Fibronectin fixation on poly (ethyl acrylate) based copolymers

N. Briz[a,b,*], C.M. Antolinos-Turpin[c,*,†], J. Alió[d,e], N. Garagorri[a,b], J.L. Gómez Ribelles[b,c], J.A. Gómez-Tejedor[c]

[a] TECNALIA, Mikeletegi Pasealekua 2, 20009 Donostia-San Sebastian, Spain

[b] CIBER de Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN)

[c] Center for Biomaterials and Tissue Engineering, Universitat Politècnica de València, Camino de Vera s/n, 46022 Valencia, Spain

[d] Universidad Miguel Hernández de Alicante, Spain.

[e] VISSUM Instituto Oftalmológico de Alicante, Spain.

[*]Both authors contributed equally to this paper.

[†]Author for correspondence

Abstract

The aim of this paper is to quantify the adhered fibronectin (by adsorption and/or grafting) and the exposure of its cell adhesive motifs (RGD and FNIII7-10) on poly (ethyl acrylate) copolymers whose chemical composition has been designed to increase wettability and to introduce acid functional groups. Fibronectin was adsorbed to poly (ethyl acrylate), poly (ethyl acrylate-co-hydroxyethyl acrylate), poly (ethyl acrylate-co-acrylic acid) and poly (ethyl acrylate-co-methacrylic acid) copolymers and covalently cross-linked to poly (ethyl acrylate-co-acrylic acid) and poly (ethyl acrylate-co-methacrylic acid) copolymers. Amount of adhered fibronectin and exhibition of RGD and FNIII7-10 fragments involved in cell adhesion were quantified with ELISA tests.

Even copolymers with a lower content of the hydrophilic component showed a decrease in water contact angle. In addition, fibronectin was successfully fixed on all surfaces, especially on the hydrophobic surfaces. However, it was demonstrated that exposure of its cell adhesion sequences, which is the key factor in cell adhesion and proliferation, was higher for hydrophilic surfaces.

1. Introduction

Cell adhesion to biomaterials is critical to many tissue engineering applications. In many instances, cells adhere to synthetic surfaces via proteins adsorbed from physiological fluids and culture media. The physicochemical properties of the material, including topography, chemistry and surface energy modulate protein adsorption; this fact plays an important role in determining the design of the biomaterial[1-6]. The amount and conformation of the adsorbed proteins affect different cellular functions including adhesion, spreading, migration and differentiation. Cell adhesion to adsorbed proteins is primarily mediated by integrin receptors. Integrins represent a widely expressed family of heterodimeric trans-membrane receptors that bind to adhesion motifs present in various extracellular matrix proteins, including fibronectin (FN), vitronectin, laminin, and collagen[7]. Several studies have demonstrated diverse cellular responses to substrates with different surface chemistries. It has been reported that surface chemistry alters the adsorption kinetics and structure of adsorbed FN[8], modifying the functional presentation of the major integrin binding domain of FN, thereby altering integrin binding and promoting cell adhesion strength[9,10].

The aim of this work was to analyze adsorption or grafting of fibronectin on poly (ethyl acrylate) (PEA) copolymers. These materials have been used in ocular implants showing excellent stability and biocompatibility. The conformation of fibronectin adsorbed on PEA substrates has been studied in the past,[11-13] these studies have shown that this polymer induces fibrillogenesis in the absence of cellular activity. It is thought that this is the process which gives rise to this polymer's very good performance in monolayer and three-dimensional cultures of different cell types such as human endothelial cord vein, HUVEC cells, conjuntival epithelial cells, fibroblasts, chondrocytes and osteoblasts[14-17].

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PEA is a very hydrophobic polymer and copolymerization with more hydrophilic co-monomers has been employed to improve wettability (see for instance reference[14] and references therein). A certain degree of wettability and hydrophilicity are fundamental requirements for materials used in cell culture supports or vehicles for cell transplants in order to favor cell seeding especially in three-dimensional scaffolds and water permeation. This research focused on the influence of material properties on fibronectin adhesion and its conformation for the exposure of some adhesion sequences that interact with integrins. The adhesion domains under study were the RGD (arginine-glycine-aspartic acid) motif which reacts with many integrins, and $FNIII_{7-10}$ sequence which consists of the RGD motif on the 10th type III repeat of FN in the presence of the proline-histidine-serine-arginine-asparagine (PHSRN) domain on the 9th type III repeat. $FNIII_{7-10}$ motif interacts with $\alpha_5\beta_1$ integrin, which controls proliferation and differentiation of osteoblasts and myoblasts, cell cycle progression, and FN matrix assembly[18].

The aim of this paper is to show the importance of the presence of a small fraction of functional groups at the surface of a polymeric substrate on protein adsorption and exhibition of adhesion sequences that cells can recognize. Fibronectin is adsorbed on the surfaces of P(EA-co-HEA), P(EA-co-AAc), P(EA-co-MAAc) copolymers while the acid functionality introduced by the AAc or MAAc groups on the substrate is employed to fix the protein on the substrate by covalent bonds by means of free amine groups of FN. The quantification of FN and the exposed domains (RGD and $FNIII_{7-10}$) was carried out by immunoassays.

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2. Materials and Methods

2.1. Substrate preparation

Polymeric substrate preparation

Seven polymer networks were prepared by copolymerization of ethyl acrylate, EA (Scharlau, 99% pure), with hydroxyethyl acrylate, HEA (Aldrich 96% pure), acrylic acid, AAc (Scharlau 99% pure), or methacrylic acid, MAAc (Scharlau 99% pure). Ethylene glycol dimethacrylate, EGDMA) (Aldrich 99% pure) was used as cross-linking agent and benzoine (Scharlau 98% pure) was used as photoinitiator. The monomers were used as received, without further purification. Mixtures of the co-monomers in the desired ratios (see Table 1) with 1 wt% EGDMA and 0.5 wt% benzoin were placed in transparent molds and polymerization took place at room temperature under UV light, producing copolymer plates around 1 mm thick. These were given a post-curing treatment at 90ºC for 24 hours in order to reach full monomer conversion. The plates were then immersed in boiling ethanol for 24 hours to extract any residual substances of low molecular weight from the samples, dried at room conditions for 48 hours and finally dried in vacuum at 60ºC until they reached a constant weight. All the resulting materials are biostable.

Table 1. Co-polymer network composition.

Reference	EA(wt %)	HEA(wt %)	AAc(wt %)	MAAc(wt %)
PEA	100	0	0	0
P(EA-co-HEA) 90/10	90	10	0	0
P(EA-co-HEA) 80/20	80	20	0	0
P(EA-co-AAc) 90/10	90	0	10	0
P(EA-co-AAc) 80/20	90	0	0	10
P(EA-co-MAAc) 90/10	80	0	20	0
P(EA-co-MAAc) 80/20	80	0	0	20

Preparation of fibronectin-coated surfaces

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Adsorbed fibronectin: Copolymers of EA containing 10 or 20 wt% HEA, AAc or MAAc were treated with a solution of 50µg/ml of human plasma FN (Sigma, Spain) dissolved in phosphate saline buffer (PBS) (Sigma, Spain) for protein adsorption experiments. A volume of 100µl was used to cover the different polymeric disks (5 mm in diameter) for 1 hour (30 minutes at 37ºC and 30 minutes at RT). Finally, disks were washed three times with PBS.

Covalently adhered fibronectin: FN was covalently cross-linked to disks of different copolymers of EA containing 10 or 20 wt% acrylic acid (AAc) or methacrylic acid (MAAc). The carboxylated copolymers were reacted with 2mM *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDAC purchased from Sigma, Spain) and 5mM *N*-hydroxysulfosuccinimide sodium salt (NHS from Sigma, Spain) in pH 5.5 2-(N-Morpholino) ethane sulfonic acid buffer (MES from Sigma, Spain) for 30 min in order to convert the carboxylic acid into a reactive ester to posterior amidation with free amine groups of FN. After a wash with pH 5.5 MES, the disks were reacted with human FN (0.5 mg/ml, Sigma, Spain) in pH 5.5 MES for 2 h and then washed once with pH 5.5 MES and twice with PBS.

2.2 Characterization of Materials

Characterization of polymeric materials

The polymer networks obtained were physically and thermally characterized by contact angle tests, water sorption and Differential Scanning Calorimetry:

Differences in water contact angle (WCA) of the different copolymers as a consequence of the presence of hydroxyl or carboxyl groups at the surface were determined using the DATAPHYSICS OCA 20. Static contact angle of a 10µl drop of water deposited on

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the solid surface; the results are the average of six measurements. Equilibrium water sorption was determined by weighing. Samples were immersed in liquid water at 37ºC to constant weight. Water content is expressed in dry basis:

$$EWC = \frac{w_{water}}{w_{dry\,polymer}} \qquad (1)$$

Differential Scanning Calorimetry (DSC) experiments were performed in an N_2 atmosphere (flow rate 600ml/min) using a Mettler-Toledo DSC 823e. Weights of specimens ranged from 5 to 10 mg. Dry samples or samples swollen to equilibrium in immersion in liquid water were sealed in aluminum pans. The temperature range in the scans performed on dry samples was from -60 to 150ºC while wet samples were scanned from -80ºC to room temperature; heating rate was 10ºC/min. Two heating scans were recorded for each batch with cooling scan at 40ºC/min between them in order to erase the effect of previous thermal history of the samples. From DSC curves, glass transition temperatures were determined from the inflection point temperature.

Quantification of FN and its exposed adhesion motives

Enzyme-Linked Immuno Sorbent Assay (ELISA) was the selected technique to quantify FN and the exposure adhesion domains, RGD and FNIII[7-10]. The reagents 3,3',5,5'-Tetramethylbenzidine (TMB), albumin from bovine serum (BSA), phosphate buffered saline (PBS) and PBS containing Tween 20 (PBST) were purchased from Sigma (Madrid, Spain). Sulphuric acid 2N (H_2SO_4) was obtained from Panreac (Barcelona, Spain). The primary antibodies used to detect fibronectin (human polyclonal anti-human fibronectin[4]) were also purchased from Sigma (Spain); the RGD (monoclonal antibody anti-human FN, cellular union domain, clone P1H11[4]) was from Chemicon (Spain) and FNIII[7-10] (HFN7.1 antibody[5]) was from Abcam (Spain). The secondary antibodies (conjugated with HRP, or horse radish

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peroxidase) Rabbit polyclonal Secondary Antibody to Mouse IgG - H&L (HRP) was obtained from Abcam (Spain) and Anti-Rabbit IgG–Peroxidase antibody produced in goat from Sigma (Spain).

FN-coated copolymeric disks were blocked with 2% BSA in PBS for 2h at 37°C and washed twice with PBS afterwards. The primary antibodies were added at a dilution of 1:50, 1:260, 1:260 for fibronectin, RGD and FNIII$_{7-10}$ respectively, and incubated for 2h at 37°C. After intensive rinsing with PBST the secondary HRP conjugated antibodies were added at a dilution of 1:45 for fibronectin antibody and 1:83 for RGD and FNIII$_{7-10}$ antibodies. Incubation was carried out for 1h at 37°C. The surfaces were rinsed with PBST, followed by addition of TMB substrate solution at RT for 15 min. The reaction was stopped by transferring part of the dye solution to a 96 well plate (Corning, USA) with a stop solution (2N H$_2$SO$_4$). The optical density (OD) was measured at 450 nm with a Powerwave XS reader (Bio Tek Instruments Inc, Winooski, VT, USA). Nine disks were analyzed for each material and each antibody, and copolymers without coatings were studied as a reference.
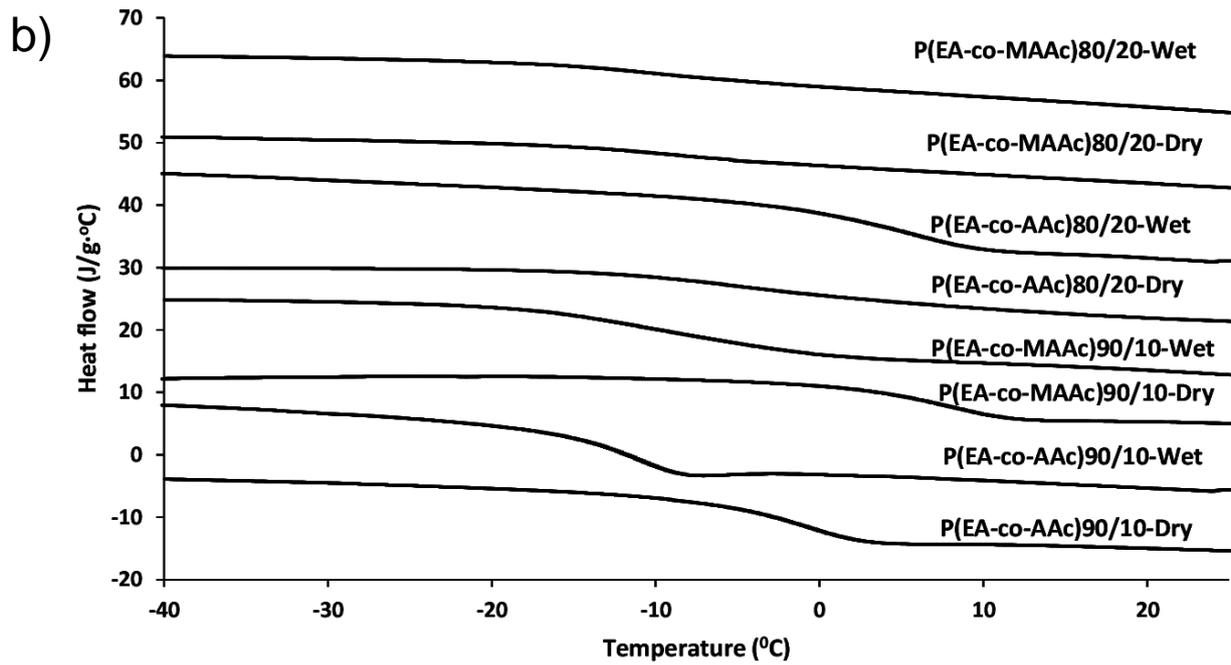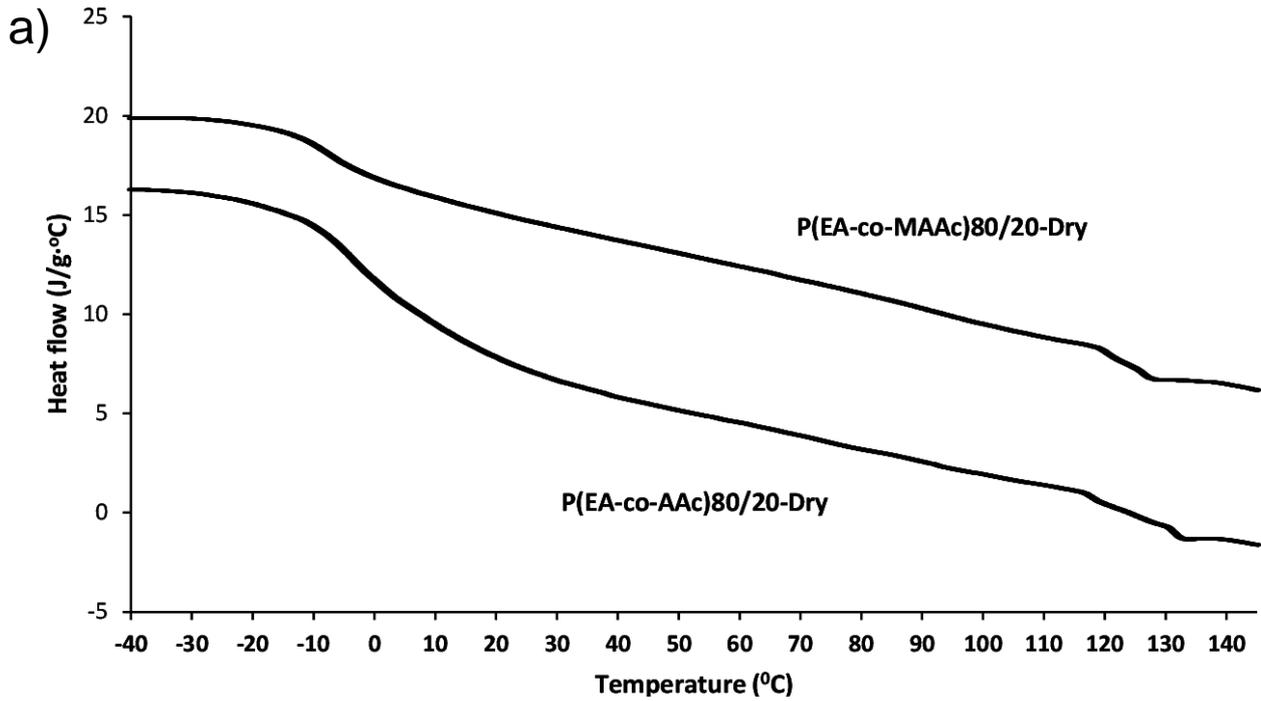
3. Results

Table 2. Contact angle, equilibrium water content (EWC) and glass transition temperatures of dry and wet samples.

Samples	Contact angle (deg)	EWC % (10.000min)	T_g Dry (°C)	T_g Wet (°C)
PEA 100	72.30 ± 0.07	1.5	-13.7± 0.5	-13.9± 0.5
P(EA-co-HEA) 90/10	69.70 ± 0.30	3.6	-9.8± 0.5	-15.1± 0.5
P(EA-co-HEA) 80/20	67.90 ± 0.05	5	-8.4± 0.5	-7± 0.5

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P(EA-co-AAC) 90/10	70.50 ± 0.06	4.2	-2.9± 0.5	-12.1± 0.5
P(EA-co-MAAC) 90/10	71.00 ± 0.04	4	6.8± 0.5	-10.2± 0.5
P(EA-co-AAC) 80/20	60.50 ± 0.18	8	-3.9± 0.5	3.8± 0.5
P(EA-co-MAAC) 80/20	71.40 ± 0.05	5	-11.9± 0.5	-13.5± 0.5

Physical and thermal characterization parameters of the different materials are listed in Table 2. Poly(ethyl acrylate), or PEA, is an amorphous material with a glass transition temperature of -13.7ºC, thus, its behavior is that of a rubber-like material at room temperature. The contact angle with water (72.3 degrees) and the water sorption capacity of only 1.5% show that this material is quite hydrophobic. Wettability and hydrophilicity of PEA can be modified by copolymerization with more hydrophilic monomers. The addition of 20% HEA increases water sorption up to 5% while contact angle decreases to 67.9 degrees. Copolymers containing 20% by weight of AAc and MAAc are more hydrophilic, with water sorption capacities of 8% and 5% respectively. The contact angle of P(EA-co-AAc) decreases to 60 degrees but in the case of P(EA-co-MAAc) the increase in wettability is more modest. Copolymer homogeneity produced by free radical polymerization was investigated by DSC (see Figure 1). The presence of phase separation in the copolymer material even in nanometric dimensions is revealed by the presence of more than one glass transition in the DSC heating thermograms. Figure 1c shows that P(EA-co-HEA) presents a single glass transition that shifts in the temperature axis with varying copolymer composition while Figure 1a shows copolymers containing AAc or MAAc present traces of a high temperature glass transition that can be ascribed to domains formed by PAAc or PMAAc blocks that associate forming a high-temperature glass transition close to that of pure PAAc or PMAAc polymers.
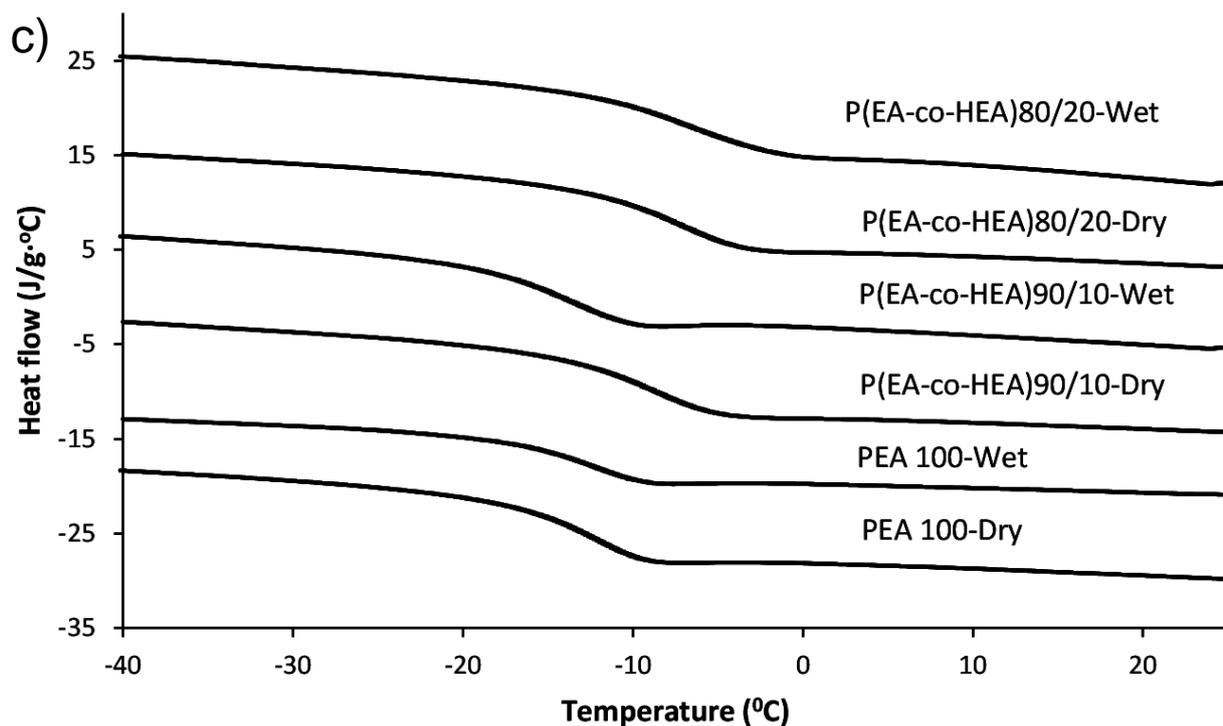
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c)

Figure 1. DSC heating thermograms of the different copolymer networks. (a) P(EA-co-MAAc) 80/20 and P(EA-co-AAc) 80/20 thermograms in the whole temperature range of the measurements (b) P(EA-co-MAAc) and P(EA-co-AAc) thermograms in the temperature range of the PEA-rich phase transition (c) P(EA-co-HEA) copolymer network thermograms.

Thermograms corresponding to wet and dry samples are presented in Figure 1b and c to show the plasticization effect due to water, which confirms the incorporation of hydrophilic groups in the polymer phase responsible for the low-temperature glass transition.

The total amount of fibronectin adsorbed (AP) on the copolymers containing hydrophilic groups is clearly smaller than on PEA substrates (Figure 2). For the grafted fibronectin (CP), Figure 2 shows that the amount of grafted fibronectin is smaller for Methacrylic Acid copolymer than for Acrylic Acid ones. Furthermore, Figure 2 shows that the amount of grafted fibronectin increases with the amount of acid groups (Methacrylic or

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Acrylic Acid) increases, as expected by the rise of reactive groups. The total RGD and $FNIII_{7-10}$ adhesion domains exhibited is also shown in Figure 2, whereas Figure 3 shows the ratio of ligand exhibited to the total amount of fibronectin adsorbed.
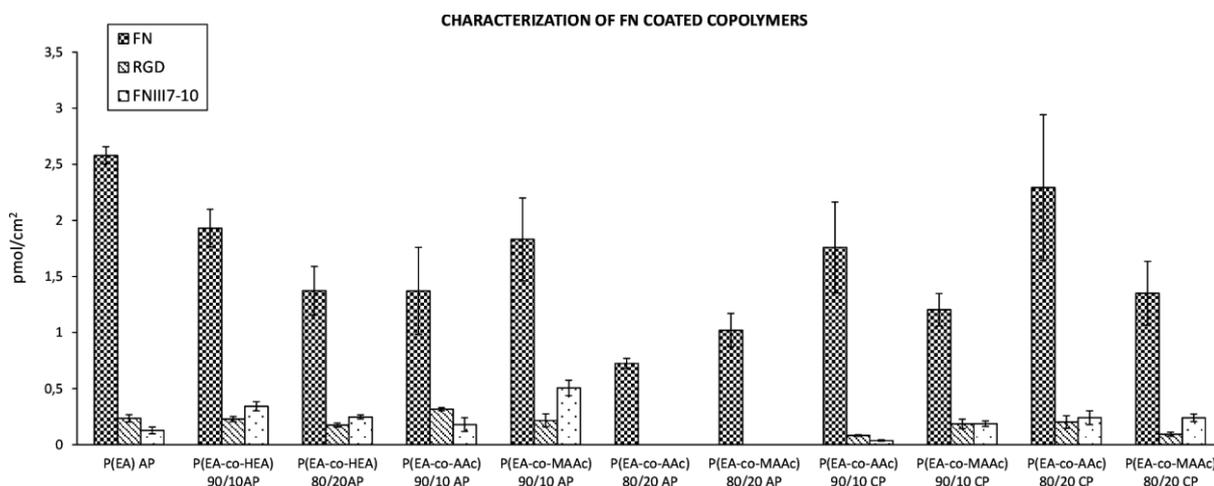

Figure 2. Amount of fibronectin adsorbed (AP) on PEA and copolymer network substrates or grafted (CP) on P(EA-co-AAc) or P(EA-co-MAAc) substrates. The total exhibition of the RGD and $FNIII_{7-10}$ adhesion domains is also shown.
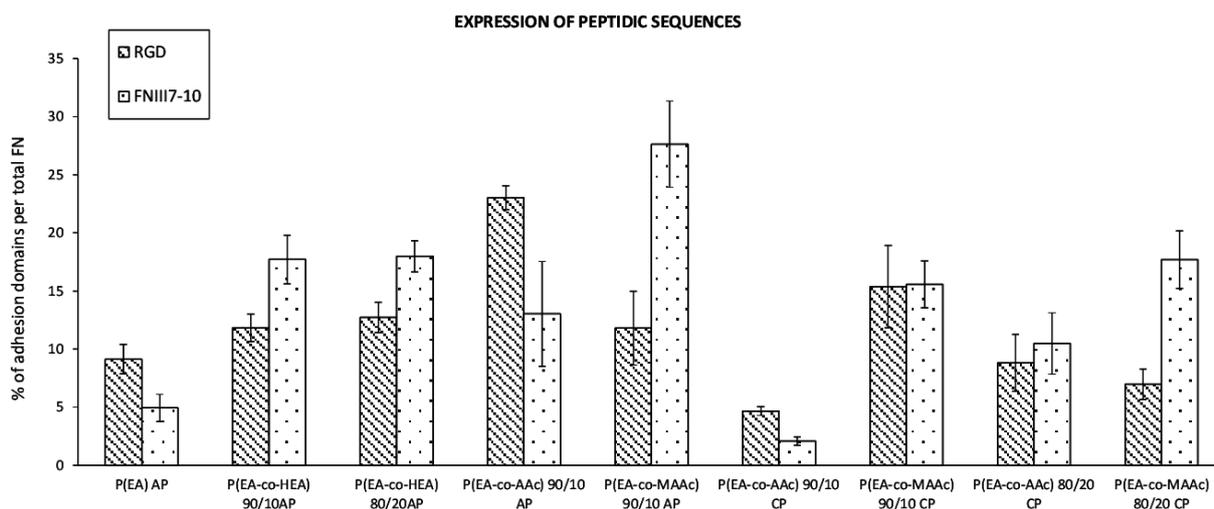

Figure 3. Exhibition of RGD and $FNIII_{7-10}$ adhesion domains relative to the total amount of fibronectin adsorbed (AP) or grafted (CP) on the substrate surface.

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4. Discussion

One of the important characteristics of copolymer networks such as the ones synthesized in this work is the homogeneity in the distribution of monomeric units of the different components at molecular level. Differences in monomer reactivity can produce the formation of copolymer chains with higher content in the most reactive component than in average. The consumption of this more reactive monomer makes those sequences of the component with lower reactivity, nearly pure, form at the end of the reaction. This fact can produce phase separation at nanometric scale, with domain sizes of just a few nanometers. These domains can be hardly observed by microscopy techniques. Nevertheless, glass transition is a phenomenon that involves cooperative motions of polymer chains in rearranging regions of just few nanometers. If phase separation takes place, the glass transition of the different phases can be detected even if the domain size is smaller than ten nanometers, while a single glass transition appears only if the components are mixed at molecular level in the whole volume of the sample (see for instance reference[19]). DSC thermograms of the dry and wet samples provide some insight into the structure at molecular level. Random copolymers show a single, broad glass transition appearing in a range of temperature situated between those of the corresponding homopolymers. On the contrary, the presence of a phase separation, even at a nanometric scale, produces two glass transitions; each of which in the range of the pure homopolymers. These glass transition temperatures are more or less shifted depending on the degree to which each component dissolves into the other. Figure 1a shows the second heating scan of the dry P(EA-co-AAc) 80/20 and P(EA-co-MAAc) 80/20 copolymer networks. Both show a glass transition in the temperature interval between -20 and 20 ºC corresponding to a PEA-rich phase but another one between 120 and 130ºC corresponding to a PAAc or PMAAc phase. The incorporation of the AAc or MAAc segment into the copolymer network is indicated by the shift in the low-temperature

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transition with respect to pure PEA as shown in Figure 1b and 1c. Table 2 shows the values of the glass transition temperature of the different samples. It is interesting to compare the glass transition temperature of the wet samples with respect to the dry ones. The introduction of water molecules in the polymer phase increases polymer chain mobility thus decreasing its glass transition temperature, what is known as plasticization. In the samples containing 10% AAc or MAAc, plasticization of the PEA-rich phase indicates the incorporation of the hydrophilic groups. Nevertheless, in the samples containing 20% AAc or MAAc, the results are not as clear-cut. In principle, higher hydrophilic monomer content in the network should result in a more pronounced plasticization, i.e. a larger shift of the glass transition towards lower temperatures but in fact this is not the case. This result may be attributable to the fact that a significant proportion of the hydrophilic segments cluster in domains separated from the PEA-rich copolymer phase. In the case of P(EA-co-HEA) networks, the glass transition temperature of both homopolymers are not so different, -13.7ºC for PEA and 5ºC (-9.8ºC) for PHEA[20]. The absence of two separate glass transitions in the DSC thermogram of the dry copolymers and the shift of the glass transition temperature with increasing HEA content seem to support the idea that a random copolymer is being formed. In P(EA-co-HEA) 90/10 this glass transition clearly shifts to lower temperatures with water absorption but the sample with 20% HEA does not (Figure 1c). The reorganization of hydrophilic domains due to the presence of water, a phenomenon known as hydrophobic interaction was shown in this system, which would explain this behaviour[20].

Since functional groups are introduced in the polymer chain by copolymerization ethyl acrylate and functional monomeric units are distributed along the polymer chains at molecular distances and thus, surface composition is expected to reflect average composition of the material. The amount of adsorbed fibronectin on the substrates diminishes significantly with

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increasing hydroxyl group content (Figure 2), a fact that correlates with the increase of equilibrium water content and the decrease of WCA. In the case of P(EA-co-HEA) substrates this result can be related to the results of the culture of human endothelial cells HUVEC on these substrates. It was shown[20] that adhesion and viability of HUVEC on the substrates required a previous coating of fibronectin, with cell numbers and viability tests decreasing sharply with the increase of HEA groups in the substrate. A similar result was found in a previous study on the culture of epithelial conjuntival cells on supports using this formulation. Cell numbers decreased sharply over culture time in supports with only 20% HEA and were not viable in supports containing 50% HEA[17]. On the other hand, it was shown that the conformation of the adsorbed FN changed with hydrophilicity. The results shown in Figure 3 show that the exhibition of RGD and $FNIII_{7-10}$ domains in P(EA-co-HEA) relative to the total amount of fibronectin adsorbed increases with HEA content and is significantly higher than in pure PEA. In addition, it was observed that the HEA copolymers with adsorbed FN were observed to favour exhibition of $FNIII_{7-10}$ domains. Exhibition of adhesion ligands was also analyzed in P(EA-co-AAc) and P(EA-co-MAAc) containing 10% of hydrophilic monomeric units. Interestingly, the exhibition of RGD sequences is predominant in P(EA-co-AAc) substrates while the opposite occurs in P(EA-co-MAAc); in any case they are higher than in PEA substrates. It can be said that in this type of substrates adhesion of chondrocytes was improved by the presence of small amounts of a hydrophilic component in the copolymer which was ascribed to improved exhibition of adhesion sequences of the proteins adsorbed from the culture medium[14].

Grafting of fibronectin on the PEA substrates is performed by reaction on the carboxyl groups exhibited by the copolymer surface; in order to achieve this, P(EA-co-AAc) or P(EA-co-MAAc) with different AAc or MAAc contents were prepared. Both series of networks

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possess physical properties that are quite similar with respect to water sorption capacity and surface energy. The difference between the two series of copolymers is essentially the mobility of the carboxyl group, which is much more restricted in the case of the methacrylic acid monomeric unit due to the proximity of methyl group bonded to the same carbon atom in the main polymer chain. In the case of the acrylic acid this methyl group is substituted by a hydrogen atom. Classical dielectric or dynamic-mechanical relaxation studies have shown that rotation around the bond that links the carboxyl group to the main chain is much easier in acrylic than in methacrylic acid[21]. The influence of this fact on the grafting of fibronectin on the copolymer surface is significant: as shown in Figure 2, the amount of fibronectin grafted on the surface is larger in the copolymer containing AAc than in those with MAAc for the same fraction of carboxyl groups. On the other hand, it was expected that an increase in the content of AAc or MAAc would result in a greater amount of fixed FN on the surface because the quantity of COOH groups are higher. However, non-significant differences have been observed, which could be due to the phase separation (shown by DSC) that results in a PEA-rich phase that becomes saturated with AAc or MAAc monomeric units while the rest forms domains which are not active for FN adhesion. In fact, it was shown that an increase of the AAc or MAAc fraction in the network from 10 to 20% does not increase water contact angle which suggests that no further carboxyl groups exists at the surface. In the case of the networks which contain AAc, the exhibition of RGD or FNIII$_{7-10}$ adhesion motifs significantly increases when the content of AAc increases from 10% to 20%. This behaviour can be also observed in the exposure of FNIII$_{7-10}$ domain in P(EA-co-MAAc). However, the exposure of RGD domain decreases when the percentage of MAAc is reduced.

Nevertheless, it is worth noting that the amount of fibronectin does not improve in the different networks with respect to fibronectin adsorbed on PEA; however, exhibition of the adhesion motifs with respect to the total fibronectin is higher. The results obtained in this

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work are consistent with others authors' results demonstrating the influence of surface chemistry in the conformation of the FN which can adopt a more globular or extended conformation depending on the distribution of hydrophilic or polar groups on the surface. Protein conformation subsequently influences cellular adhesion[7,9,10,20,22].

4. Conclusions

We have tested the ability of PEA based copolymer substrates to sustain the fibronectin coating and its proteic conformation through the study of the exposure of adhesion motifs that are essential for cell adhesion and viability. It could be concluded that in the case of adsorbed FN, low hydrophilic group content on the surface rapidly decreases the amount of fibronectin and increases the exposure of adhesion motifs. However, in the case of covalently adhered FN, the quantity of FN is higher when the surface is more hydrophilic; it seems, however, that the exposure of adhesion domains does not depend on wettability. The exposure of FNIII$_{7\text{-}10}$ domain in grafted FN was shown to depend on the composition of copolymer, being higher in MAAc surfaces than in AAc surfaces and in surfaces with a high content of monomeric units of this type. Generally speaking, it seems that the increment in functional groups (OH and COOH) improves the exposure of adhesion motifs (except the RGD domain in P(EA-co-MAAc)), being higher in surfaces with OH. Moreover, the fixation of FN by covalent bonds does not result in either higher protein content or a greater percentage of exposed adhesion domains on the surface with respect to FN adsorption.

5. Acknowledgements

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