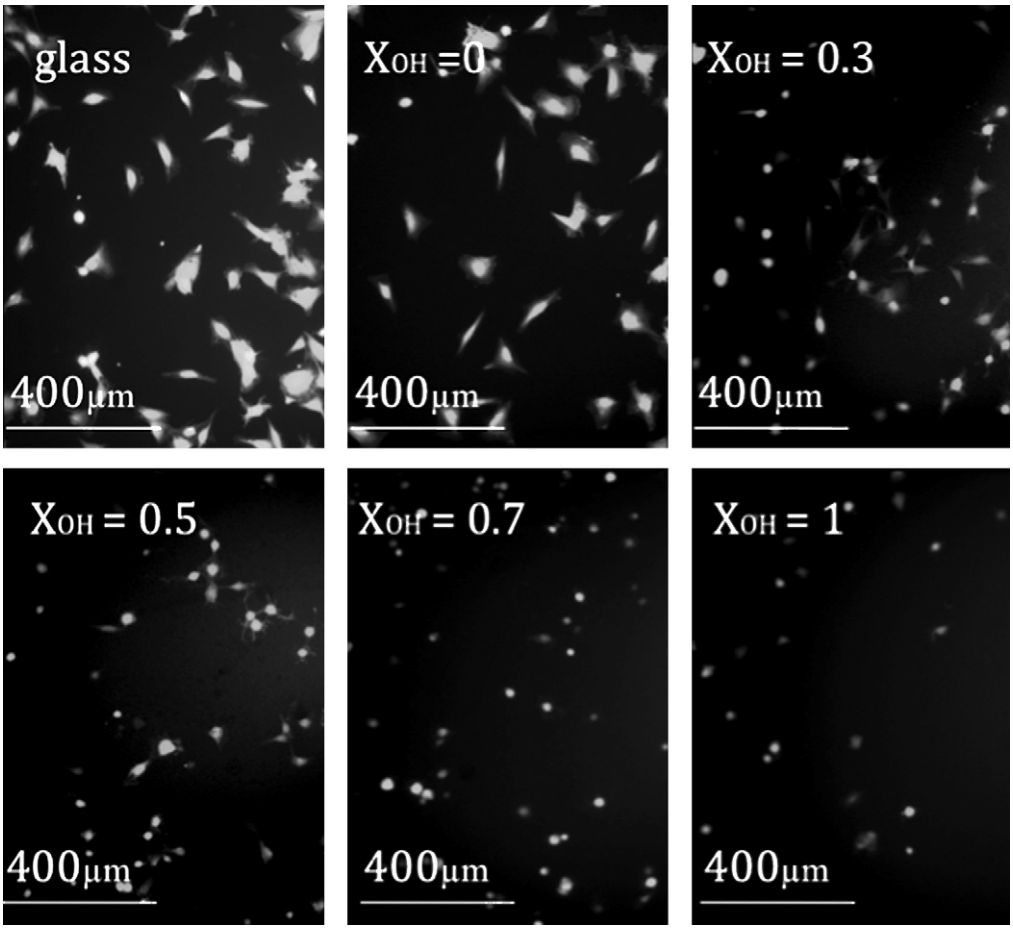

Fig. 5. Fluorescence microscopy images for FDA vital staining of fibroblast on the different substrates and the control glass. The density of −OH groups (x_{OH}) is shown on each picture for the different substrates.

fraction of hydroxy groups increases, leading for the more hydrophilic substrates to only 4% of the surface covered by the protein.

Fig. 4 shows the monotonic decrease for the fraction of the surface covered by the protein as the amount of −OH groups in the system increases as obtained from image analysis of AFM results after adsorption from a solution of concentration 10 μg/ml.

Other adhesive proteins were previously investigated on this family of polymers concerning protein adsorption as obtained by AFM. Interestingly, all of them show particular features that reflect the difficulty in establishing general rules concerning the effect of material properties on protein adsorption. For example fibronectin and fibrinogen tend to organise into a network after adsorption on the more hydrophobic substrates (−OH content lower the 20%) and globular aggregates were found as the fraction of −OH groups in the system increases [30,31], which does not happen for VN adsorption (Fig. 3). Besides even if the amount of adsorbed fibrinogen follows the same trend as it is shown for VN (Fig. 3), the amount of adsorbed fibronectin follows a non-monotonic dependence with the fraction of hydroxy groups in the system (almost the same amount of fibronectin was found for OH_0 and OH_{100} but in very different supramolecular organizations on the surface). Nevertheless, laminin adsorption takes place in a similar fashion as VN does, but only for the more hydrophobic polymers; as the fraction of −OH groups increases laminin self-interacts giving rise to a network of the protein on the substrate for intermediate hydroxy contents [32], which does not happen for VN adsorption for any copolymer composition.

3.2. Biological activity

It is well documented that the biological activity of many proteins is dependent on the physicochemical properties of the underlying substrate. Here we studied the initial response of human dermal fibroblasts to our model copolymers after coating with VN from a solution of concentration 10 μg/ml (in absence of serum). Fig. 5 shows the overall morphology of adhering fibroblasts onto the different VN-coated substrates compared to control glass (as positive control, also coated with 10 μg/ml VN solution). To disregard the effect of protein secreted by cells we focused on the initial cell–material interaction within only 2 h of cell culture. Protein secretion must be really low during this time framework if any. It is convenient to remark here that the same experiment was done without coating the substrates surface with any protein (plane materials) and cells were not able to adhere on any substrate.

We have used FDA staining to confirm that only viable cells are considered (esterases convert it into the fluorescent form) and we have observed fibronectin matrix formation in long term culture [33]. Moreover, cell viability on OH_{70} and OH_{100} substrates was assessed by collecting cells after 3 h of culture and seeding them on control TCPS plates, where they adhered and expanded normally [31].

Cell adhesion and spreading depend strongly on the OH fraction on the surface. Well spread cells, similar to those on the control glass, are observed on the most hydrophobic substrate OH_0; as the fraction of OH groups in the material increases, both the number of attached cells and their spreading diminish. Only rounded cells are observed on the more hydrophilic systems (OH_{70} and OH_{100}). The development of the actin cytoskeleton is also strongly affected by the underlying polymer; that is to say, by the activity of the VN layer adsorbed on the substrate (Fig. 6). Following the same trend well-developed actin fibres are observed on both the control glass and the hydrophobic substrate (OH_0), while the state of maturation of the cytoskeleton diminishes as the fraction of OH groups increases. Less stressed fibres are observed on the OH_{30} substrate, followed by the only peripheral distribution of actin on the OH_{50} substrate which reflects delayed cell spreading and presumably an altered tensional activity of the cell on these substrates [34]. Higher

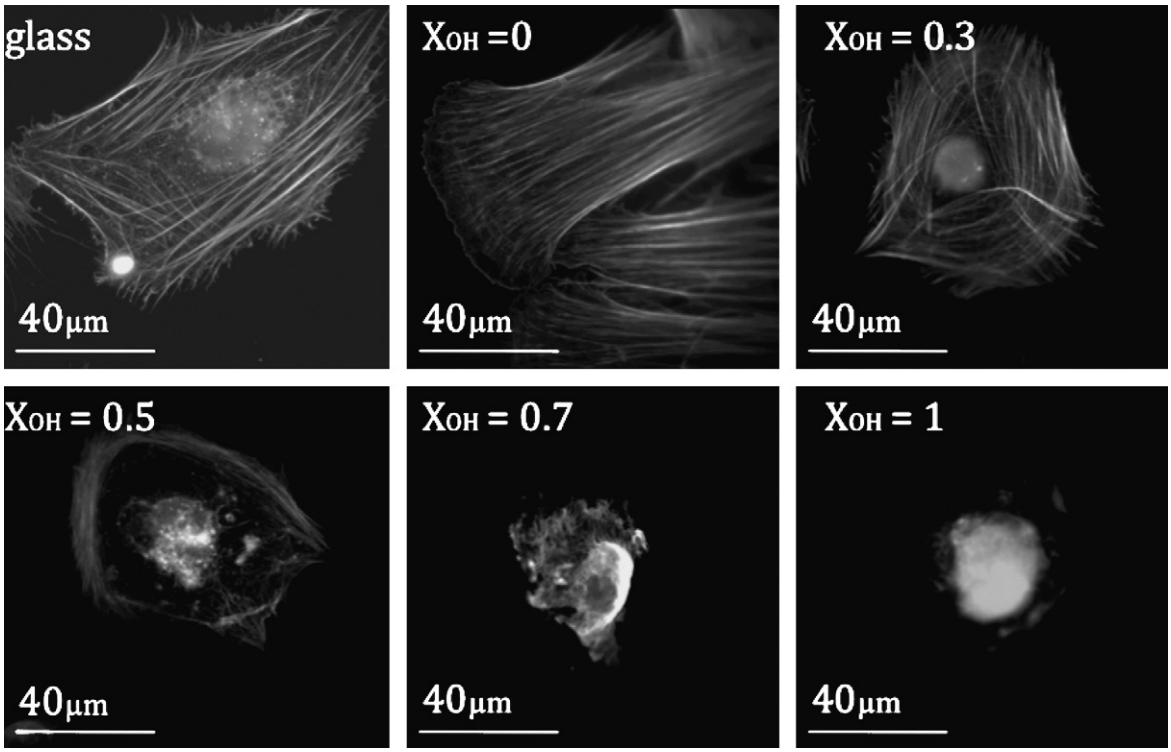

Fig. 6. Fluorescence microscopy images for actin cytoskeleton development of fibroblast on the different substrates and the control glass. The density of −OH groups (x_{OH}) is shown in each picture for the different substrates.

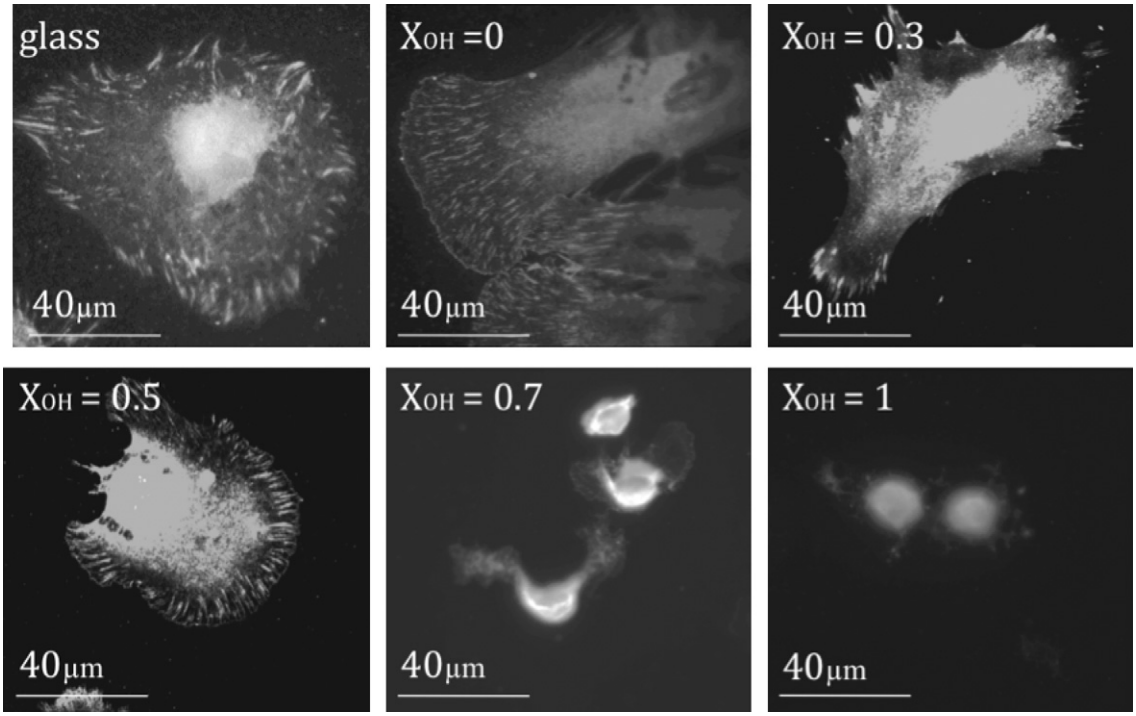

Fig. 7. Fluorescence microscopy images for focal adhesion formation of fibroblast on the different substrates and the control glass through immunofluorescence for vinculin. The density of −OH groups (x_{OH}) is shown in each picture for the different substrates.

fractions of OH groups on the substrate, leading to a very low amount of adsorbed VN, results in rounded cells lacking any actin organization, at least at the scale available by optical microscopy. This by no means excludes the very early stages of actin polymerization at the nanoscale.

The observed alteration in the overall cell morphology relates to the efficiency of the initial cell adhesion to the surfaces. Indeed, as shown in Fig. 7 the vinculin staining revealed well-defined focal adhesion complexes on the VN-coated substrates up to intermediate hydroxy contents (OH_{50}), including the control glass. Even if vinculin is expressed also in the more hydrophilic substrates, it is not organized into focal contacts but randomly distributed.

As stated above, this family of substrates was previously investigated with other matrix proteins and their biological activity was also correlated [30–32]. For instance, when coated with fibronectin, both protein adsorption and its biological activity were different. On the one hand FN adsorption takes place in a non-monotonic fashion leading to similar amounts of adsorbed protein on the more hydrophilic and hydrophobic substrata. FN tends to self-organise on the OH_0 surface leading to the so-called substrate induced assembly of FN into networks accompanied with very good cellular interaction [30]. This phenomenon was also found when fibrinogen was adsorbed on the OH_0 (PEA) substrate but it does not happen for VN adsorption, which reflects the specificity of the substrate–protein interaction. More importantly, however, is the enhanced VN activity on this family of substrates as compared with either FN or FG. For instance, for the substrate with intermediate OH content (OH_{50}) the interaction between FN domains and the substrate surface keeps the protein molecules in a globular-like shape that lead to poor cell adhesion and, consequently, to delayed spreading and focal adhesion formation [33]. A similar situation is found for cell interaction after fibrinogen adsorption on the OH_{50} substrate, on which less cell adhesion and cytoskeleton development were found [31]. Nevertheless, VN adsorption on the substrate with intermediate hydroxy content still results in pronounced cell adhesion, spreading and focal adhesion developments, even if the amount of adsorbed protein is significantly lower than on the more hydrophobic substrates.

The relationship between vitronectin adsorption and focal adhesion development could be discussed in terms of the mechanistic model proposed by Kato and Mkrsich [35]. This model proposed that each polymerization event of receptors could be considered to be a consequence of either a nucleation or growth mechanism. Nucleation refers to the aggregation of some integrin receptors into a cluster that does not dissociate (nucleation of focal adhesions). This cluster becomes irreversible because an intracellular network of proteins cross-links the integrin receptors together. On the other hand, growth describes the subsequent process of individual receptors (and smaller, mobile clusters) diffusing to the nucleated clusters (growth of focal adhesions). According to this model, as the amount of adsorbed VN on the surface decreases the growth of fewer focal adhesions is enhanced over nucleation. Higher number of focal contacts was formed on $-OH_0$ (and the control glass) distributed throughout the cytoplasm (favoured nucleation). As the fraction of hydroxyl groups increases (and the surface density of adsorbed VN decreases, Fig. 4) only some larger focal contacts distributed along the cell periphery are found (favoured growth). Moreover, there is minimum density of VN molecules on the substrate for focal adhesions to be developed that is not reached after VN adsorption on the $-OH_{70}$ and OH_{100} polymers.

4. Conclusions

VN supramolecular organization and distribution were investigated by AFM on model surfaces with controlled −OH density. Globular protein molecules are observed on the different substrates in increasing amount as the hydrophobicity of the substrate increases (the fraction of −OH groups decreases). Moreover, as the concentration of the solution from which the protein is adsorbed

increases, protein–protein interactions are enhanced leading to the formation of supramolecular aggregates (3–5 molecules) on the substrate. The biological activity of the protein on the different surfaces was also assessed after adsorption and the FDA vital staining, actin cytoskeleton development and focal adhesion formation equivocally show that VN is active on substrates containing up to 50% of OH groups, thus showing an enhanced biological activity of the protein as compared to other soluble matrix proteins such as fibronectin and fibrinogen on the same substrates.

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