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Fibronectin activity on substrates with controlled —OH density

Dencho Gugutkov,[1] George Altankov,[1,2] José Carlos Rodríguez Hernández,[3,4] Manuel Monleón Pradas,[3,4,5] Manuel Salmerón Sánchez[3,4,5]

[1]*Institut de Bioenginyeria de Catalunya, Barcelona, Spain*
[2]*ICREA (Institució Catalana de Recerca i Estudis Avançats), Barcelona, Spain*
[3]*Networking Research Center on Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Valencia, Spain*
[4]*Center for Biomaterials and Tissue Engineering, Universidad Politécnica de Valencia, 46022 Valencia, Spain*
[5]*Regenerative Medicine Unit, Centro de Investigación Príncipe Felipe, Autopista del Saler 16, 46013 Valencia, Spain*

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Abstract: Adhesion of human fibroblast to a family of fibronectin (FN) coated 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. Cell adhesion and spreading surprisingly decreased as the fraction of —OH groups on the surface increased. AFM studies of FN conformation revealed formation of a protein network on the more hydrophobic surfaces. The density of this network diminished as the fraction of —OH groups in the sample increased, up to a maximal —OH concentration at which, instead of the network, only FN aggregates were observed. The kinetics of network development was followed at different adsorption times. Immunofluorescence for vinculin revealed the formation of well-developed focal adhesion complexes on the more hydrophobic surface (similar to the control glass), which became less defined as the fraction of —OH groups increased. Thus, the efficiency of cell adhesion is enhanced by the formation of FN networks on the substrate, directly revealing the importance of the adsorbed protein conformation for cell adhesion. However, cell-dependent reorganization of substrate-associated FN, which usually takes place on more hydrophilic substrates (as do at the control glass slides), was not observed in this system, suggesting the increased strength of protein-to-substrate interaction. Instead, the late FN matrix formation—after 3 days of culture—was again better pronounced on the more hydrophobic substrates and decreased as the fraction of —OH groups increase, which is in a good agreement with the results for overall cell morphology and focal adhesion formation. © 2009 Wiley Periodicals, Inc. J Biomed Mater Res 92A: 322–331, 2010

Key words: cell adhesion; fibronectin; fibroblast; extracellular matrix; AFM

INTRODUCTION

Cell adhesion to synthetic materials is mediated by extracellular matrix proteins, which adsorb on the substrate on contact with physiological fluids *in vivo* or culture medium *in vitro*.[1–3] The concentration, distribution, and strength of interaction of the adsorbed protein layer are influenced by the nature of the underlying substrate. Besides the large variety of physical and chemical modifications developed to enhance the biocompatibility of materials, relatively little is known about the fate of adsorbed proteins. Soluble matrix proteins such as fibronectin (FN) and vitronectin often behave rather complex on biomaterials interface. FN is a glycoprotein that forms dimers, consisting of two subunits of 220 kDa, linked by a single disulfide bond near the carboxyl termini.[4,5] The importance of FN as a mediator of cell adhesion to a substrate was early recognized.[6] There is a line of investigations showing that FN not only adsorbs but, on adsorption, it undergoes active removal and fibril-like reorganization governed by the adhering cells.[7–9] This is presumably a process that is connected with the ability of cells to form their own extracellular matrix,[10] but it plays a distinct role for biocompatibility of materials and points to the active role of adhering cells.[7–10]

Cells interact with the adsorbed protein layer via integrins—a family of transmembrane receptors that

Correspondence to: M. Salmerón Sánchez; e-mail: masalsan@fis.upv.es

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govern the interaction of cells with the extracellular matrix (ECM). Integrin-mediated adhesion is a complex process that involves integrin association with the actin cytoskeleton and clustering into supramolecular complexes, focal adhesions, which contain structural proteins (vinculin, talin, tensin, etc.) and signaling molecules.[11,12]

Integrin-FN interaction, governed mainly by the $\alpha_5\beta_1$ dimer, often leads to the reorganization of the adsorbed FN into extracellular matrix fibrils.[13] β_1 integrin-mediated FN fibrillogenesis on the surface of a synthetic material depends on the wettability of the surface: fibroblasts are able to remove and reorganize adsorbed FN from some hydrophilic surfaces but not on other more hydrophobic ones.[7–9] Cell-mediated FN reorganization on a synthetic material seems to be an important factor to determine the biocompatibility of a material, because poor cell adhesion and spreading has been found in cases when integrin-mediated rearrangement of FN did not occur during ECM formation.[7,10]

Many studies have shown the importance of FN in promoting cell adhesion and regulating cell survival and phenotype expression on different surfaces.[14–21] The hydrophobic/hydrophilic nature of the surface is able to modulate FN conformation,[14] which is said to adsorb preferentially on hydrophobic surfaces[22] and that it undergoes greater extension of its dimer arms on hydrophilic than on hydrophobic glass[23] in a conformation which favors the cell-material interaction.[24] It is well documented that physicochemical properties of biomaterials surfaces have a great impact on protein adsorption and subsequent adhesion and proliferation of cells.[2,14,15,19–21] In particular, the substrate wettability, as characterized by water contact angle, has been found to be a clue parameter.[2,7–10,20] In general, wettable surfaces support cellular adhesion, which presumably is connected with the appropriate conformation of adsorbed proteins.[20] However, this is not always straightforward because materials with very high wettability, which usually bind much water—like hydrogels—do not support the adsorption of proteins and cell adhesion.[25] It seems that several factors collectively contribute to cellular interaction, and it is often difficult to distinguish them; for example, surface chemistry,[11,12,14,15] surface charge,[25,26] and even the micro/nano surface roughness[27,28] have been shown to influence FN adsorption and its biological activity.

This work investigates the role of —OH groups on cell-to-substrate interaction using human fibroblasts and a set of FN-coated surfaces with controlled fraction of hydroxyl groups. Cell adhesion and spreading, overall cell morphology and focal adhesions formation, as well as the fate of substrate associated FN, including its initial reorganization by the cells and subsequent fibrillar matrix formation, are studied and correlated with the conformation of adsorbed FN as directly observed by atomic force microscopy.

MATERIALS AND METHODS

Substrate preparation

Copolymer sheets were obtained by polymerization of a solution of both monomers ethyl acrylate, EA, (99% pure, Aldrich, Steinheim, Germany) and hydroxyethyl acrylate, HEA (96% pure, Aldrich), with the desired proportion, using 0.1 wt % of benzoin (98% pure, Scharlau, Barcelona, Spain) as photoinitiator and a 2 wt % of ethyleneglycol dimethacrylate EGDMA (Aldrich, 98% pure) as crosslinking agent. The polymerization was carried out 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 (hereafter, $-OH_x$ will refer to the sample with fraction x of HEA in the copolymer). 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.

Small disks were cut from the polymerized sheets to be used in the protein adsorption and cell adhesion studies. The samples were sterilized with gamma radiation (25 kGy) before the experiments.

Atomic force microscopy

AFM 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 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.

Fibronectin from human plasma (Roche, Mannheim, Germany) was adsorbed on the different substrates by immersing the material sheets in 20 μg/mL physiological solution (NaCl 0.9%) for 10 min.

The influence of the adsorption time on the conformation of the adsorbed protein was investigated by immersing the PEA ($-OH_0$) sheet in protein solutions at different times: 10 s, 30 s, 1 min, 3 min.

After protein adsorption, samples were rinsed three times in the physiological solution to eliminate the nonadsorbed protein. Remaining drops on the surface were dried by exposing the sample to a nitrogen flow for 2–3 min. AFM was performed in the tapping mode in air immediately after sample preparation. Both height and phase magnitudes were recorded for each image.

TABLE I
Equilibrium Water Content (EWC) and Water Contact Angle (WCA) for the Different Substrates

x_{OH}	EWC (%)	WCA (°)	R_{max} (nm)	RMS (nm)
0	1.7 ± 0.4	89 ± 1	24.3	3.1
0.3	7.6 ± 0.9	80 ± 2	8.5	1.5
0.5	18.2 ± 1.7	67 ± 1	10.6	1.1
0.7	40.6 ± 0.4	55 ± 1	17.1	1.5
1	134 ± 5	45 ± 2	6.9	1.2

Mean values and their standard deviations are reported for both EWC and WCA. The last two columns show the roughness parameters (R_{max}, the difference between the highest and lowest heights; RMS, root mean square, the standard deviation of the height values) for the different samples calculated on 1 × 1 μm^2 before fibronectin adsorption.

Contact angle measures

Water (Sigma-Aldrich, reagent grade) contact angle experiments were performed making use of the optical contact angle measuring device DataPhysics OCA 10 (Filderstadt, Germany). The sessile drop method was used to evaluate the static contact angle with constant drop volumes at room conditions. Measurements were performed in triplicate for each sample after gamma radiation.

Cells

Human dermal fibroblast cell line CCD-25SK was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in Dulbecco's modified Eagle medium (Gibco, 11960-044) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 2 m*M* L-glutamine, and 1 m*M* sodium pyruvate (Gibco, 11360-039), in a humidified atmosphere of 5% CO_2 in air. Around confluence the cells were detached with trypsin-EDTA (Gibco, 25200-072) that was inactivated with FBS after 5 min. The cells were then re-plated or used for experiments.

For cell experiments, the polymer substrates were sterilized in 70% ethanol during 10 min and then copiously rinsed with sterile phosphate buffered saline solution (PBS). The samples were placed individually in 24-well tissue culture plates with a culture area of 1.9 cm^2 (Nunc, 142475).

Cell adhesion

To investigate initial cell adhesion and morphology, 2.0 × 10^4 cells/well were seeded to each sample in 2.0 mL serum free medium. One set of samples had been precoated with FN (20 μg/mL) for 30 min at 37°C. After 2 h of incubation, the living cells were labeled with fluorescein diacetate (FDA) by adding 10 μL/mL from a stock of 1 mg/mL FDA in acetone to the medium. Under these conditions, the vital cells convert FDA in a fluorescent analogue via their esterases. Representative pictures of the adhered cells were then taken with a fluorescent microscope (Nikon, Eclipse E600) using the green channel. At least three representative pictures of each sample were made. Cell density and cell spreading were quantified by image analysis by calculating the average cell area on the different substrates.

FITC-fibronectin reorganization

Human plasma FN (Sigma, F2006) was dissolved in 0.1*M* sodium bicarbonate buffer (pH = 9.0) at 1 mg/mL, then 10 μL of fluorescein isotiocianate (FITC; Sigma, F7378) dissolved in dimethylsulfoxide to 10 mg/mL was added and left for 2 h at room temperature. The labeled FN was separated from nonconjugated dye on a Sephadex G-25 desalting column equilibrated with PBS. The final protein concentration was estimated by measuring the absorbance at 280 nm, while the degree of FITC-labeling was calculated against the absorbance at 494 nm. Aliquots were then stored at −20°C.

The ability of fibroblasts to reorganize adsorbed FITC-FN (i.e., early matrix) was monitored by coating all samples with 40 μg/mL for 30 min at 37°C, then rinsing with PBS twice, before seeding with 2.0 × 10^4 cells/well in serum containing medium. After 4 h of incubation, the samples were fixed using 3% paraformaldehyde, mounted in Mowiol (Sigma, 324590) and viewed and photographed with a fluorescent microscope (Nikon, Eclipse E600). As a positive control, a regular round shaped glass coverslip was used (Menzel GmbH, 15 mm diameter).

Fibronectin matrix formation

The ability of fibroblasts to secrete and deposit FN into the extracellular matrix fibrils (i.e., late matrix) was examined via immunofluorescence. For that, 3.0 × 10^4 cells/well were cultured on the different substrates for 3 days in serum containing medium. At the end of incubation, the cells were rinsed with PBS three times before fixed with 3% paraformaldehyde for 5 min. The samples were then washed as above and saturated with 1% bovine serum albumin (BSA) for 15 min. Subsequently, they were stained with a polyclonal rabbit anti-FN antibody (Santa Cruz, 9068) dissolved in 1% BSA in PBS for 30 min, followed by goat anti-rabbit Cy3-conjugated secondary antibody for 30 min before washed and mounted with Mowiol.

Focal adhesions formation

All samples were fixed with 3% paraformaldehyde (5 min) and permeabilized 0.5% Triton X-100 in PBS (5 min) before saturation with 10% albumin in PBS. Immunofluoresecence for vinculin was performed according to standard protocol (30 min, 37°C) applying mouse monoclonal anti-vinculin antibody 1:100 (Invitrogen) followed by goat anti-mouse Cy2 conjugated secondary antibody (Sigma) before washing and mounting with Mowiol.

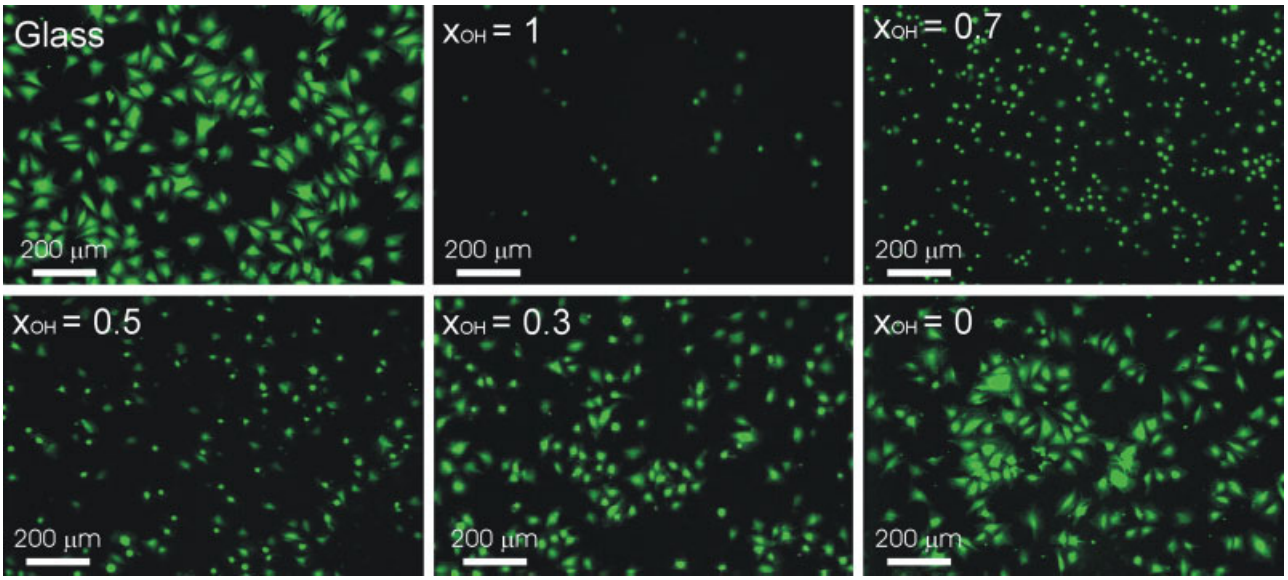

Figure 1. FDA vital staining of fibroblast on the different substrates and the control glass. The density of —OH groups (x_{OH}) is shown on each picture for the different substrates. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

RESULTS

Figure 1 shows the overall cell morphology on the different FN-coated substrates and the control glass after 2 h of culture. Cell density and spreading depend strongly on the —OH fraction on the surface (Table II). However, most elongated 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, the number of attached cells reduces and spreading diminishes. As expected, the substrate wettability clearly drops (see Table I) with reducing the —OH density. Only rounded cells are observed on the more hydrophilic systems ($—OH_{50}$ and $—OH_{70}$) even if the cell density does not differ significantly among them.

The organization of cellular proteins involved in the formation of focal adhesion complexes provides an opportunity to learn more about the effectiveness of cell-to-substrate interaction. Figure 2 shows the

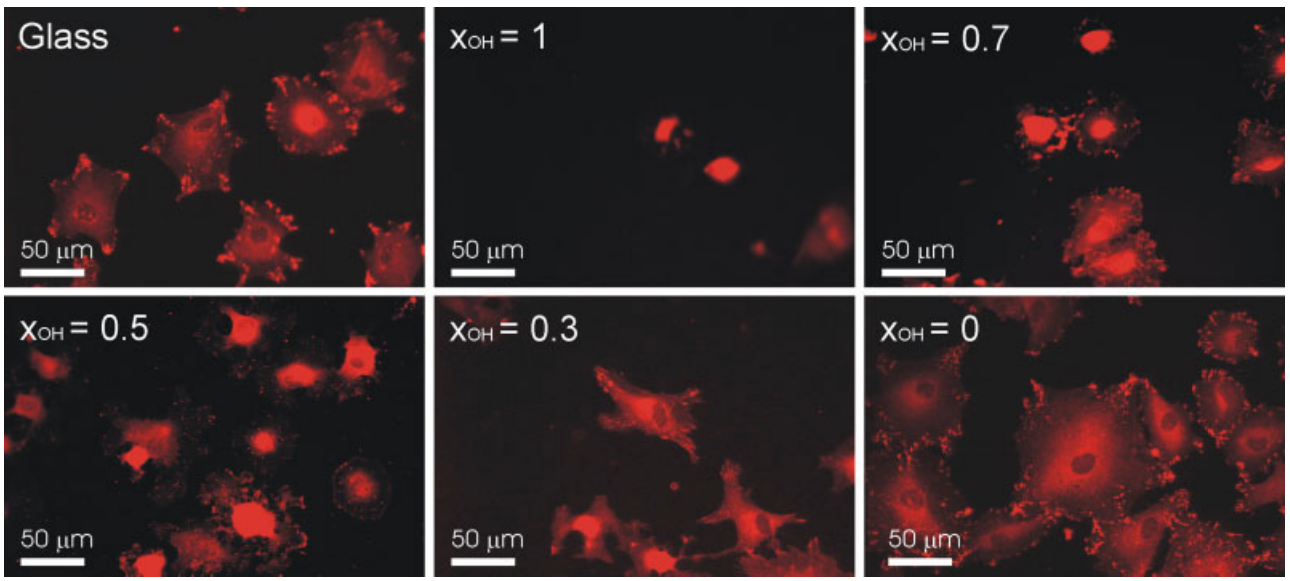

Figure 2. Focal adhesion formation of fibroblast on the different substrates and the control glass through inmunofluorescence for vinculin. The density of —OH groups (x_{OH}) is shown on each picture for the different substrates. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

distribution of vinculin in fibroblasts adhering on the different model substrates. Well-defined focal adhesions were found only on the more hydrophobic substrates (—OH_0, —OH_{30}, Fig. 2) and on the control glass (Fig. 2). Even if vinculin is expressed also in the more hydrophilic substrates, it is not organized into focal contacts but randomly distributed along the cell periphery.

Late FN matrix formation, for example after 3 days of culture, was also followed via immunofluorescence on the different samples. It was found that the cells are able to synthesize and deposit FN matrix fibrils on some of the material surfaces (Fig. 3) (hereafter, the term fibril will refer to the assembly of individual FN molecules through protein–protein interactions, which constitute a stable supramolecular entity. The formation of FN fibrils, the so-called fibrillogenesis, is a process either mediated by integrins or, as it is accounted for in the paper, induced by the substrate). However, FN fibrils could not be found on the more hydrophilic samples [—OH_{100} and —OH_{70}, Fig. 3(b,c)] while on the sample with intermediate composition, —OH_{50}, the fibroblasts deposit only small fibrils, located mostly beneath the cells [Fig. 3(d)]. As the hydroxyl fraction decreases the FN deposition increases, which moreover is organized into a typical matrix-like structure [Fig. 3(e,f)] similar to those on the control glass [Fig. 3(a)].

Figure 4 shows the cellular reorganization of adsorbed FITC-FN after 2 h of culture. As inferred from the homogeneous fluorescence of the substrate, no reorganization of FN takes place whatever the hydroxyl fraction of groups in the sample, that is, FN reorganization does not depend on the hydrophilicity for this family of substrates [Fig. 4(b–f)]. Conversely, FN reorganization is well pronounced on the control glass surface, an effect well documented in previous studies,[7–9] as indicated by the dark areas of FN removal by the cells and organization into bright fibril-like structures [Fig. 4(a)].

Figure 5 shows the AFM images of the FN adsorbed on the most hydrophobic substrate (—OH_0) after different adsorption times (10, 30, 60, and 180 s) from 20 μg/mL protein solution. Previous studies show that AFM phase signal reveals protein conformation when substrata's surfaces are not completely smooth (i.e., when their roughness blurs the small features due to adsorbed proteins).[28] The overall FN conformation on the surface changes as adsorption time goes by. At the very beginning of the adsorption process [Fig. 5(a), 10 s], isolated globular FN molecules are homogeneously distributed on the material. After 30 s of adsorption, FN globular molecules tend to align suggesting the initial formation of intermolecular connections, which result in protein–protein contacts through the surface [Fig. 5(b)]. FN conformation on Figure 5(c) reveals the formation of a protein network on the material after 60 s of adsorption. Increasing the adsorption time results in thickening the fibrils, which make up the protein network [Fig. 5(d), 3 min].

Surface density of —OH groups (x_{OH}) influences FN conformation on the substrates. Figure 6 shows protein conformation and distribution after adsorption for 10 min on the different substrates at different magnifications from a 20 μg/mL protein solution, which was the concentration previously employed for coating the substrates in cell adhesion studies. The more hydrophobic surfaces induce the formation of protein networks, whose density decreases as the fraction of —OH groups increases. FN network is well developed on the PEA (—OH_0) substrates [Fig. 6(a,g)]. Protein molecules with elongated shape, in the form of long fibrils, are still formed on the —OH_{30} surface, but only weakly connected protein filaments are identified [Fig. 6(b,h)]. Higher amounts of hydroxyl groups (from $x_{OH} = 0.5$ on) prevent the formation of a protein network on the materials surface and only disperse (nano) aggregates of the protein are observed on the —OH_{50}, —OH_{70} and PHEA substrates. Isolated globular molecules, homogeneously dispersed throughout the surface lacking FN–FN interactions, are observed on the control glass [Fig. 6(f,l)]. It must be remarked here the perfectly, ring-shaped, FN conformations observed on the control glass which is suggested to be a consequence of the protein-material interaction on hydrated surfaces.[28]

DISCUSSION

The initial cell-material interaction is a process driven by the soluble ECM proteins such as fibronectin and vitronectin, which rapidly adsorb on their surface.[2,10,20,29,30] The quantity, distribution, conformation and strength of interaction between these ECM proteins and the material's surface determine the cell response to artificial substrates.[7,8] In solution, before adsorption, FN is in a compact conformation[5,31]; substrate chemistry is known to influence FN conformation on a synthetic material leading to either the extension of the protein arms when favorable interactions between the surface chemistry and the protein takes place or, on the contrary, keeping the overall compact solution in other cases.[14,21]

To learn more about the particular role of —OH groups on the surface behavior of adsorbed matrix proteins, we have copolymerized 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 copoly-

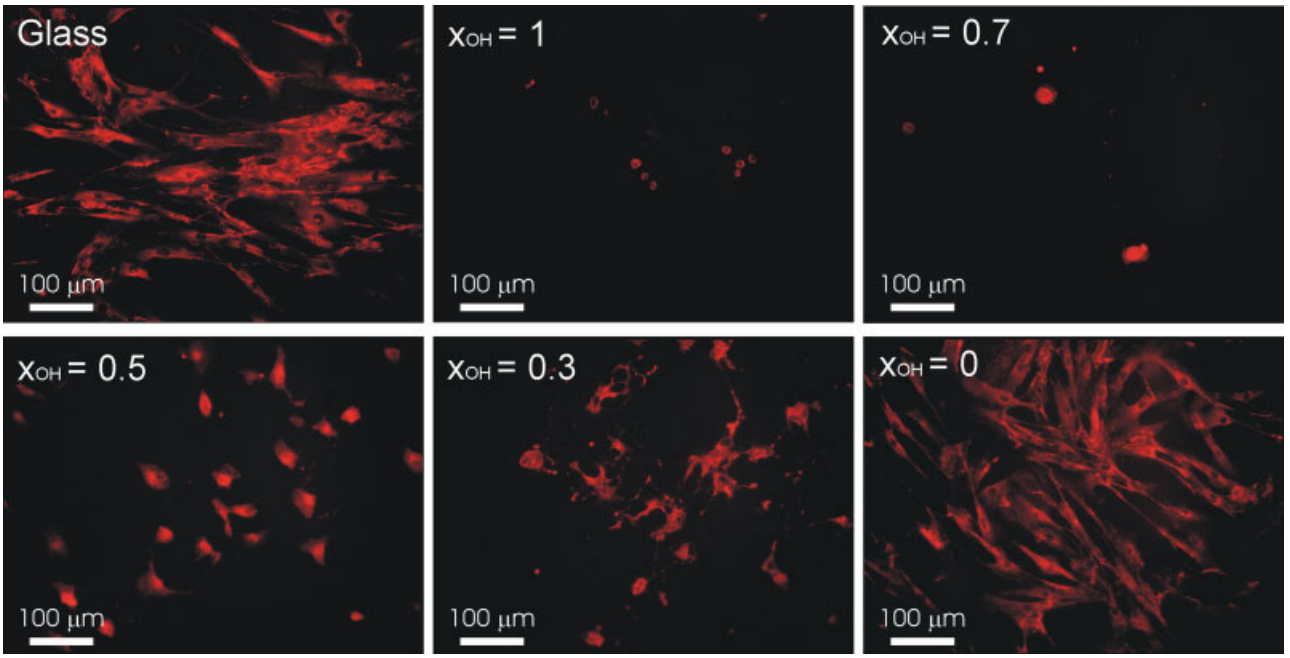

Figure 3. Fibronectin matrix formation by fibroblast on the different substrates and the control glass after 3 days of culture. The mass fraction of —OH groups (x_{OH}) is shown on each picture for the different substrates. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

merization gives rise to a substrate in which the surface density of —OH groups can be varied without modifying any other chemical functionality of the system. Substrate's hardness, sheet thickness, and roughness are known to influence protein adsorption. Our substrates were sheets ~1 mm thickness in the rubber state (room temperature is well-above the glass transition temperature), so their moduli are those of an elastomer (~1 MPa, independently of composition).[32] Moreover, the effect of gamma radiation on the physico-chemical properties of the system was shown to be negligible for this system.[33]

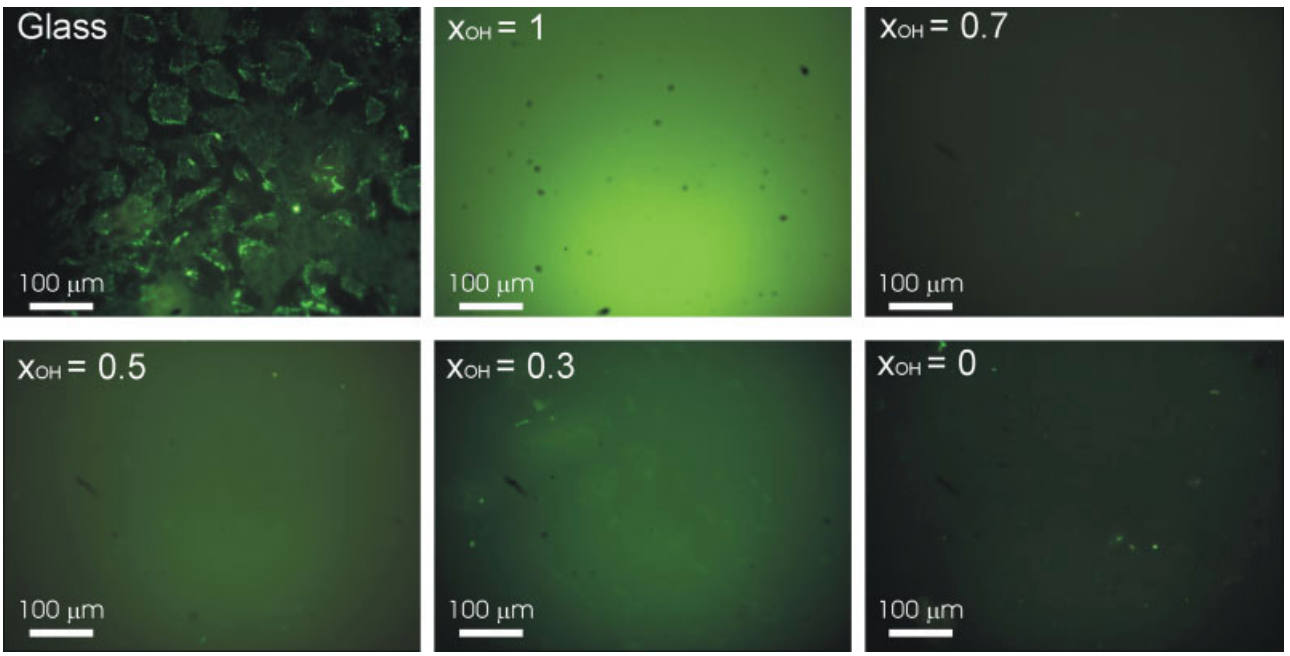

Figure 4. Reorganization of fluorescent fibronectin by fribroblast on the different substrates and the control glass after 2 h. The mass fraction of —OH groups is shown on each picture for the different substrates. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

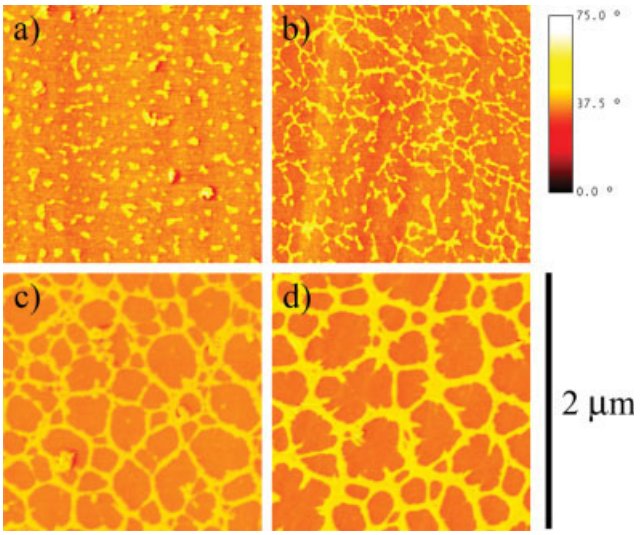

Figure 5. Dynamics of fibronectin fibrillogenesis on PEA (—OH_0) as revealed by the phase magnitude in AFM. The protein was adsorbed from a solution of concentration 20 μg/mL for different times (a) 10 s (b) 30 s (c) 1 min, and (d) 3 min. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

The concentration of —OH groups determines both the wettability and the hydrophilicity of the substrate, whereas the surface roughness remains unaffected (Table I). The interaction of the protein domains with the chemical functionalities of the substrate and with water determines the molecule's adsorbed conformation. That is to say, proteins will perceive not only the differences in the —OH groups of the polymeric structure, but also strong differences in the content of water molecules on the surface.

In contrast with results of previous studies,[7–10] dramatically altered fibroblast adhesion and focal adhesions formation were found on the most hydrophobic substrates. It was surprising, as it is generally agreed that some wettability enhances cellular interaction.[2,7–10] However, this is valid for moderate wettable substrata (about 30–60° WCA), and further decrease of WCA is either ineffective or leads to diminish cellular interaction.[2,34,35] Materials with too high wettability, like hydrogels[34] or PEG grafted substrates,[18,21] which carry too much water, do not support cell adhesion, because they acquire repelling properties for proteins and cells. Conversely, too high hydrophobicity also does not favor cellular interaction because may induce conformational changes of FN molecules resulting in altered biological properties.[2,10,20] The relatively high equilibrium water content of pure PHEA in our system could explain the low cell attachment. It is noteworthy, however, that the values for the wettability of these samples (WCA 45°) correspond to values that are optimal for the cellular interaction in other systems.[2,10,20,35] Conversely, surfaces with about 90° WCA, characteristic for pure PEA, and where the best cellular interaction was found, usually abrogate cellular interaction.[7–10,20,35] All this points to the different behavior surface associated FN in our system.

Indeed, we found that FN is able to form a network on the more hydrophobic surfaces (OH_0, —OH_{30}) whose density decreases as the fraction of the hydroxyl groups on the surface increases. FN fibrillogenesis, for example, the formation of a FN matrix, has been described as a process driven only by cells,[7] which occurs when integrins interact with the RGD domains of the FN molecule and extend their subunits giving rise to the formation of fibrils.[13] In this way, the FN that is synthesized by fibroblasts assembles into a fibril network,[6,14,15,17] this cell-medi-

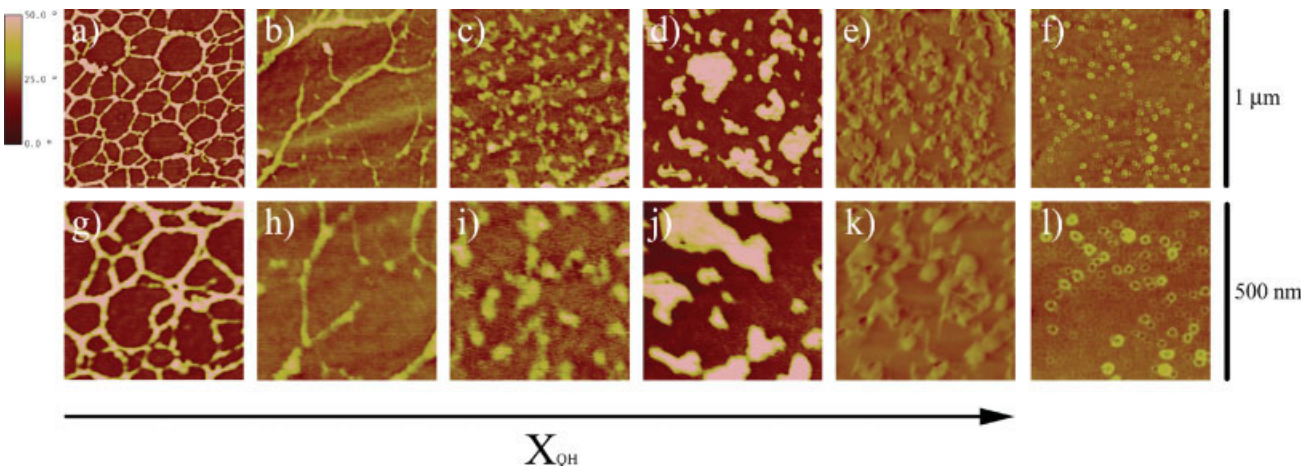

Figure 6. Fibronectin conformation as revealed by the phase magnitude in AFM. The protein was adsorbed for 10 min from a solution of concentration 20 μg/mL on substrates with increasing fraction of —OH groups. (a,g) PEA; x_{OH} = 0, (b,h) x_{OH} = 0.30, (c,i) x_{OH} = 0.50, (d,j) x_{OH} = 0.70, (e,k) x_{OH} = 1, (f,l) control glass. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

ated FN assembly obviously also takes place on our model surfaces (Fig. 3). During this assembly, however, FN needs to undergo distinct conformational changes[14,15,17] which on adsorption to the substrate can be limited. This may explain why materials surfaces affect FN matrix formation.[8] A line of previous studies suggests that to be biocompatible, materials need to adsorb proteins loosely, for example, in such a way that cells can easily remove and organize in matrix fibrils.[7–10] Our results, however, show that FN fibrillogenesis can take place as a consequence of the sole interaction between the protein molecules and a material surface, that is, without the need of cell involvement. Moreover, this material-induced fibrillogenesis is a dynamic process that depends strongly on the amount of protein adsorbed on the substrate and the time: as the FN is adsorbed its organization changes from a globular-like morphology to a more elongated one, and finally, the formation of the protein network takes place (Fig. 5). It seems, however, that from a certain concentration of hydroxyl groups on (x_{OH} = 0.5 or higher), the interaction between FN domains and the substrate surface keeps the protein molecules in a globular-like conformation, and the protein network is not formed anymore. We cannot discard the formation of FN globular aggregates in the more hydrophilic samples as a consequence of the drying process, which could lead to lateral reorganization of the adsorbed layer at the air-liquid interface; this process could be favored on very hydrophilic surfaces due to the absence of strong enough protein-surface interactions to prevent the protein relaxation during water release.[36] Nevertheless, this dehydration-generated change of conformation does not take place on the more hydrophobic substrates, on which the substrate-induced fibrillogenesis is stable.

It is noteworthy that the biological activity of the above observed FN network differs significantly from what we know for FN behavior on other set of surfaces.[10] The strength of the interaction between the protein and the substrate depends on the conformation of the protein after the adsorption process. Besides, it has been suggested that protein adsorption takes place more strongly on hydrophobic substrates.[35] Nonetheless, it is suggested that the formation of a protein network on the most hydrophobic substrate (—OH_0) must increase the strength of the material-protein interaction: cells are able to adhere and spread in a similar way as on the control glass [Fig. 1(a)] although residing on strongly hydrophobic substrate [—OH_0, Fig. 1(f)]; however, cell mediated FN-reorganization could only take place on the control glass but not on —OH_0 substrate [Fig. 4(a)], which suggests that FN is rather strongly bound compared to control glass, where it adsorbs more loosely.[7–10] Nevertheless, focal adhesion contacts are well developed on both surfaces (Fig. 2). Thus, very different FN conformations on the substrate may result in a similar cell response, which suggests that protein conformation and the strength of cellular interaction cannot be simply correlated (or even considered independently) when one tries to understand cell-material interactions, that is, excellent cell adhesion may occur even if cells are unable to reorganize adsorbed FN before rendering their own matrix as long as the protein-material interaction lead to the adequate protein conformation of the substrate.

TABLE II
Cell Density and Spreading Area as Calculated From Image Analysis of Figure 1

x_{OH}	Cell Area (μm^2)	Cell Density ($cells/mm^2$)
0	27 ± 17	142
0.3	21 ± 8	144
0.5	13 ± 4	112
0.7	7 ± 2	175
1	14 ± 5	26
Glass	43 ± 14	150

As the amount of —OH groups on the surface increases cell adhesion and spreading diminish (Table II), as well as the number of focal adhesion points. On the —OH_{30} substrate, preadsorbed FN is still able to form interconnected fibrils in a network-like fashion which correlated with relatively good cell spreading [Fig. 1(e)] and focal adhesion formation [Fig. 2(e)] even if cell-mediated FN reorganization cannot take place on the substrate [Fig. 4(e)]. Higher amounts of hydroxyl groups (above 50%, —OH_{50}, —OH_{70}, —OH_{100}) lead to poor cell adhesion and, consequently, to poorer spreading and focal adhesion formation [see Figs. 1, 2, and 4(b–d)]. AFM data show that on these substrates FN adsorption takes place in a globular-like conformation and there is no protein network formation [Fig. 5(d–f)]. For this group of substrates, cell-mediated FN reorganization does not take place presumably because of the conformation of the adsorbed FN is not good enough to allow initial cell adhesion, which are required before starting the matrix reorganization process.[10,37]

Late FN matrix formation-after 3 days of culture-was again excellent on the more hydrophobic substrates and decreased as the fraction of —OH groups increased in good agreement with cell adhesion and focal adhesions formation. Cells are also able to secrete their own extracellular matrix on the control glass. Altogether this suggests that even unable to organize the preadsorbed FN on the substrate, the fibroblasts respond on this FN network, presumably because the conformation of the protein provides the adequate signals which stimulate their normal matrix-forming activity.[10]

Our results suggest that the distinction between hydrophilic and hydrophobic features of a substrate is insufficient to explain the general trends underlying the cell-material interaction, and more factors must be taken into account. For instance, it has been reported that the ability of fibroblasts to secrete ECM proteins is greatly reduced on hydrophobic substrates[9] even if cell adhesion takes place, what clearly differs from the results in this work. Rather, fibroblast functional behavior on a synthetic substrate depends in a subtle way on the particular substrate chemistry that influences the process of protein adsorption. Both protein conformation on the substrate and the intensity of the protein-material bond play a fundamental role on cell behavior: the adequate protein conformation on the substrate—leading to a substrate induced FN fibrillogenenis—results in excellent cell adhesion and matrix formation (for low —OH contents in this work), even if preadsorbed FN cannot be removed by cells. Alternatively, if protein conformation is good enough so as to support initial cell adhesion, cells will be able to remove the initial FN layer and secrete their own extracellular matrix (as it happens in the control glass). Higher —OH fractions in the substrate lead to inadequate protein conformation on the substrate, which does not support good cell adhesion and consequently leads to diminished functionality.

CONCLUSIONS

FN conformation depends on the substrate density of hydroxyl groups. On the more hydrophobic substrates, FN is capable of establishing FN–FN interactions, which lead to the formation of a protein network, directly revealed by AFM, enhancing the protein-material interaction. The kinetics of FN fibrillogenesis was observed by AFM after different adsorption times. The presence of a protein network on the material favors fibroblast adhesion and late matrix formation, even if cells are not able to reorganize the preadsorbed FN layer. Higher amounts of hydroxyl groups on the substrate diminished cell adhesion and functionality: protein conformation is not adequate to cluster integrins and to direct adequate signals to the cell interior.

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