

Controlled Assembly of Fibronectin Nanofibrils Triggered by Random Copolymer Chemistry

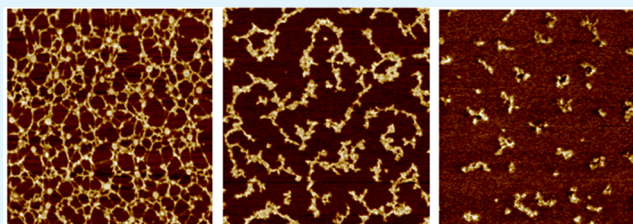
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ABSTRACT: Fibronectin fibrillogenesis is the physiological process by which cells elaborate a fibrous FN matrix. Poly(ethyl acrylate), PEA, has been described to induce a similar process upon simple adsorption of fibronectin (FN) from a protein solution—in the absence of cells—leading to the so-called material-driven fibronectin fibrillogenesis. Poly(methyl acrylate), PMA, is a polymer with very similar chemistry to PEA, on which FN is adsorbed, keeping the globular conformation of the protein in solution. We have used radical polymerization to synthesize copolymers with controlled EA/MA ratio, seeking to modulate the degree of FN fibrillogenesis. The physicochemical properties of the system were studied using dynamic–mechanical analysis, differential scanning calorimetry, and water contact angle. Both the degree of FN fibrillogenesis and the availability of the integrin binding region of FN directly depend on the percentage of EA in the copolymer, whereas the same total amount of FN was adsorbed regardless the EA/MA ratio. Cell morphology adhesion and differentiation of murine C2C12 were shown to depend on the degree of FN fibrillogenesis previously attained on the material surface. Myogenic differentiation was enhanced on the copolymers with higher EA content, i.e. more interconnected FN fibrils.



KEYWORDS: fibronectin, fibrillogenesis, biointerface, bioactive substrates, cell adhesion, cell differentiation

1. INTRODUCTION

The chemical and physical properties of biomaterials determine the cell/material interaction.^{1–3} The first biological event which occurs on the material surface when biomaterials are either studied *in vitro* or implanted *in vivo* is protein adsorption from culture media or physiological fluids, respectively.^{4–6} Differences in protein adsorption and configuration on the substrate play a pivotal role in cellular behavior, including cell adhesion, proliferation, migration, and differentiation.^{7–9}

Integrins are the major receptors for proteins adsorbed on a material surface and trigger cell adhesion and activate diverse intracellular signaling pathways to regulate cell fate.^{10,11} Integrins are also involved in assembling fibrillar matrix networks from soluble secreted matrix proteins, e.g. the formation of fibrillar fibronectin matrices.¹²

Fibronectin (FN) is organized into a fibrillar network through direct interactions with cell surface receptors.¹³ FN fibrillogenesis is a cell-mediated matrix assembly process during which FN undergoes conformational changes that expose fibronectin-binding sites and promote intermolecular interactions needed for fibril formation.¹⁴ FN fibrillar matrices have diverse functional properties, provide binding sites for other ECM proteins, act as a structural support for cells, and transduce cell signaling.^{13,15}

Several studies have demonstrated that surface chemistry is a key parameter that determines the amount and conformation of adsorbed FN.^{16–18} In previous works, we have shown the ability of certain material surfaces (in particular poly(ethyl acrylate) – PEA) to promote the organization of fibronectin into physiological-like (nano)networks, a process that we named material-driven fibronectin fibrillogenesis.^{19–22} There, the adsorption of individual FN molecules on the material surface allows the exposure of FN self-assembly sites to drive FN–FN interaction and the organization of (nano)networks at the material interface, in the absence of cells. After FN adsorption on PEA, a biologically active FN (nano)network is spontaneously assembled on the material surface. This phenomenon does not occur on poly(methyl acrylate) (PMA), which differs from PEA in one single carbon in the side chain, where FN is adsorbed, keeping the globular conformation.^{20,21} Other ways to induce the formation of fibronectin fibrils on a material surface, mostly triggered by force, have been reported elsewhere.^{20,23–25}

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Here, a series of copolymers with controlled ethyl acrylate/methyl acrylate (EA/MA) ratio were synthesized, seeking to modulate the degree of FN fibrillogenesis. We investigated FN adsorption and conformation as a function of the amount of EA in the copolymers. Afterward, we assessed the effect of the degree of fibrillogenesis on cell adhesion and cell differentiation using C2C12 myoblasts.

The main novelty of this work is that we now have a way to control the degree of this process of organization of fibronectin into nanonetworks by using the percentage of ethyl acrylate in a random copolymer that consists of EA (which polymer induces fibrillogenesis) and MA (which polymer adsorbs FN in a globular shape). Overall, this paper shows that the composition of the copolymer, i.e. the EA fraction, determines the degree of fibrillogenesis, and, in turn, this controls cell fate, including cell differentiation.

2. MATERIALS AND METHODS

2.1. Materials. Copolymers sheets (ca. 0.4 mm of thickness) were obtained by radical polymerization of a solution of ethyl acrylate (EA), methyl acrylate, MA, (Sigma-Aldrich, Steinheim, Germany) and merging ratios of EA/MA of 70/30, 50/50 and 30/70 wt % using 1% wt benzoin (98% pure, Scharlau) as photoinitiator. The reaction was carried out in ultraviolet light for 24 h. After polymerization, low molecular weight substances were extracted from the material by drying in vacuum to constant weight. Besides, pure poly(ethyl acrylate), PEA, and poly(methyl acrylate), PMA, sheets were prepared in the same way. Thereafter, materials with the aforementioned composition will be named as PEA100, PEA70, PEA50, PEA30 and PMA100.

Thin films were prepared using a spin-coater (Brewer Science). Each one of the synthesized polymers and copolymers was dissolved in toluene at concentration between 4 and 6 wt %. Spin-casting was performed on 12 and 25 mm glass coverslips at 3000 rpm for 30 s. Samples were dried in vacuum at 60 °C before further characterization. The obtained films are not porous and have approximately 500 nm of thickness.

2.2. Physico-chemical characterization of materials. **2.2.1. Differential scanning calorimetry.** Differential scanning calorimetry (DSC) measurements were performed in a Mettler Toledo 823e apparatus on samples between 5 and 10 mg. Nitrogen gas was purged through the DSC cell with a flow rate of 20 mL/min. After erasing the effects of any previous thermal history by heating to 100 °C, the samples were subjected to a cooling scan down to -60 °C at 10 °C/min, followed by a heating scan from that temperature up to 100 °C at a rate of 10 °C/min. The glass transition temperature (T_g) was calculated from the cooling scan as the inflection point of the specific heat capacity (c_p) versus temperature; which coincides with the minimum in the temperature derivative of c_p .

2.2.2. Dynamic-mechanical analysis (DMA). Dynamic-mechanical analysis was performed using a Seiko DMS210 apparatus at a frequency of 1 Hz in the tension mode. The temperature dependence of the storage modulus (E'), loss modulus (E''), and loss tangent ($\tan \delta$) was measured in the temperature range -70 to 70 °C at a heating rate of 2 °C/min. The samples for these measurements were prismatic (ca. 15 × 8 × 1 mm).

2.2.3. Water contact angle on the different substrates. The surface wettability was investigated by water contact angle (WCA) measurements. To measure the static contact angles, a 3 μ L drop was deposited onto the surface and stabilization allowed (~10 s). When the three-phase boundary is in motion, the angles formed are called dynamic contact angles and they are further subdivided into advancing and receding contact angles. The advancing contact angles was measured as drop expanded, whereas the receding contact angle whole the liquid was removed from the drop. The difference between the advancing and the receding contact angle is called hysteresis (H). Measurements were carried out using a Theta optical tensiometer

(Biolin Scientific). All measurements were performed six times for each substrate.

2.3. Atomic force microscope (AFM) experiments. Atomic force microscopy (AFM) was performed in a Multimode AFM equipped with NanoScope IIIa controller from Veeco (Manchester, UK) operating in tapping mode in air. Nanoscope 5.30r2 software version was used for image processing and analysis. 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 600 mV and the amplitude set point A_{sp} was 1.8 V. The ratio between the amplitude set point and the free amplitude A_{oo}/A_0 was kept equal to 0.8.

In order to study the topography of the surfaces, roughness of spin-coated samples was measured from AFM images (size 1 μ m × 1 μ m). R_q (root-mean-square average of height deviations taken from the mean image data plane) was calculated using the roughness subroutine in the Nanoscope software.

To analyze FN organization on material surfaces, samples prepared by spin-coating were covered with a solution of concentration 5 or 20 μ g/mL for 10 min (FN from human plasma (Sigma-Aldrich) and PBS). After adsorption, the samples were rinsed in PBS to eliminate the nonadsorbed protein.

Image processing and fractal dimension (F_D) calculation via the box-counting method (BCM)²⁶ were performed by using FraCLac and ImageJ software.^{27,28}

2.4. FN adsorption. FN from human plasma was adsorbed from a solution of concentration 20 μ g/mL in DPBS for 1h (for complete saturation) on 12 mm samples. After adsorption, supernatant was collected and quantified by Micro BCA Protein Assay Kit (Thermo Scientific). The adsorbed FN was obtained as the difference between the amount of FN in the initial solution and in the supernatant.

2.5. FN conformation. The relationship between the degree of FN fibrillogenesis and the availability of FN domains on the material surface was investigated by means of an enzyme-linked immunosorbent assay (ELISA). FN was adsorbed from a solution with a concentration of 20 μ g/mL in DPBS for 1h on spin coated substrates (12 mm). Then, samples were rinsed with DPBS to eliminate the nonadsorbed protein. After adsorption, samples were blocked in 1% BSA/DPBS and incubated with primary antibody Anti-Fibronectin cell binding domain (1:500 MAB1937, Merck-Millipore) in blocking solution for 1h at 37 °C. Samples were then rinsed in 0.5% Tween-20/DPBS and incubated with alkaline phosphatase conjugated secondary antibody (1:5000) for 1 h at 37 °C followed by incubation with 4-methylumbelliferyl phosphate substrate (Sigma-Aldrich) for 45 min at 37 °C. Reaction products were quantified using a fluorescence plate reader (Victor III, PerkinElmer) at 365 nm/465 nm.

2.6. Cell culture. Murine C2C12 myoblasts (Sigma-Aldrich) were maintained in Dubelcco's Modified Eagle Medium (DMEM) supplemented with 20% fetal bovine serum and 1% penicillin-streptomycin in a humidified atmosphere at 37 °C and 5% CO₂. Cells were subcultured before reaching confluence (approximately every 2 days).

For cell adhesion, myoblasts were seeded at low density (5000 cells/cm²) onto different materials after coating with 20 μ g/mL of FN in DMEM supplemented with 1% penicillin-streptomycin and in absence of serum in medium.

In myoblast differentiation experiments, C2C12 cells were seeded at confluence density (20000 cells/cm²) onto the materials coated with 20 μ g/mL of FN in DMEM supplemented with differentiation medium in absence of serum (1% insulin-transferrin-selenium and 1% penicillin-streptomycin). For the experiments including contractility inhibitors, blebbistatin was added to the culture medium at 10 mM after 2 h of culture and maintained for 4 days.

2.7. Cell adhesion experiments. After 3 h of culture, C2C12 cells were washed in DPBS (Gibco) and fixed in 10% formalin solution (Sigma) at 4 °C. Cells were incubated with permeabilizing buffer (103 g/L sucrose, 2.92 g/L NaCl, 0.6 g/L MgCl₂, 4.76 g/L HEPES buffer, 5 mL/L Triton X-100, pH 7.2) for 5 min, next blocked in 1% BSA/DPBS and incubated with primary antibody against vinculin (Sigma,

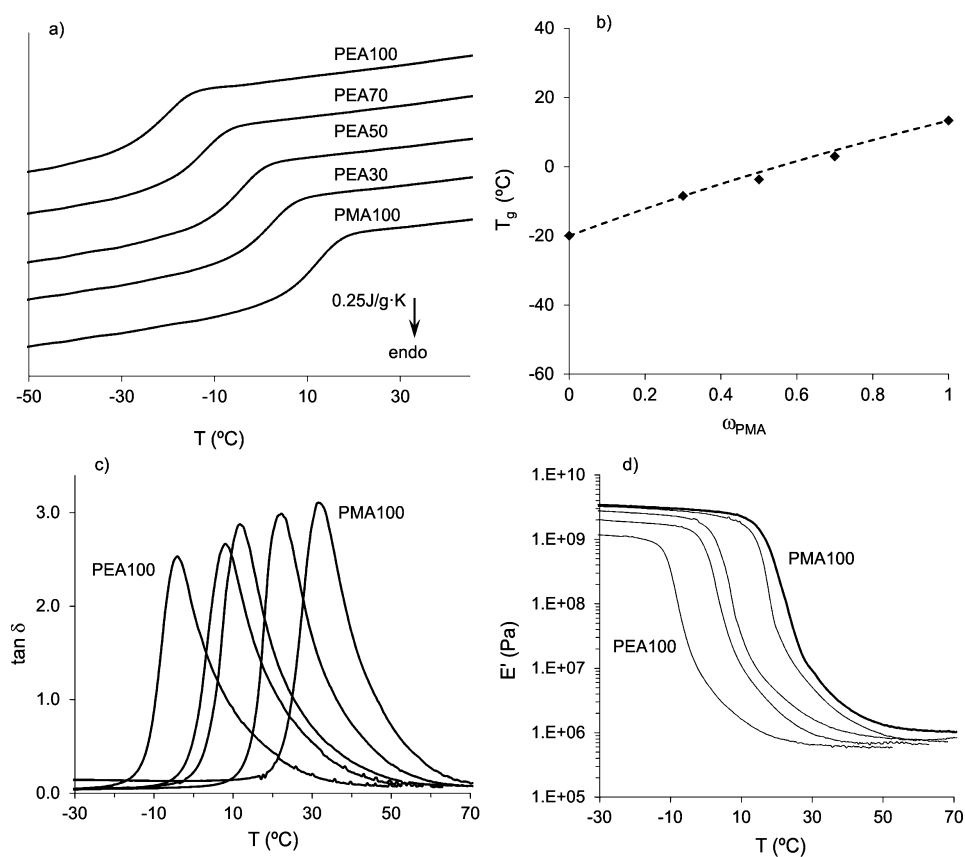


Figure 1. (a) DSC thermograms at a cooling rate of 10 °C/min. (b) Couchman–Karasz prediction and experimental glass transition values (T_g). (c) Mechanical loss tangent ($\tan \delta$) and (d) elastic modulus (E') measured at 1 Hz.

1:400). Samples were then rinsed in DPBS/0.5% Tween-20. Cy3-conjugated antimouse secondary antibody in 1% BSA/DPBS (Invitrogen) and BODIPY FL phalloidin (Invitrogen) were used. Finally, samples were washed and mounted in Vectashield with DAPI (Vector Laboratories). Eclipse 80i (Nikon) fluorescent microscope was used for cellular imaging. After imaging, cell morphology was analyzed by ImageJ software.

2.8. Focal adhesion kinase activity. After 3 h of C2C12 cells culture on samples, total protein extraction was performed by lysing the cells with RIPA buffer (50 mM Tris-HCl pH 7.4, 1% nonidet p-40, 0.25% Na-deoxycholate, 150 mM NaCl and 1 mM EDTA) supplemented with protease inhibitor cocktail tablets (Roche). The lysates were concentrated with Microcon YM-30 Centrifugal Filters units (Millipore) and separated in 8%-SDS PAGE under denaturing conditions as described elsewhere.²⁹ Proteins were transferred to a PVDF membrane (GE Healthcare) using a semidry transfer cell system (Biorad), and blocked by immersion in 5% skimmed milk in PBS. To analyze the expression patterns of FAK and phosphorylated FAK on tyrosine 397 (pFAK), the blots were incubated separately with primary antibody against FAK (Millipore, 1:2500), pFAKs (Millipore, 1:250) and α -Tubuline (Abcam, 1:20000) as a loading control in PBS/0.1% Tween-20/2% skimmed milk for 1h at room temperature and washed with PBS/0.1% Tween-20. The blots were subsequently incubated in HRP-conjugated secondary antibody (GE Healthcare) diluted 1:20000 in PBS/0.1% Tween-20/2% skimmed milk. The Supersignal West Femto Maximum Sensitivity Substrate (Pierce) was used prior to exposing the blots to X-ray film. Image analysis of the western bands was conducted using ImageJ software.

2.9. Myogenic differentiation experiments. C2C12 cells were cultured on FN-coated materials for 4 days under differentiation conditions and immunostained for sarcomeric α -actinin. Cells were fixed in 70% ethanol/37% formaldehyde/glacial acetic acid (20:2:1) for 10 min and then blocked in 5% goat serum in DPBS for 1 h. Samples were sequentially incubated with primary antibody anti-

arcomeric α -actinin (Abcam, 1:200) and secondary antibody antimouse AlexaFluor-488 (Invitrogen, 1:200) for 1h, and then washed with DPBS/0.5% Tween 20. Finally the samples were mounted with Vectashield containing DAPI. Eclipse 80i (Nikon) fluorescent microscope was used for cellular imaging. Myogenic differentiation was calculated as the percentage of cells positive for sarcomeric α -actinin using ImageJ software.

2.10. Statistics. All experiments were performed at least three times unless otherwise noted. Data were reported as mean - standard error. To establish if our data follow a normal distribution, D'Agostino-Pearson omnibus test was resorted.

Results were analyzed by one-way ANOVA using GraphPad Prism 6.0. If treatment level differences were determined to be significant, pairwise comparisons would perform using a Tukey in case of normal distribution of data or a Dunn's test in the contrary case. A 95% confidence level was considered significant.

3. RESULTS

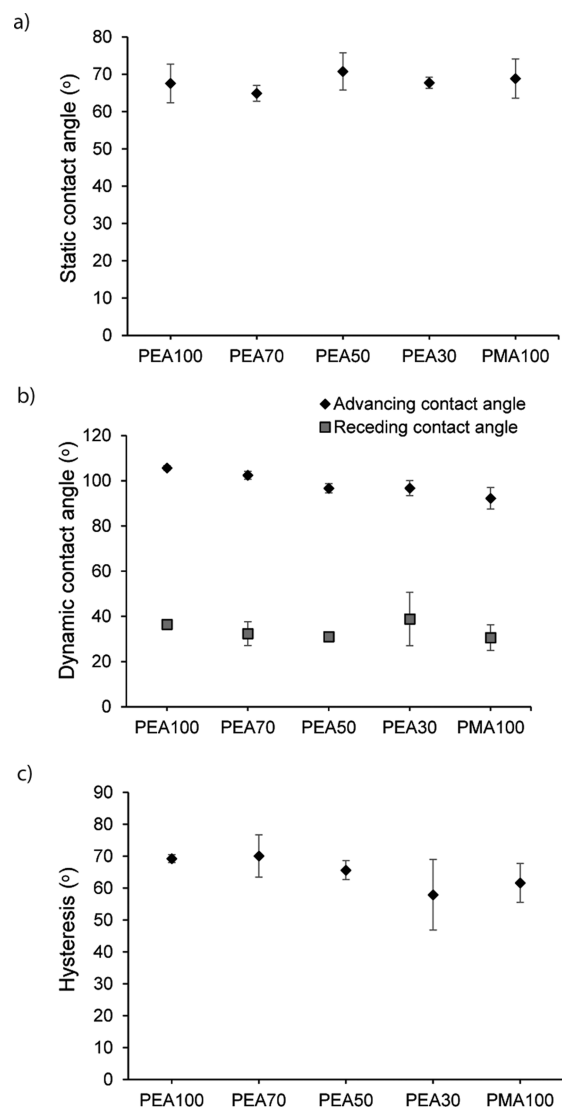
3.1. Polymers and copolymers physicochemical properties. Figure 1a shows the cooling DSC thermograms for the pure homopolymers and copolymers. Only one glass transition was found in the EA/MA copolymers, located between those of the corresponding homopolymers. As the amount of MA increases, the glass transition temperature (T_g) increases according to the copolymer composition (Table 1). The dynamical-mechanical spectra, elastic modulus (E'), and loss tangent ($\tan \delta$) is depicted in Figure 1c and 1d. Pure polymers show a single maximum in $\tan \delta$ and a drop in E' , which corresponds to the main relaxation, related to the glass transition. Likewise, copolymers present one single relaxation process, placed between those of the corresponding homopolymers. The magnitude and temperature of the maximum of \tan

Table 1. Glass Transition Temperature (T_g), Elastic Modulus (E') at 37 °C, and Roughness Parameter (R_q)

Sample	T_g (°C)	E' (kPa)	R_q (nm)
PEA100	-20 ± 1	620 ± 5	0.288
PEA70	-8 ± 1	750 ± 5	0.316
PEA50	-4 ± 1	1100 ± 10	0.291
PEA30	3 ± 1	2130 ± 10	0.294
PMA100	13 ± 1	3100 ± 10	0.265

δ depend on composition: the higher the amount of EA units, the lower the intensity and temperature of this main relaxation. The elastic modulus measured at 37 °C for the copolymers presents values in between both homopolymers (Table 1), increasing monotonically as the amount of MA in the system does. Static and dynamic water contact angle measurements (Figure 2a) are quite similar throughout the copolymers with comparable hysteresis, which confirms no differences in wettability regardless of the EA/MA ratio.

Surface topography, characterized by the roughness parameter R_q obtained from AFM images, shows no significant differences

**Figure 2.** Water contact angle on the different substrates: (a) static contact angle; (b) dynamic contact angle; and (c) hysteresis.

between copolymers (Table 1). All the spin-coated surfaces are rather smooth at the nanoscale and homogeneous for the copolymer series.

3.2. FN organization on material surfaces. AFM images (Figure 3a) show FN distribution on the copolymers after the adsorption of FN from solutions with concentration of 5 and 20 $\mu\text{g}/\text{mL}$. Different degrees of fibrillogenesis were found in dependence of the EA/MA ratio in the copolymers. As expected, PEA100 shows a complete interconnected (nano)-network of FN fibrils from the solution of concentration 20 $\mu\text{g}/\text{mL}$, whereas PMA100 presents a globular FN conformation after adsorption. The density of interconnected fibrils decreases with higher amount of MA units in the copolymers, resulting in a lower degree of fibrillogenesis compared to PEA 100. If FN is adsorbed from a solution of concentration 5 $\mu\text{g}/\text{mL}$ (Figure 3a), this fact becomes even more evident. In particular, the fibrillar organization is virtually lost on PEA50 and PEA30, whereas the surfaces with higher ratio EA/MA show FN fibrils, but lacking full interconnection between them.

3.2.1. FN conformation on material surfaces. The quantification of the amount of adsorbed FN on the copolymers showed no significant differences throughout the series (Figure 4a). Nevertheless, ELISA with the monoclonal antibody mAb1937 (against FNIII₈) suggests different availability of the integrin binding site of FN in dependence of the EA/MA ratio. FNIII₈ is better exposed on PEA100, and it decreases monotonically as the amount of MA in the copolymer increases (Figure 4b).

3.3. Myoblast adhesion on FN coated substrates. Cell adhesion was studied on the copolymers coated with FN (with different degree of FN fibrillogenesis) after 3 h of culture in serum free conditions and under low density seeding to minimize cell–cell contacts (5000 cells/ cm^2). Immunofluorescence for the focal adhesion protein vinculin was performed. Attached cells with well developed focal adhesions were observed on all substrates regardless of the composition of the copolymer, i.e. the degree of FN fibrillogenesis on the material surface. Cells were slightly bigger but with similar shape (circularity) on copolymers with higher content of EA and then higher degree of FN fibrillogenesis (Figure 5).

We then investigated the starting of signaling cascades on the different copolymers by quantifying the phosphorylation of FAK in tyrosine 397. Results in Figure 6a show that the degree of FN fibrillogenesis attained on the different copolymers has no influence on total FAK expression. However, the level of phosphorylation of FAK (pFAK) was higher on copolymers with higher EA content, i.e. on which FN is assembled with higher degree of fibrillogenesis (Figure 6b).

3.4. Myoblast differentiation. In order to analyze the influence of different degrees of FN fibrillogenesis on myoblast differentiation, cells were cultured for 4 days in ITS containing medium and afterward stained for sarcomeric α -actinin, a protein expressed in differentiated myoblasts.³⁰ Myoblast differentiation depends on the degree of fibrillogenesis (Figure 7). PEA and copolymers with high EA content show high levels of differentiated myoblasts (even higher than collagen, which is the gold standard for myoblast differentiation). However, as the degree of fibrillogenesis decreases (lower EA contents), the fraction of differentiated cells decreases up to PMA100, on which FN is adsorbed in a globular shape and the percentage of differentiated cells is the lowest one. This result shows a direct relationship between myoblast differentiation and the distribution of FN into (nano)networks at the material interface.

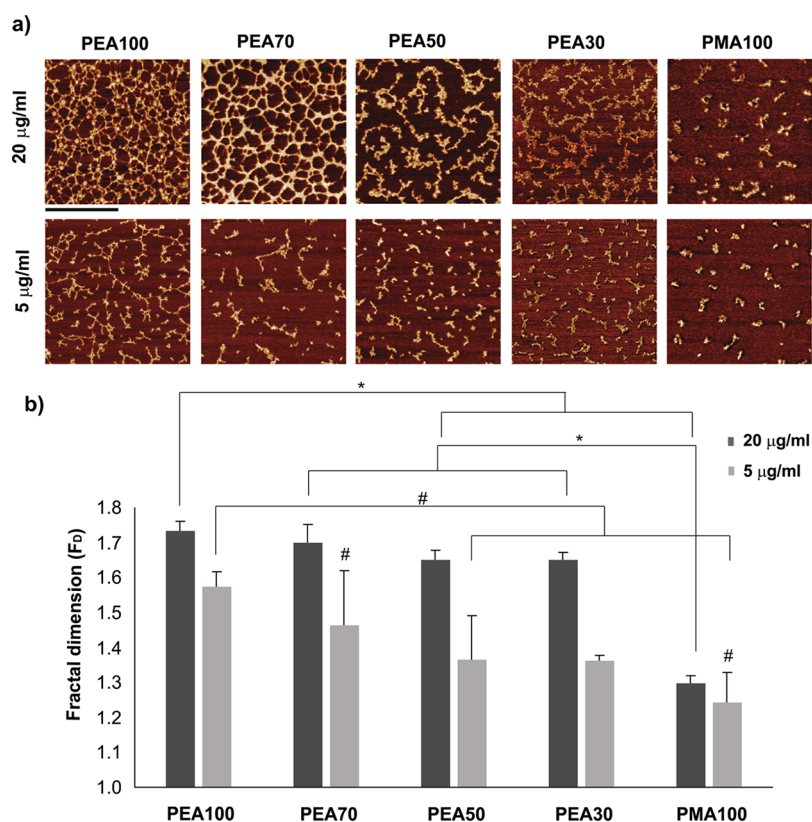


Figure 3. Material-driven degree of FN fibrillogenesis. (a) AFM images (phase magnitude) of FN adsorbed on material substrates for 10 min from solutions with a concentration of 5 and 20 µg/mL. The scale bar is 0.5 µm. (b) Fractal dimension (F_D) calculated from AFM images.

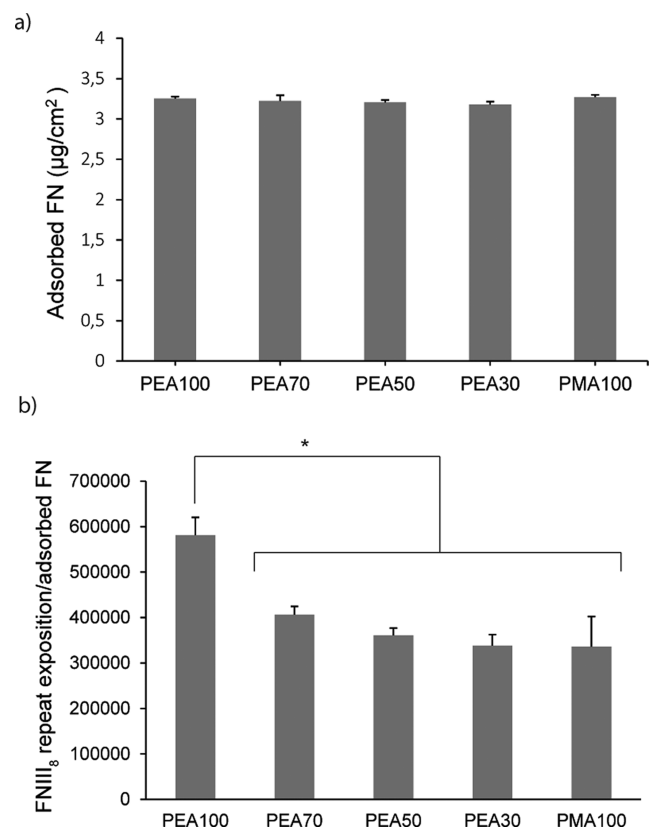


Figure 4. Fibronectin conformation on material surfaces. (a) Surface density of adsorbed FN after 1 h. (b) Exposition of FNIII₈ repeat on surfaces obtained by enzyme-linked immunosorbent assay (ELISA).

Blebbistatin is a small molecule which binds to myosin heads to form a complex with low affinity for actin and inhibit cell contractility.³¹ We have used blebbistatin to assess the role of contractility in the ability of different degrees of FN fibrillogenesis to trigger differentiation. Cell differentiation remained constant regardless of the presence of blebbistatin only for copolymers with low EA content, i.e. on which FN is adsorbed with low degree of fibrillogenesis or in a globular shape (PMA) (Figure 8). However, higher degrees of FN fibrillogenesis (on PEA100 and PEA 70) stimulated cell contractility and then differentiation, as the addition of blebbistatin reduced significantly the level of differentiation.

4. DISCUSSION

The terminal model (first-order Markov model) of copolymerization enables the estimation of copolymer composition after the polymerization process has finished.^{32,33} The ratio of molar fraction of monomers F_1/F_2 in the copolymer can be obtained from the molar fraction of monomers f_1 and f_2 in the reaction mixture and the monomer reactivity ratios r_1 and r_2 according to the expression:

$$\frac{F_1}{F_2} = \frac{f_1(f_1 r_1 + f_2)}{f_2(f_2 r_2 + f_1)} \quad (1)$$

The reactivity ratios for EA and MA are 0.6 and 0.96, respectively.³⁴ The higher MA reactivity ratio means that MA radicals have a higher propensity to react with MA monomers rather than with the EA ones. This behavior can be seen in Figure 9, where the straight line depicts the ideal random copolymers ($r_1 \cdot r_2 = 1$), the two monomers with equal reactivities. This ideal situation leads to a final composition of

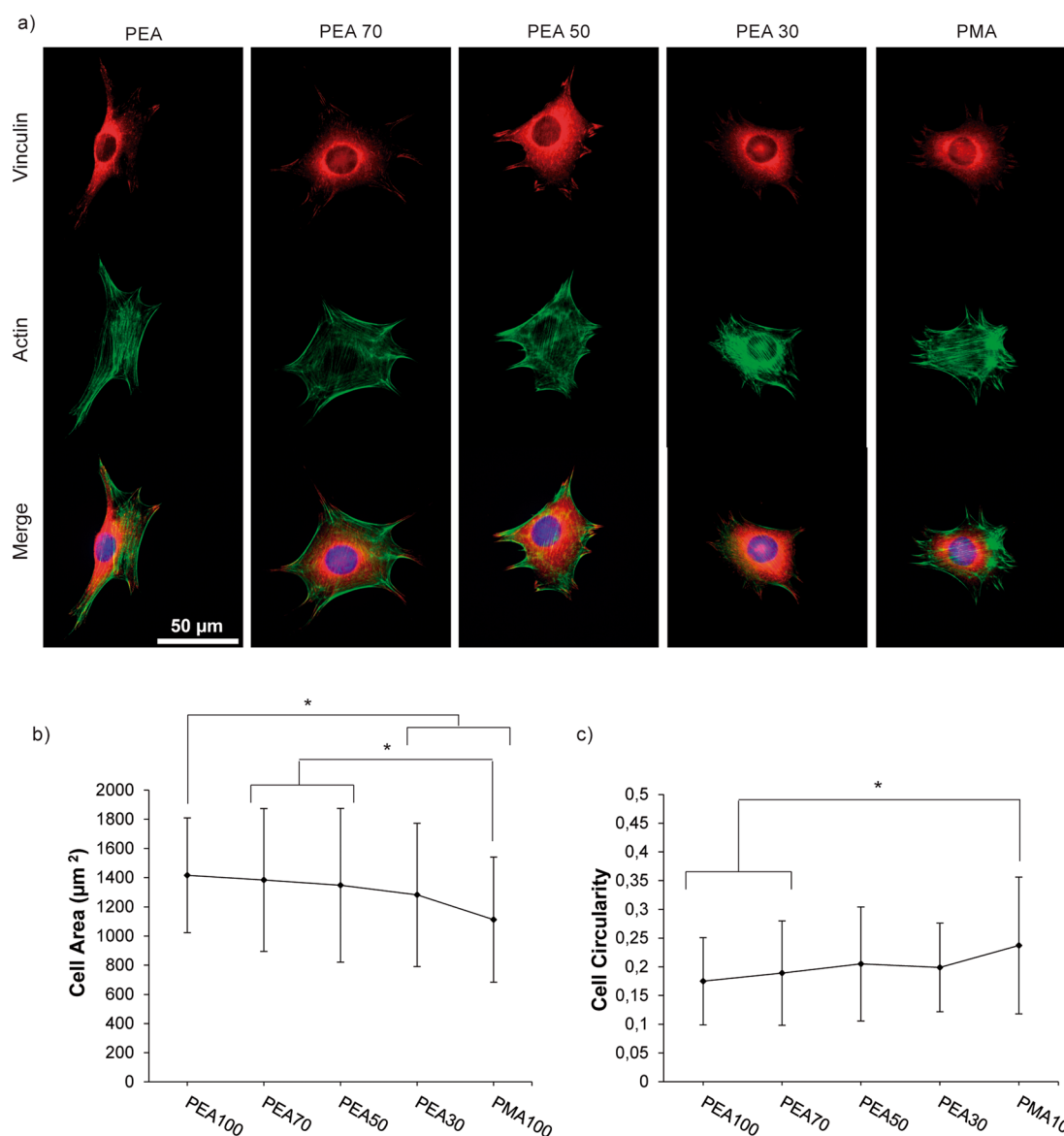


Figure 5. Adhesion of C2C12 cells after 3 h culture on FN coated materials. (a) Immunofluorescence for vinculin. (b) Cell area and (c) cell circularity.

the copolymer chain that matches the comonomer solution, as well as a random distribution of comonomers along the copolymer chain. The reactivity ratios r_1 and r_2 are not too different for MA and EA. Then, the prediction of the terminal model (eq 1) is slightly below the straight line (Figure 9), which means that the fraction of EA units in the copolymer chain is lower than the fraction of EA units in the reacting mixture. Figure 9 shows that the fraction of EA units for the 70:30, 50:50, and 30:70 comonomer solutions turns into 66%, 45%, and 26%, respectively; i.e., the copolymer chain is less rich in EA than the original solution. This also means that the number of MA units in the copolymer chain is above the fraction of MA in the solution. The higher tendency of EA to bind MA units together with the tendency of MA to bind MA units might result in a copolymer chain that is not perfectly random distributed, but where small blocks of MA are preferentially present.

Only one single glass transition and one main relaxation can be seen in the copolymers, which suggests no phase separation in the EA and MA domains within the system. The length of

cooperativity at the glass transition has been evaluated in the order of a few nanometers in amorphous polymers.³⁵ This size determines an order of magnitude for the size domains that can show an independent glass transition in a phase-separated system. Although the terminal model suggested the potential presence of small blocks of MA in the copolymer, their size must not be large enough to produce an independent glass transition. Consistent with this approach, the copolymers present one single glass transition process located between those of the corresponding homopolymers according to its composition, following the F_{EA}/F_{MA} ratio.

The composition dependence of the glass transition temperature was also compared with the prediction of the Couchman–Karasz equation for miscible polymer blends or copolymers:³⁶

$$T_g = \frac{(1 - \omega_{PMA})\Delta c_{p-PEA}T_{g-PEA} + \omega_{PMA}\Delta c_{p-PMA}T_{g-PMA}}{(1 - \omega_{PMA})\Delta c_{p-PEA} + \omega_{PMA}\Delta c_{p-PMA}} \quad (2)$$

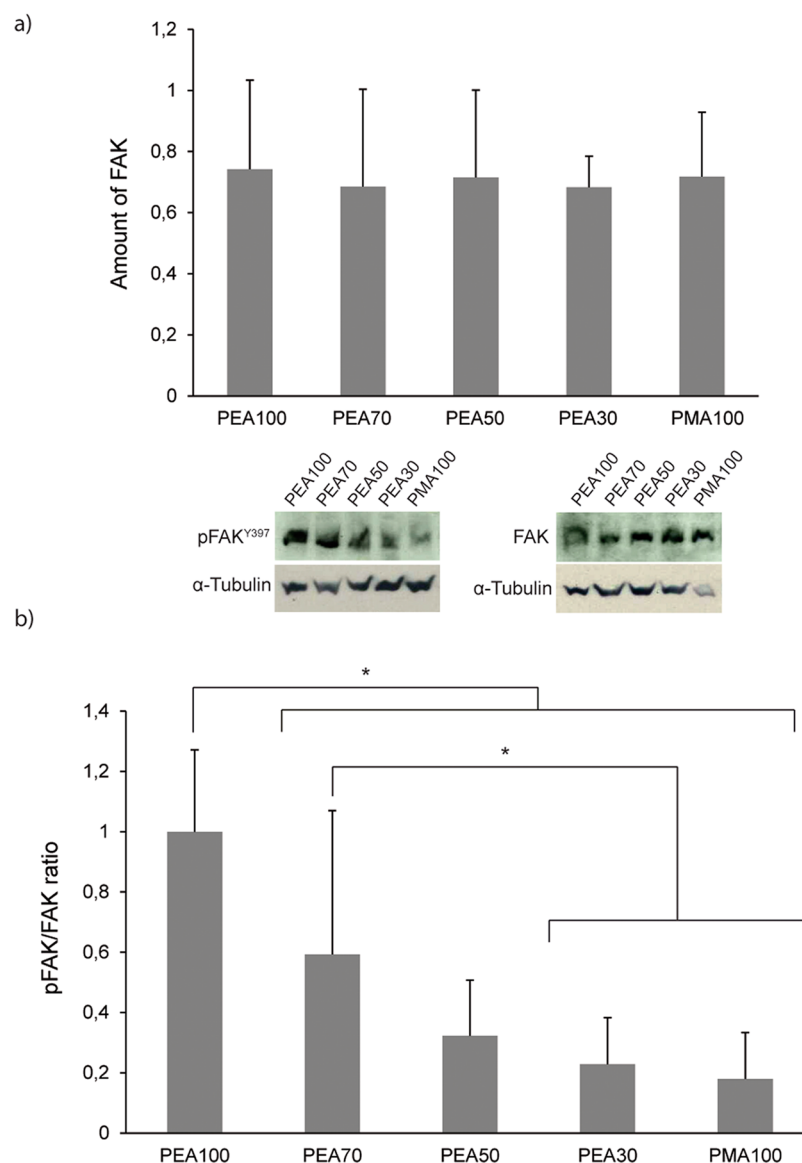


Figure 6. Expression of focal adhesion kinase (FAK) on C2C12 cells after 3 h culture on FN coated materials. (a) FAK expression and bands from Western blot. (b) Determination of FAK activity obtained as pFAK/FAK ratio.

where ω_{PMA} is the mass fraction of MA in the copolymer, Δc_{p-PEA} and Δc_{p-PMA} are the specific heat capacity difference at T_g for both homopolymers, and T_{g-PEA} and T_{g-PMA} are the glass transition temperature of the PEA100 and PMA 100 homopolymers, respectively. The theoretical data predicted from the model are in good agreement with the experimental results (Figure 1b), which further confirms the homogeneity of the copolymers.

We have shown that the composition of the copolymer, i.e. the fraction of EA, determined the degree of fibrillogenesis and that this is the key parameter driving cell differentiation. Adhesion, migration, and differentiation processes are dependent on both surface chemistry and stiffness^{37–39} in addition to the surface topography and wettability.⁴⁰ Cells have an ability to sense and probe the stiffness of their surroundings, as they adhere to and interact with the local ECM.⁴¹ The elastic modulus (E') of the EA/MA copolymers varies between 0.6 and 3 GPa (Table 1). The elastic modulus for skeletal muscle tissue is 12 kPa.⁴² Then, all copolymers can be considered as stiff surfaces from the myoblast's point of view, and we can

disregard the mechanical properties of the substrate stiffness, to play any role on cell behavior. Furthermore, surface properties such as roughness (Table 1) and wettability (Figure 2) remain constant through the copolymers, which disregards these parameters as the key to explain cellular experiments.

The material-driven FN fibrillogenesis on PEA was found to be a time- and concentration-dependent process, whose dynamics were followed via AFM at different adsorption times⁴³ or at increasing protein concentration (for a fixed adsorption time).¹⁹ Based on these observations, the process was explained to include the following sequence of events:²⁵ (i) Conformational change of FN upon adsorption on PEA that involves unfolding and efficient arm extension of FN upon interaction with chemical groups of the substrate (a vinyl backbone with $-\text{COOCH}_2\text{CH}_3$ side chain that carries negative charge). (ii) Enhanced FN–FN interaction as the adsorption process continues, that involves the amino-terminal 70 kDa fragment, the hallmark of cell mediated FN fibrillogenesis.⁴⁴ (iii) Finally, new FN molecules are preferentially adsorbed in close contact to the proteins already present in the substrate,

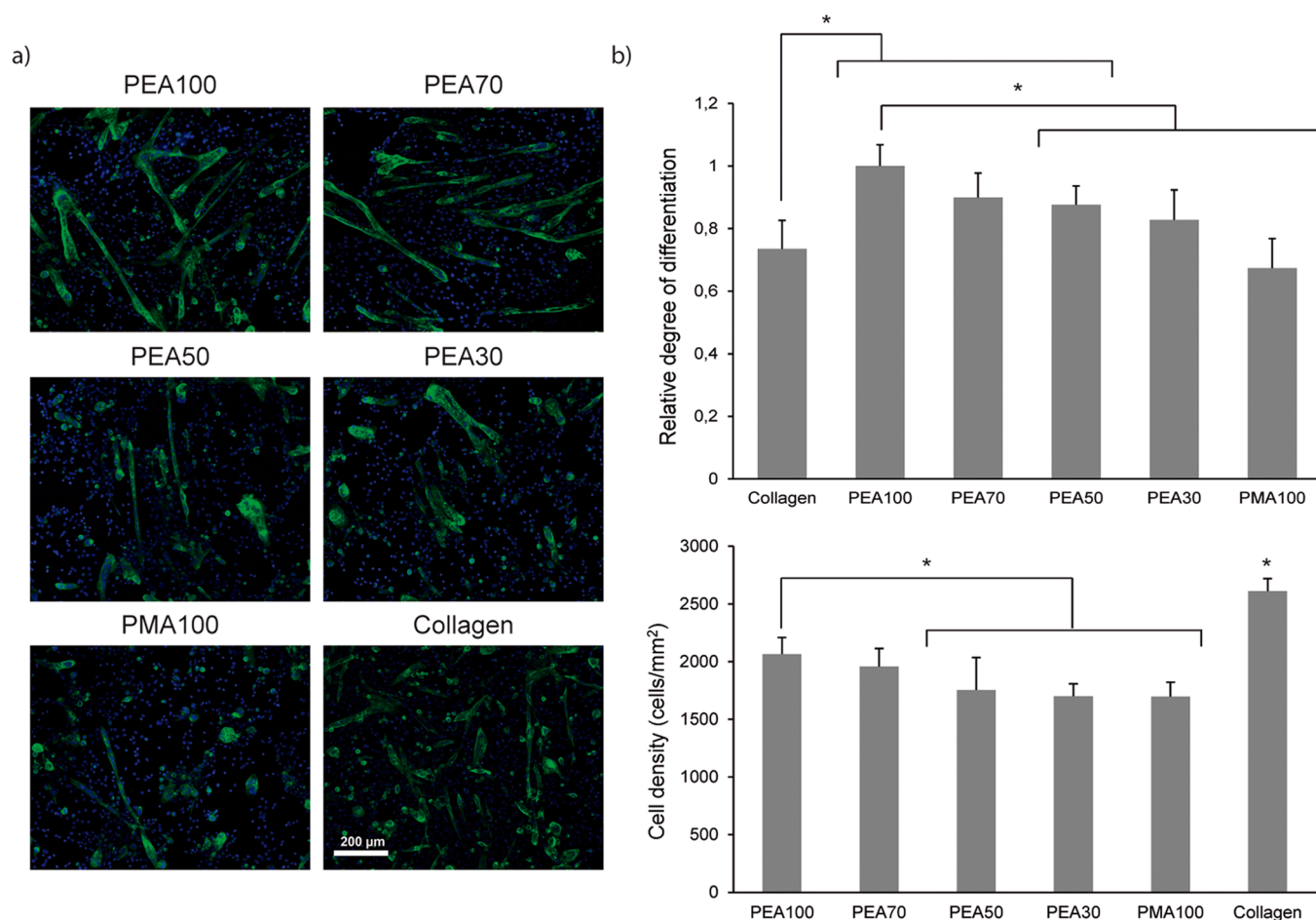


Figure 7. Myogenic differentiation after 4 days of culture on the copolymers. (a) Immunofluorescence of sarcomeric α -actinin (green) and nuclei (blue). (b) Fraction of differentiated cells normalized using the level of differentiation on PEA100. The figure at the bottom shows the cell density on the copolymers after 4 days.

probably as a consequence of the presence of polar oriented FN molecules. The formation of a network on PEA is not a property unique to FN. For example, a similar network was found for fibrinogen⁴⁵ and collagen IV,⁴⁶ but only globular-isolated molecules were observed after laminin⁴⁷ and vitronectin adsorption.⁴⁸

Unlike PEA, FN is adsorbed in a globular shape on PMA, a polymer which differs from PEA in only one methyl group in the side chain.^{19,21} The organization of FN after adsorption on the copolymers EA/MA led to different degrees of fibrillogenesis (Figure 3a), with fibrils attaining different degrees of interconnection. Protein interconnection degree, quantified by the fractal dimension (Figure 3b), shows the relationship between the EA/MA ratio and the degree of FN fibrillogenesis. That is to say, the EA ratio in the copolymers is a way to control the degree of FN fibrillogenesis at the material interface. It has been previously shown that the degree of fibrillogenesis can be modulated on PEA by adsorbing FN from solutions of different concentrations or by using different adsorption times.^{20,22} However, in both cases the total amount of FN on the surfaces changes as well, which does not happen with the copolymers shown in this work (Figure 4a). The fact that we can control the degree of fibrillogenesis by tuning the composition of the surface further supports the concept that the organization of FN at the interface is a consequence of specific protein–material interactions. As we now have

random copolymers of EA and MA, the fraction of EA groups on the surface is a reflection of the composition of the system, and since only these EA groups promote the organization of fibronectin, the phenomenon occurs (at the nanoscale) but with lower length scales, i.e. lower degree of fibrillogenesis.

In addition, different degrees of FN fibrillogenesis involve also a different conformation of the protein on the surface, as revealed by the increased availability of the integrin binding region, as the interconnection between fibrils on the material surface does (Figure 4b). This means that, for intermediate degrees of fibrillogenesis (e.g., PEA50), the protein interface at the material interface consists of FN fibrils, with the molecule in a good conformation to have the integrin binding regions available, and other nonfibrillar molecules on which the integrin binding site is not so well exposed. The superposition of these two kinds of fibronectin conformations results in simultaneous controlled FN organization into fibrils, which, moreover, present different affinities to bind integrins. As a consequence, cells on substrates with higher degree of FN fibrillogenesis result in better cell adhesion, with slightly longer stretched cells and higher number of stress actin fibers (Figure 5).⁴⁹ In turn, cell signaling is activated more efficiently (Figure 6b) as a consequence of the higher density of available integrin binding regions as fibrillogenesis progresses (Figure 4b). This is well correlated with classical experiments that show a correlation

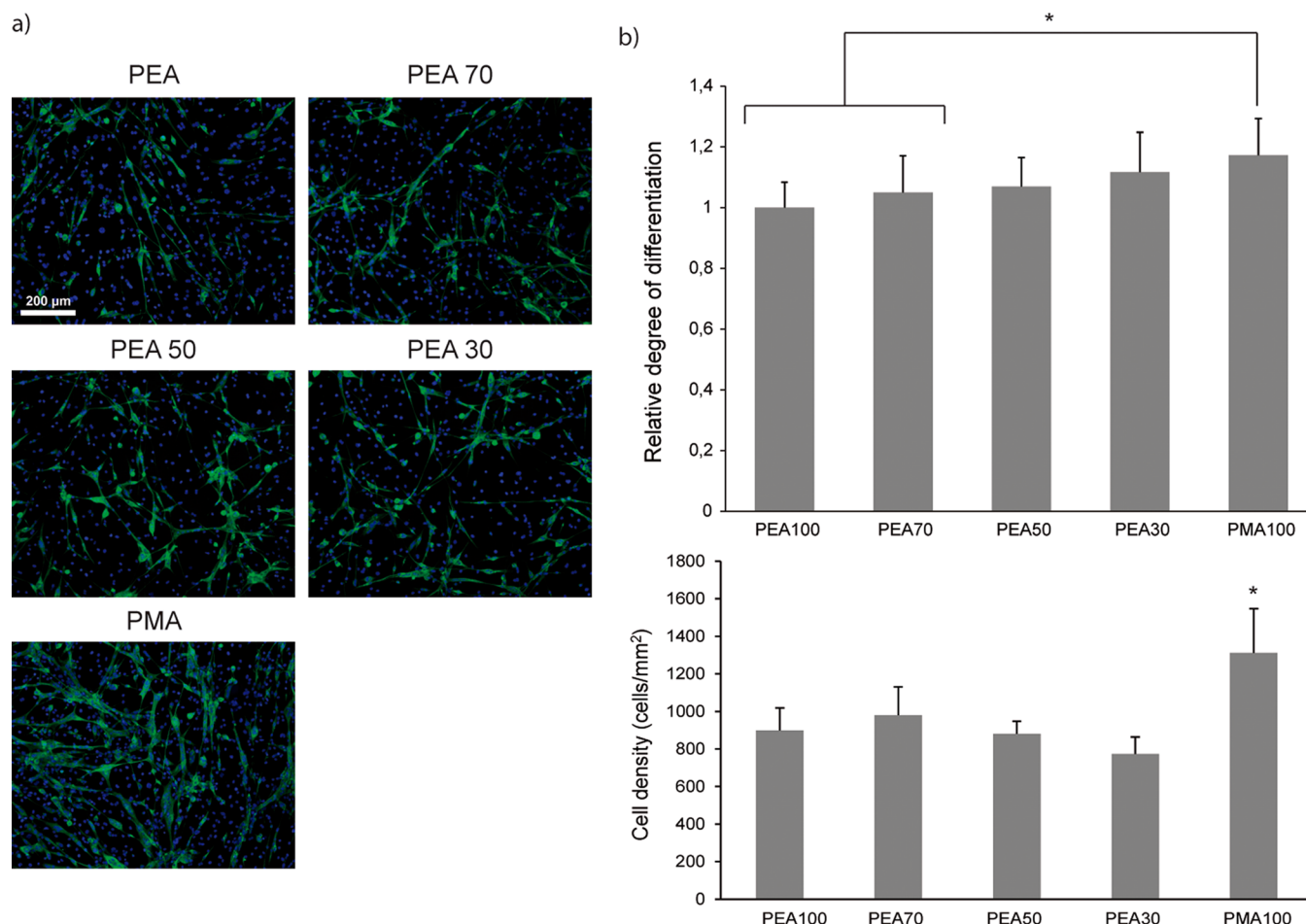


Figure 8. Myogenic differentiation on the copolymers after 4 days of culture with blebbistatin at 10 μM . (a) Immunofluorescence of sarcomeric α -actinin (green) and nuclei (blue). (b) Fraction of differentiated cells normalized using the level of differentiation on PEA100. The figure at the bottom shows the cell density on the copolymers after 4 days.

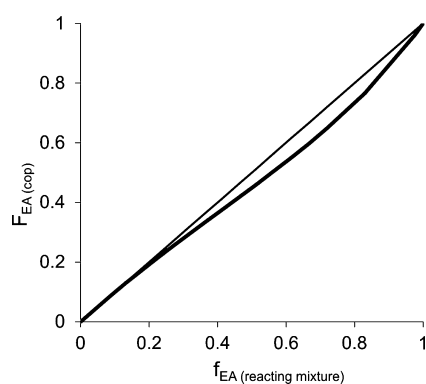


Figure 9. Molar fraction of EA units in the copolymer as predicted by the terminal model, $F_{EA(cop)}$ as a function of the molar fraction of EA monomers in the reacting mixture, f_{EA} . The straight line gives the composition of a random copolymer where the fraction of monomers in the reacting mixture remains after polymerization.

between fibronectin conformation, cell adhesion, and signaling using model surfaces.^{16,50}

Myoblast differentiation occurs more effectively on substrates with higher degree of FN fibrillogenesis (Figure 7). On the one hand, there is a direct relationship between myoblasts adhesion and differentiation, as previously shown.⁵¹ Variations of integrin

expression and signaling mediated by focal adhesion kinase positively influence myogenic differentiation.^{21,52,53}

On the other hand, activation of cell contractility is necessary to promote differentiation. Contractile forces generated for actomyosin machinery result from dynamic interactions between actin and nonmuscular myosin as a consequence of phosphorylation of myosin light chain II by several kinases as Rho associated kinases or Myosin Light Chain kinase.⁵⁴ Myosins constitute a superfamily of ATP-dependent motor proteins that play important roles in several cellular processes that require force and translocation⁵⁵ and within the cell regulate several functions, including skeletal muscle contraction and differentiation.⁵⁶ In the myoblast differentiation and fusion process, cells produce large amounts of contractile machinery proteins such as a myosin heavy chain, two myosin light chains, and two subunits of tropomyosin,⁵⁷ demonstrating the importance of contractile machinery in myoblast differentiation. We have previously shown that the organization of FN into (nano)networks on PEA enhanced myoblast differentiation by activating cell contractility.²¹ Here, we show that when blebbistatin is added to culture medium (Figure 8), cell differentiation drops significantly only on copolymers which show a higher degree of FN fibrillogenesis, whereas it has no effect for cells on globular FN (PMA) and moderate degrees of fibrillogenesis (PEA30). This suggests a direct dependence

between the degree of fibrillogenesis, the activation of cell contractility, and the level of cell differentiation.

5. CONCLUSIONS

We have shown that material-driven FN fibrillogenesis originally described on PEA can be modulated by copolymerizing EA with MA units. Increasing the amount of EA in the copolymer leads to well-organized FN (nano)networks that show better availability of the integrin binding region of FN. This fact, together with the ability of FN (nano)networks to enhance cell contractility, leads to better cell adhesion, cell signaling, and myoblast differentiation by increasing the degree of FN fibrillogenesis on the material surface.

We have shown that the organization of fibronectin into nanofibrils provides a robust platform to induce cell differentiation.⁵⁸ Materials that control the degree of FN fibrillogenesis upon simple adsorption can be incorporated into novel strategies to modulate cell response in the field of tissue engineering. This series of materials can be used to coat the surface of 3D scaffolds, which results in a system that is a biomimetic of the extracellular matrix, with controlled density of fibrils tailored but the composition of the underlying material. This platform has the potential to become an effective approach to engineer advanced microenvironments that control the number of cell adhesion sites as well as the amount of growth factors used in the system to promote cell differentiation in vitro and tissue repair in vivo. We have recently submitted a patent application that presents the use of the system in tissue engineering applications, in particular musculoskeletal repair and regeneration.⁵⁹

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Notes

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