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HYDROPHILIC SURFACE MODIFICATION OF ACRYLATE-BASED BIOMATERIALS

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Abstract

Acrylic polymers have proved to be excellent with regard to cell adhesion, colonization and survival, *in vitro* and *in vivo*. Highly ordered and regular pore structures thereof can be produced with the help of polyamide templates, which are removed with nitric acid. This treatment converts a fraction of the ethyl acrylate side groups into acrylic acid, turning poly(ethyl acrylate) (PEA) scaffolds into a more hydrophilic and pH sensitive substrate, while its good biological performance remains intact. To quantify the extent of such a modification, and be able to characterize the degree of hydrophilicity of PEA, PEA was treated with acid for different times (4, 9 and 17 days), and compared with poly(acrylic acid) (PAAc) and a 90/10 %wt. EA/AAc copolymer (P(EA-co-AAc)). The biological performance was also assessed for samples immersed in acid up to 4 days and the copolymer, and it was found that the incorporation of acidic units on the material surface was not prejudicial for cells. This surface modification of 3D porous hydrophobic scaffolds makes easier the wetting with culture medium and aqueous

solutions in general, and thus represents an advantage in the manageability of the scaffolds.

Keywords: scaffold, nitric acid, ethyl acrylate, acrylic acid, surface modification

1. Introduction

Poly(ethyl acrylate) (PEA) is a polymer with a low hydrophilicity, elastomeric at body temperature. Its mechanical properties are close to those of soft biological tissues. PEA has shown a very good biological performance *in vitro* [1-7] and *in vivo* [8].

Different porous architectures thereof can be produced by employing a porogen template with the negative of the pore structure, which after the polymerization is removed by dissolution: scaffolds with interconnected spherical pores [1, 8, 9], unidirectional cylindrical channels [10], or orthogonally intersecting cylindrical channels [8, 10, 11]. For the latter, a porogenic template made of polyamide-6 (PA-6) sintered fabrics is used, which was removed by dissolution in nitric acid. The present paper focuses on the effects of this production step, which involves immersion of the polymer in nitric acid for a time long enough to dissolve the porogen, on the physico-chemical and biological properties of the PEA matrix. The use of acid in the production of these scaffolds becomes necessary because this particular pore architecture is obtained by sintering PA-6 fabrics, which are easily available commercially, and acids are generally the sole good solvents for many semicrystalline polymers such as PA-6. Acids dissolve the fabrics template, but respect the insoluble PEA network. Moreover, this process simultaneously improves the polymer hydrophilicity, enhancing the wettability and cell colonization of the scaffold.

It is well known that the presence of certain chemical groups on the surface of the substrates can modulate their biological outcome [12-14]. In particular, the presence of acid groups improves wettability, which can facilitate the seeding of porous materials

[15], but also the presence of surface charges are reported to affect the deposition of ECM-proteins [16-18] that would translate into a better cell-material interaction or differentiation for certain cell lines [5, 19, 20]. Indeed, the preconditioning of hydrophobic PEA scaffolds with a hydrogel has been found to improve significantly cell seeding and migration to colonize them homogeneously [21-23].

To the best of our knowledge, there is no previous systematic characterization of the nitric acid effect on PEA. In the present study PEA was prepared and exposed to an acid treatment of the sort it would suffer during the typical preparation steps of the above-mentioned type of scaffold, as described in [10, 11, 24, 25]. EA being the ethyl ester of acrylic acid, it was hypothesized that the effect the nitric acid has on PEA was the hydrolysis of a number of its side chains transforming ethyl acrylate units into acrylic acid and giving rise to a random copolymer containing EA and AAc units, thus reverting the process of acid-catalysed esterification of acrylic acid. In order to prove this hypothesis, acid-treated films were compared with untreated PEA, poly(acrylic acid) (PAAc) and a P(EA-co-AAc) copolymer. The effect of the acid treatment on the biological performance of the materials was evaluated *in vitro*, and compared with that of untreated PEA, to complete the characterization.

2. Materials and methods

2.1. Materials

PEA scaffolds with interconnected cylindrical orthogonal pores were obtained following the procedure described in [25]. Briefly, porogenic templates were previously obtained by sintering 8 layers of nylon fabrics with a nominal thread diameter of 150 μm and a mesh opening of 300 μm (SAATI S.A.). The reactant mixture, consisting of ethyl acrylate (99%, Sigma-Aldrich), 0.1 wt.% of azo-bis-isobutyronitrile (99%,

Fluka), AZBN, as thermal initiator, and 2 wt% of ethyleneglycol dimethacrylate (98%, Sigma-Aldrich) as cross-linker, was injected in a porogen template, polymerized for 24 h at 60°C, and postpolymerized for 24 h more in an oven at 90°C. Next, the nylon templates were removed by dissolution in nitric acid (30%, Aldrich), repeatedly changed, during 4 days to dissolve the nylon template. Then, scaffolds were washed in boiling water for 16 h (the water was changed every 8 hours) to remove nitric acid traces. The scaffolds obtained were cut, dried under vacuum and observed by scanning electron microscopy.

PEA films were obtained by UV polymerization of reactant ethyl acrylate monomer (EA; 99%, Sigma-Aldrich), using 1% of benzoin as initiator (Scharlab) and 2% of ethyleneglycol dimethacrylate (98%, Sigma-Aldrich) as crosslinker. The monomeric mixture was stirred during 15 min, injected in a glass flat mould, polymerized for 8 h in an UV oven and post-polymerized for 24 h at 90°C. To remove any unreacted species, samples were washed in boiling ethanol (EtOH; 99%, Scharlab) during 24 h with renewal every 8 h. Analogously, PAAc films were obtained from acrylic acid monomer (99%, Sigma-Aldrich). The same procedure was followed to obtain EA-co-AAc copolymers in a 90:10 weight proportion. After the polymerization and washing steps, 1 mm-thick samples were cut either in (0.5 x 2.5cm²) bars or punched as discs of 5, 7 or 10 mm diameter. PEA samples were immersed in a HNO₃ (65% extra pure, Scharlab)/water 1/1 vol. solution and placed in a shaker for 4 (PEA-4d), 8 (PEA-9d) or 17 days (PEA-17d). At withdrawal, samples were dried with a filter paper and then rinsed for 16 h in boiling water (with a change after 8 h). Next, they were left overnight in fresh water and dried thoroughly.

2.2. Methods

2.2.1. Scanning electron microscopy (SEM)

The scaffolds morphology was characterized with a SEM device (Hitachi S-4800) at 10 kV. Both samples' surfaces and inner sections were observed, after sputter-coating with gold.

2.2.2. Fourier Transformed infrared spectroscopy (FTIR)

A Thermo Nicolet Nexus FTIR (Thermo Fischer Scientific Inc., Waltham, MA, USA) spectrometer was employed to determine the surface composition of the samples before and after acid treatments. Spectra were obtained in the ATR mode, with a resolution of 8 cm^{-1} , between 500 and 4000 cm^{-1} as the average of 68 scans.

2.2.3. Swelling kinetics experiment

Samples of the treated and non-treated materials were swollen to equilibrium by immersion in different media to study their pH-sensitivity in terms of the degree of swelling. These media were pure water, a 0.2 M NaOH aqueous solution, and aqueous solutions with constant ionic strength but different pH (prepared as described in [26]): Na_2HPO_4 , $\text{H}_3\text{C}_6\text{H}_5\text{O}_7$ and KCl were used to obtain solutions with ionic strength of 1.0 M and pH of 2, 4.6 and 8, respectively. Samples dried under vacuum were weighed before immersion and at selected time points. After withdrawal, the liquid remaining on the surface was carefully removed prior to weighing. The water uptake was calculated in triplicate as: $WC = \frac{W_t - W_0}{W_0}$, where WC is the water content, W_t is the weight of the disc swollen at time t and W_0 is the weight of the dry disc. The equilibrium water content, EW_C , is the attained final constant value of WC .

2.2.4. Density measurements

Density of the materials was determined with a Mettler Toledo AE-240 balance combined with a density accessory kit through Archimedes' principle. Samples were weighed in air and in n-octane (95%, Fluka), and their densities were calculated in triplicate as: $\rho = \frac{\rho_{N-oct} \cdot W_{air}}{W_{air} - W_{N-oct}}$, where ρ_{N-oct} is the density of n-octane (0.703 g/cm³), W_{air} is the weight of the disc in air and W_{N-oct} is the weight of the disc immersed in n-octane.

2.2.5. Differential scanning calorimetry (DSC)

DSC measurements were carried out in a Mettler Toledo DSC823e (Columbus, OH, USA) device, to determine the thermal properties of the samples. Specimens were heated in 30 μ L pierced pans from -100°C to 200°C at 10°C/min, with a nitrogen flux of 60 mL/min. The glass transition temperature (T_g) was determined as the midpoint, and the heat capacity increase (ΔC_p) was obtained for that T_g .

2.2.6. Dynamic-mechanical thermal analysis (DMTA)

DMTA measurements were performed in a Seiko DMS210 instrument (Seiko Instruments Inc., Chiba, Japan) to determine the influence of the acid on the mechanical properties of PEA. Measurements were done from -60°C to 180°C at 2°C/min, at 1 Hz in the tensile mode. Samples were rectangular, 5 mm wide.

2.2.7. Contact angle measurements

The contact angle of 3 μ L drops of extra pure water on the surface of the materials was determined in the sessile-drop mode with a Dataphysics OCA instrument (Filderstadt,

Germany). Samples were characterized in their dry state and after swelling in water for 48 h, with 10 water drops each.

2.2.8. Thermogravimetric analysis (TGA)

TGA scans were conducted in a TA-SDT Q600 device (TA Instruments, New Castle, DE, USA), from 35 to 1000°C at 10°C/min and with a 50 mL/min nitrogen flux. 10 mg samples were dried under vacuum and 60°C for 24 h before measurements.

2.2.9. Toluidine blue staining

A colorimetric characterization of the nitric acid effect was carried out by immersing discs in a 0.1% toluidine blue (Sigma-Aldrich) aqueous solution for 30 s, followed by a gentle rinse with distilled water to remove the excess of staining.

2.2.10. Sterilization and conditioning

Prior to cell culture, discs were washed for 30 min in an EtOH/H₂O 70/30 mixture in an ultrasound bath (Bandelin Sonorex Digitec). Next, they were rinsed with fresh EtOH/H₂O and dried thoroughly. Samples were then sterilized with UV light followed by several DPBS (Dulbecco's Phosphate Buffered Solution, Sigma) rinses, and kept overnight in fresh DPBS before fibroblasts culture, or in culture medium containing foetal bovine serum (FBS) for cultures with human umbilical vein endothelial cells (HUVECs).

2.2.11. Cell culture

L929 mouse fibroblasts (C34/An connective tissue, Sigma Aldrich) in their 18th passage and HUVECs (Gibco) in their 6th passage were used. Fibroblasts were cultured

with DMEM 4.5g/l glucose (Gibco) containing glutamine (Invitrogen), 10% of FBS (Invitrogen) and 1% of penicillin/streptomycin (P/S; Life technology), until confluence. Then, cells were trypsinized with trypsin-EDTA (Invitrogen) for 3 min at 37°C, collected and centrifuged at 1000 rpm for 5 min, re-suspended, counted and diluted to the desired cell density. Cells were seeded on 5 or 7 mm diameter samples as 20 or 40 μ L droplets, each one containing 5000 or 9800 cells, respectively. Bidimensional continuous samples, rather than tridimensional scaffolds, were used in cultures as simpler models, so as to avoid cells loss while performing cuts, stainings and rinsings and reveal more clearly differences in the biological performance of the different chemical compositions. The samples were incubated for 30 min, to allow cells to attach before filling the wells with 180 or 400 μ L of fresh medium, respectively. The medium was changed every day.

HUVECs were cultured with Medium 200 (Invitrogen) supplemented with LSGS (low serum growth supplement, Invitrogen) and gentamicin (Gibco) and P/S until confluence. Then, cells were trypsinized, collected and centrifuged at 180 g for 7 min. Suspensions of the desired densities were prepared: 20 μ L droplets containing 7700 cells for 5 mm diameter samples and 15000 cells in 40 μ L droplets for the 7 mm diameter discs. 30 min after seeding, the medium was completed and renewed every day.

2.2.12. Cell viability assay

After 6 h, 1, 3 and 5 days of culture MTS assays ((4,5-dimethylthiazol-2-yl)-5-(3 carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, Cell titer 96 Aqueous One Solution cell proliferation assay Promega) were performed to study the seeding efficiency and the cell proliferation rate, 3 replicates per time. Briefly, the samples were

rinsed with DPBS and moved to a new 96-well plate. 200 μ L of the MTS/phenol red-free medium mixture was added to each well and incubated in the dark. After 3 h, 100 μ L aliquots were placed in new wells and their absorbance was measured in a Victor Multilabel Counter 1420 spectrophotometer (Perkin Elmer, Waltham, MA; USA) at 490 nm. Cells cultured directly in polystyrene wells (or on PEA discs in the case of HUVECs) were employed as positive controls. MTS reactant solution incubated in wells with acellular materials worked as negative control.

2.2.13. Haematoxylin and eosin staining

At the selected time points samples were fixed with formaldehyde as previously described. Before staining, they were rinsed with distilled water for 3 min followed by other 3 min in tap water. Samples were next soaked in a haematoxylin solution according to Mayer (Sigma-Aldrich) and incubated for 10 min at RT, followed by the addition of a bluing solution (tap water, NaHCO_3 1% w/v (Fluka) and MgSO_4 0.0667% w/v (Scharlab) for 90 s. Once removed, type B eosin (Sigma) was added and incubated for 10 min at RT. Finally, samples were rinsed with tap water, mounted with a glycerol (Sigma)/water 85/15% vol. mixture, coverslipped and observed with a petrographic microscope (Nikon).

2.2.14. Statistical analysis

Results are expressed as mean \pm standard deviation from at least three replicates. Data were analysed pair wise with an ANOVA test with Statgraphics Centurion XVI.I. Results are statistically significant unless noted otherwise, except for the MTS results that are explained in detail in the figure caption. Significance was assigned at p-values < 0.05.

3. Results

3.1. Morphological characterization

Interconnected cylindrical orthogonal pores can be clearly observed in a cross section image of the PEA scaffold (Fig. 1-A), as well as several layers (8) of cylindrical crossed pores in parallel and perpendicular planes (Fig. 1-B). The bulk porosity of the scaffolds was $76.4 \pm 6.1\%$. They were flexible and with pore diameters ($149.08 \pm 17.02 \mu\text{m}$) excellent for cell colonization.

3.2. Physico-chemical and mechanical characterization

The FTIR spectra evidence changes occurred in the chemical structure of PEA by the HNO_3 treatments (Fig. 2-A). As expected, the 3200 cm^{-1} wide band (characteristic for the hydroxyl groups) appears for all acid-treated acrylates but not for the untreated PEA (highlighted in the Figure). And in the particular case of PEA-17d the 3500 cm^{-1} band is present as well (pointed out in the Figure). The PAAc spectrum exhibits both 3500 and 3200 cm^{-1} bands. In the P(EA-co-AAc) spectrum, only the 3200 cm^{-1} band is shown.

The symmetric and asymmetric stretches of CH_x (CH_2 , CH_3) groups appear as peaks in the 2962 and 2888 cm^{-1} region for all materials. For the PAAc, as for the PEA-17d although more moderately, another wide band is seen in the 2800 to 2500 cm^{-1} region (pointed out in the Figure), which could be attributed to the carboxylic acid group. This band was not evident for the copolymer.

The 1715 cm^{-1} peak is present and very well defined for all studied materials, since it is characteristic for carbonyl bonds in the carboxyl group. For the PEA-9d and PEA-17d, a

slight shift of this peak towards lower wavenumbers and a new one at 1600 cm^{-1} are perceived. In the particular case of the PAAc, the peak shift is more evident and the new peak is broader.

Samples either untreated or treated for 4 days with nitric acid show a slightly different behaviour in water, but a dramatically different behaviour when swollen in a basic solution (Fig. 3-A) in a preliminary assay. For untreated PEA, there are no significant differences in the water content when swollen in water or in the NaOH solution; moreover, the swelling in NaOH(aq) did not increase significantly from 1 h on. In contrast, the swelling of acid-treated PEA changed from a WC around 16% after 1 h up to 130% after 24 h. When swollen in water, these samples had a WC after 24 h only slightly higher than the untreated ones: 2.78% vs. 1.14%.

The effect of the duration of the acid treatment on the swelling degree was further characterized by using media with different pH but constant ionic strength. The goal of these tests were to reveal the chemical modifications occurred during the preparation of the materials. These conditions are not, thus, representative of physiological media. In physiological media, the swelling of each network will vary as a function of pH, ionic strength and temperature, and can be somewhat tailored with its crosslinking degree.

When the samples were swollen at pH 2 (Fig. 3-B), the *EWC* increased slightly with the acid-treatment: 1% for untreated PEA up to 5% for PEA-17d. The PEA-9d samples showed quasi-identical values to P(EA-co-AAc). No significant differences between the *EWC* of the copolymer and PEA treated for 4 and 9 days were observed. The *EWC* attained by PAAc was much higher (50%), and P(EA-co-AAc) (2.5%) appeared to be more hydrophilic than PEA but was far from the values of PAAc. Similar results and

values were obtained for pH 4.6. However, when the materials were swollen in a basic medium, *i.e.* pH 8, their behaviour changed dramatically except for the untreated PEA, which maintained an *EWC* around 1%. The PEA-4d nearly triples its *EWC* at this pH reaching a value around 6%, PEA-9d reached an *EWC* of nearly 60%, and 240% was the value attained by PEA-17d. Much higher values were obtained for PAAc (650%), and P(EA-co-AAc) reached an intermediate 60%.

The swelling of PEA was independent of the medium pH, with a moderate value of *EWC*. For the acid-treated samples, as for the copolymer and PAAc, there were no remarkable differences between the *EWC* attained at pH=2 and at 4.6, but at pH=8 these materials showed a hydrophilic behaviour, which was proportional to the immersion time for the treated series or to the fraction of carboxyl lateral groups for the reference materials. Interestingly, the PEA-9d and the copolymer swelled at all pH values following the same trend and attaining a similar *EWC*.

The density was also sensitive to the nitric acid exposure. The longer the treatment, the greater the density of the resulting material (Fig. 4-A), changing gradually from 1.134 g/cm³ for untreated PEA up to 1.180 g/cm³ for PEA-17d. The densities of PEA-4d and PEA-9d were closer to that of the copolymer (1.158 g/cm³); in fact, no statistically significant difference was observed between PEA-9d and the copolymer. As expected, PAAc exhibited a much higher density, 1.289 g/cm³.

A displacement of the glass transition towards higher temperatures with the duration of the acid treatment was observed, shifting from 260K for PEA up to 295.4K for PEA-17d (Fig. 5). The DSC thermogram of P(EA-co-AAc) is intermediate to those of PEA-

4d and PEA-9d, its glass transition temperature (271.21 K) lying between the respective values for these samples. By contrast, PEA-17d behaved similarly to PAAc, although this last's T_g was a bit lower, 294.14K.

The specific heat capacity jump at the glass transition (Δc_p) slightly increased with the time of acid treatment, from 0.39 J/gK for PEA up to 0.453 J/gK for PEA-17d. Again, the PAAc value was the highest (0.769 J/gK), and that of the copolymer (0.423 J/gK) was of the order of acid treated PEA.

The acid treatments did also influence the viscoelastic behaviour, Fig. 6. At temperatures below 0°C, all materials displayed a high storage modulus (10^9 - 10^{10} Pa) typical of glassy polymers. The PEA main relaxation was observed in the interval from -10°C to 30°C; it was shifted to higher temperatures, in the range from -5 to 40°C in P(EA-co-AAc) and much higher in PAAc, from 15°C to 60°C. In the case of the acid-treated polymers, the relaxation shifted from that of PEA towards that of P(EA-co-AAc) for PEA-4d, to greater temperatures for PEA-9d and up to values close to those of PAAc for PEA-17d. After this relaxation, the elastic modulus was in all cases in the order of MPa, typical of an elastomer.

As the inset in Fig. 6-B shows, the temperature at which $\tan\delta$ achieves its maximum value (T_α) increases with the duration of the acid treatment. The maximum for P(EA-co-AAc) is located between those for PEA-4d and PEA-9d, and that of PAAc is the highest and close to that of the PEA-17d.

The water contact angle on the surface was also affected by the chemical modifications occurring in the PEA matrix, but was revealed only when the polyacrylates were in their swollen state. Dry acid-treated PEA discs exhibited a slightly lower contact angle than untreated PEA, indicating that after the acid exposure the PEA becomes more wettable (Fig. 7-A). As expected from their chemistry, that includes lateral acid groups, both P(EA-co-AAc) and PAAc exhibited low contact angles, even below those of the treated samples. Surprisingly, despite only 10%wt of the monomeric units in P(EA-co-AAc) contain an acid group, its surface appeared to be more wettable than PAAc, whose units, all of them, contain such group. The differences between untreated and acid-treated PEA and PAAc were not statistically significant. However, when the contact angles were measured after a previous swelling (Fig. 7-B), the results were different. The contact angle on swollen PEA was still high but somewhat lower than in its dry state, and those of the treated samples decreased with the immersion time in acid up to 9 days. After swelling, the average contact angle of PAAc was lower than that of P(EA-co-AAc), as expected given their composition. PEA-4d behaved as P(EA-co-AAc) and PEA-9d had a contact angle close to that of PAAc (there were no statistically significant differences between them). Swollen PEA-17d showed again an anomalous behaviour.

The thermal degradation of PEA is also affected by the acid treatment (Fig. 4-B): the onset of the weight loss is shifted toward lower temperatures and the main thermal degradation of PEA, which occurs between 325°C and 425°C, gradually changes with the duration of the acid treatment up to 260°C-440°C for PEA-17d. Once more, PEA-4d and PEA-9d exhibited an intermediate behaviour between those of PEA and the copolymer, undergoing the main thermal degradation in the 295°C-440°C interval. The weight of the solid residue slightly increased with the acid treatment time, from 3% for

PEA up to 5.4% for PEA-17d. Although PEA-17d shows a decomposition thermogram not dissimilar to that of P(EA-co-AAc), its solid residue coincides with that of PAAc.

After staining with toluidine blue, samples were gradually bluer with either the treatment time in nitric acid (Fig 7. A-D) or the fraction of acrylic acid groups (Fig 7. E, F). Untreated PEA acquired a light purplish shade. The colouring of PEA-4d and PEA-9d was very similar to that of P(EA-co-AAc) and already in the blue tonality; while PAAc was the most stained sample.

3.3. Biological experiments

The MTS assay after the fibroblasts culture (Fig. 8) showed no statistically significant differences among the different materials and positive control, regarding the initial adhesion. In all cases (except for P(EA-co-AAc) at day 1), there is a significant difference between the acellular control and the cellular samples indicating that cells attached to the substrates and are viable at longer times. In general, untreated PEA exhibited a better behaviour as compared with the other polymers, and exhibited a closer behaviour to the control (plate well), increasing the absorbance with time. In the case of PEA-4d and P(EA-co-AAc), the absorbance increase was more moderated. Since PEA reached absorbance values close to those of the positive control in fibroblasts culture, in the following HUVECs culture it was used as positive control.

The MTS results for HUVECs culture (Fig. 9) show a similar tendency. In this case the values obtained were independent of the treatment up to 1 day: absorbance levels did not increase significantly in any sample. Afterwards, HUVECs showed increased viability significantly on PEA surfaces and less on PEA-4d, whereas no statistically significant absorbance increase was observed for P(EA-co-AAc).

After staining with haematoxylin-eosin, slight differences between samples were observed. In general, better results were found with fibroblasts (Fig. 8) than with endothelial cells. Fibroblasts viability was suitable, and even several layers of cells were arranged on specific areas of the surfaces after 5 days of culture. A very good initial adhesion was also observed for P(EA-co-AAc). Despite the better results obtained in the MTS assay with control wells, the stained cells shape and density suggests that the investigated materials induce a better initial adhesion than the glass cover, and cell viability on the treated PEA or the copolymer does not seem to be worse.

Haematoxylin–eosin staining images of HUVECs (Fig. 9) show a better initial attachment on PEA as compared to the other polymers; nonetheless, after 5 days cells had properly proliferated and exhibited an elongated morphology on all studied substrates. At low magnification, it can be observed that cells tend to form scattered circular structures from the first time point, which is only few hours after the seeding, and grow into more complex structures, formed by more cells in this circular disposition contacting between them with time.

4. Discussion

The PEA matrix polymer of scaffolds prepared as in [9, 10, 25] suffers a surface modification as a consequence of the template rinsing process. In order to characterize these modifications, PEA was exposed to nitric acid aqueous solutions for different times, and compared with PAAc and a copolymer. This has allowed to identify the physico-chemical changes produced and to quantify them.

The exposure of PEA polymer to nitric acid aqueous solutions seemed to change a number of ethyl acrylate monomer units into ethyl acrylate ones, by replacement of a hydrogen for the ethyl group, thus reverting the esterification process by which ethyl acrylate monomer results from acrylic acid [27].

The emergence of new bands on the FTIR spectra (Fig. 2-A) at 3500 and 3200 cm^{-1} agrees with such substitution. The different behaviour exhibited by untreated and acid-treated samples when swollen in water or in a basic aqueous medium (NaOH 0.2 M) (Fig. 2-A), indicates that the material becomes more hydrophilic and, more importantly, pH sensitive when treated with HNO_3 . The fact that a dramatic increase of the *WC* occurs at basic pH for the acid-treated acrylates, proportional to the immersion time, as happens in the control polymers containing acid side groups PAAc and P(EA-co-AAc) (Fig. 3-B), confirms the presence of acid groups in the treated samples, which are dissociated in basic media turning polymers into superabsorbents [28]. This gradual modification was visually supported by toluidine blue staining (specific for acid groups) (Fig. 7). The darker and more bluish samples were those where carboxylic acid groups were introduced, either by the acid treatment or by the co-polymerization with AAc.

Considering the results obtained with different techniques, it can be stated that the behaviour of PEA-4d and PEA-9d is similar to that of the 90:10 P(EA-co-AAc) copolymer, as the last exhibited an intermediate behaviour between them. PEA-9d attained the same equilibrium values and followed the same trend as P(EA-co-AAc) in the swelling study (Fig. 3-B) at all pH values; moreover, the PEA-4d dynamic-mechanical main relaxation (Fig. 6) is very close to that of P(EA-co-AAc). But it is PEA-9d whose degradation thermogram resembles more that of the copolymer (Fig. 4-

B). Visually, with the toluidine blue staining, the colouring of P(EA-co-AAc) lies between those of PEA-4d and PEA-9d (Fig. 6).

The PEA-17d exhibited an erratic behaviour. For instance, in the swelling study (Fig. 3-B), it reached the highest values (except for the PAAc) outside the trend, independently from the pH value, while its density (Fig.4-A) was again below the PAAc density but greater than those of the other materials. Its glass transition (Fig. 5) and $\tan \delta$ maximum temperature (Fig. 6) were close to those of PAAc. Despite the fact that the thermal degradation profiles of PEA-17d and PAAc are very different (Fig. 4-B), the percentages of solid residue left are very similar. This anomalous performance suggests that such a long treatment in nitric acid induces other chemical modifications besides the random hydrolysis of side chains, which would require a further characterization.

The nitric acid treatment has an effect on the PEA surface properties and therefore on the wettability. The water contact angle (Fig. 7) measured in the dry state experienced scarce changes with the increase of the acid treatment duration, and surprisingly the copolymer with only a small fraction of AAc groups showed a lower contact angle than PAAc. This can be attributed to the acid side groups that reduce chain mobility, leading to a vitrification of the materials at room temperature, which leads to a poorer exposure of hydroxyl groups at the surface. On the contrary, the plasticising effect of water improves chain mobility; thus contact angle values exhibited the expected trend when the materials were in their swollen states, with a proportional reduction of the angle with the immersion time in acid or the increase of AAc units. The exception was, once more, PEA 17-d.

If it is assumed that the acid treatment affects PEA only by transforming ethyl acrylate units into acrylic acid units, the density and DSC results combined with mathematical models can be used to estimate the fraction of each component in the treated materials. With the measured densities and considering the mixture of both homopolymers as ideal, for which: $\frac{1}{\rho} = \frac{1}{\rho_1} \cdot \omega_1 + \frac{1}{\rho_2} \cdot \omega_2$, where ρ is the density of the mixture, ω_1 and ω_2 are the mass fractions of components 1 and 2, and ρ_1 and ρ_2 are their densities, the fraction of each component in the treated PEA was estimated. Firstly, it was verified for the copolymer (whose composition was known) that the ideal mixture was a good model for this system (its measured density was 1.158 g/cm³ and the calculated one was 1.148 g/cm³, which was considered close enough). For PEA-4d, a fraction of acrylic acid units, ω_2 , of 0.09 was obtained, 0.18 for PEA-9d and 0.32 for PEA-17d.

It is also possible to determine the fraction of each component in the mixture from its glass transition temperature, T_{gmix} , those of the pure homopolymers PEA and PAAc (T_{g1}, T_{g2}), and their heat capacity jumps at Tg ($\Delta C_{p1}, \Delta C_{p2}$) by using Couchman-Karasz's expression [29]:

$$\ln(T_{gmix}) = \omega_1 \cdot \Delta C_{p1} \cdot \ln(T_{g1}) + \omega_2 \cdot \Delta C_{p2} \cdot \ln(T_{g2}) \Delta C_{p1} \cdot \omega_1 + \Delta C_{p2} \cdot \omega_2$$

If the ratio T_{gPEA} / T_{gPAAc} is close to 1, which is here the case, this expression can be simplified by removing the logarithms to:

$$T_{gmix} = \frac{\omega_1 \cdot \Delta C_{p1} \cdot T_{g1} + \omega_2 \cdot \Delta C_{p2} \cdot T_{g2}}{\Delta C_{p1} \cdot \omega_1 + \Delta C_{p2} \cdot \omega_2}$$

With the DSC results and the Couchman-Karaszc simplified expression, findings quite different to the previous ones were obtained. For PEA-4d the estimated acrylic acid fraction was 0.27, for PEA-9d it was 0.44, and for PEA-17d a value of 0.87 was obtained. These discrepancies with the values predicted from densities could be attributable to the low T_g measured for PAAc, as compared to values previously reported [30].

The biological experiments show that the modification of the PEA does not compromise its use as a scaffold: an increase in hydrophilicity of the scaffold improves the seeding of cells and promotes cell migration towards the interior. In fibroblasts cultures on films, for all times, PEA, PEA-4d and P(EA-co-AAc) showed a great density of cells, even sometimes higher than on the control, with extended cell morphology (Fig.8). Previous works reported that substrates containing small fractions of carboxylic acid groups enhanced keratocytes attachment and proliferation [31]; in other works, their presence led to greater densities of fibronectin or oriented it with more accessible binding sites, what enhanced cell attachment [32].

We could attribute the better outcome of PEA over P(EA-co-AAc) or PEA-4d to an initial variation of the pH medium, because of the presence of side acid groups, that might affect cells metabolism reducing absorbance levels. The reason for the stained samples showing a greater density than anticipated by MTS results could be found in the volume of medium in which they were cultured: 200 μ L for the samples employed for MTS assays, whereas 400 μ L for the stained samples. Probably, this increase of medium was enough to buffer the pH variation induced by the acid groups of the samples.

HUVECs are very sensitive cells, and nonetheless they showed to be viable and assembled into circular structures on all the materials (Fig. 9-C,F,I). The good outcome could be favored by the conditioning step in medium containing FBS; indeed, previous works reported that the presence of COOH groups enhanced the deposition of fibronectin and albumin encouraging endothelial cells growth [33]. Analogously, works report that human mesenchymal stem cells (hMSCs) can grow on substrates containing acrylic acid units after being coated with collagen [20, 34]. These positive results open the possibility to obtaining vascular-like structures when HUVECs are seeded in these three-dimensional PEA scaffolds.

5. Conclusions

Immersion of PEA in nitric acid aqueous solutions, as is employed for scaffolds production, transforms the homopolymer into a random copolymer of ethyl acrylate and acrylic acid, with a proportion of both that depends on the immersion time. By comparing the physico-chemical properties of PEA with different times of exposure with those of a P(EA-co-AAc) copolymer it was concluded that the weight fraction of monomer units converted was about 10% after 4 days. As a consequence of these changes, PEA becomes more hydrophilic, exhibits pH-dependent swelling, and is capable of vitrifying at physiological temperature in the dry state. Nevertheless, the chemical modifications occurred at these times do not impair the biological performance of the material, which continues to be excellent even with very sensitive cells. If immersion in nitric acid solution lasts longer, other chemical changes that have not been identified might take place.

Acknowledgements

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Figure captions

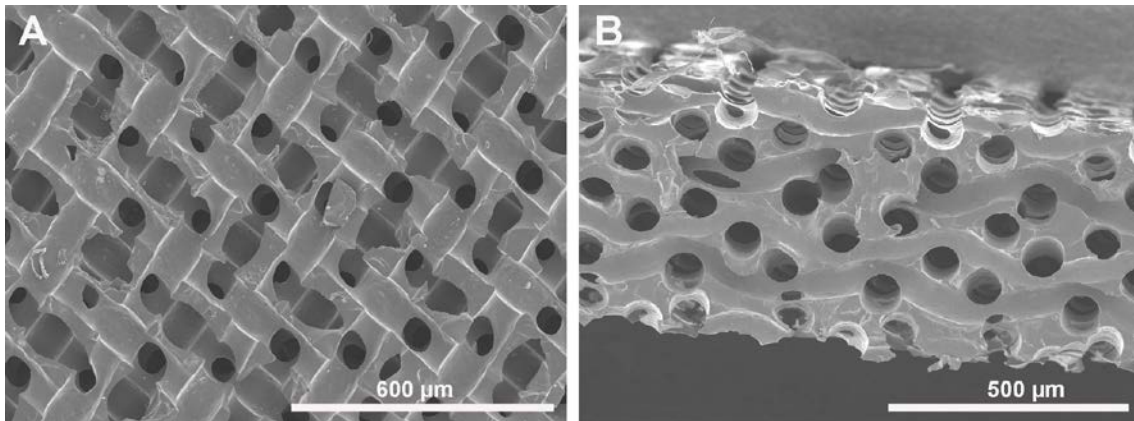


Figure 1. SEM images of the PEA scaffolds with cylindrical orthogonal pores: (A) surface, (B) cross-section. Scale bare: 600 μm (A) and 500 μm (B)

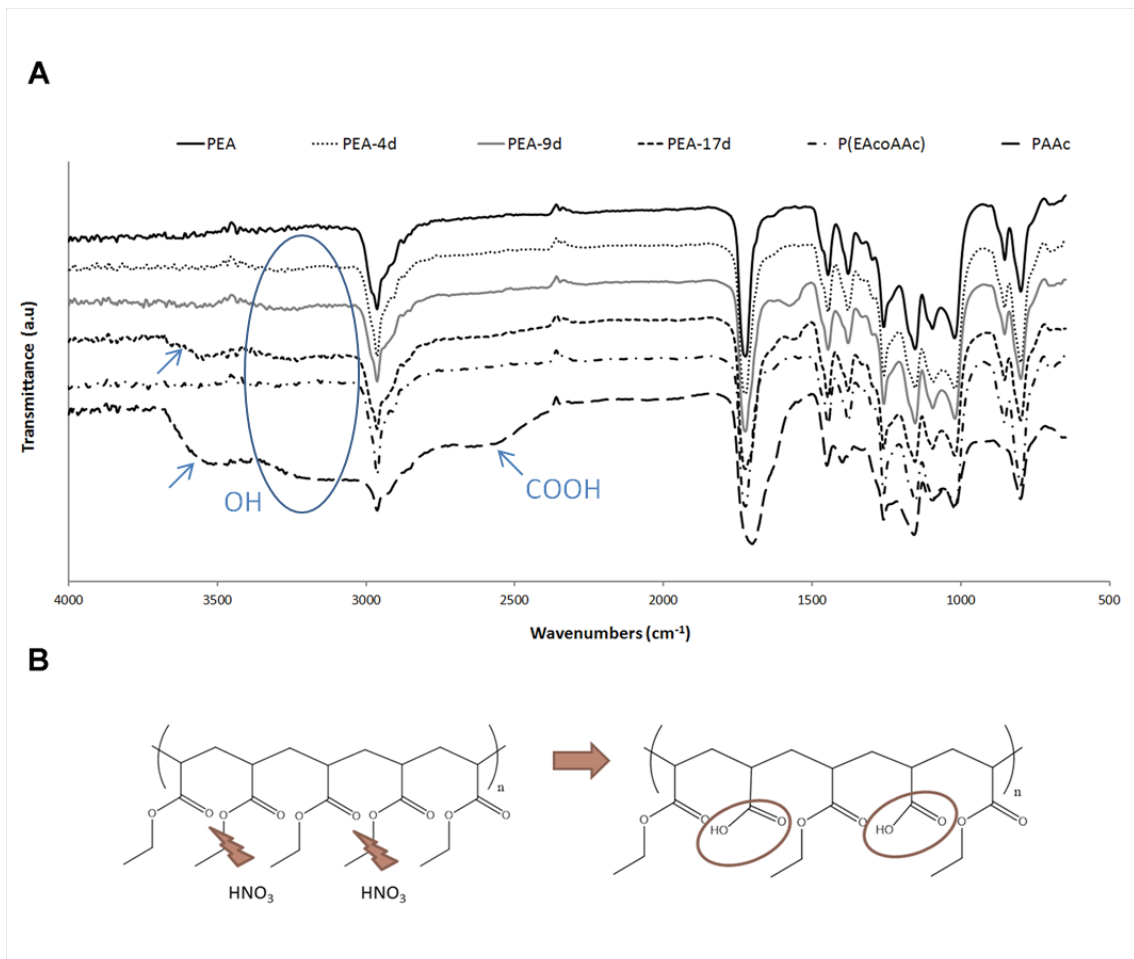


Figure 2. (A) FTIR spectra of PEA, PEA treated for 4, 9 and 17 days in nitric acid, PAAc and the copolymer P(EA-co-AAc), in the 500 to 4000 cm⁻¹ region. (B) Scheme of the reaction taking place during the HNO₃ treatment

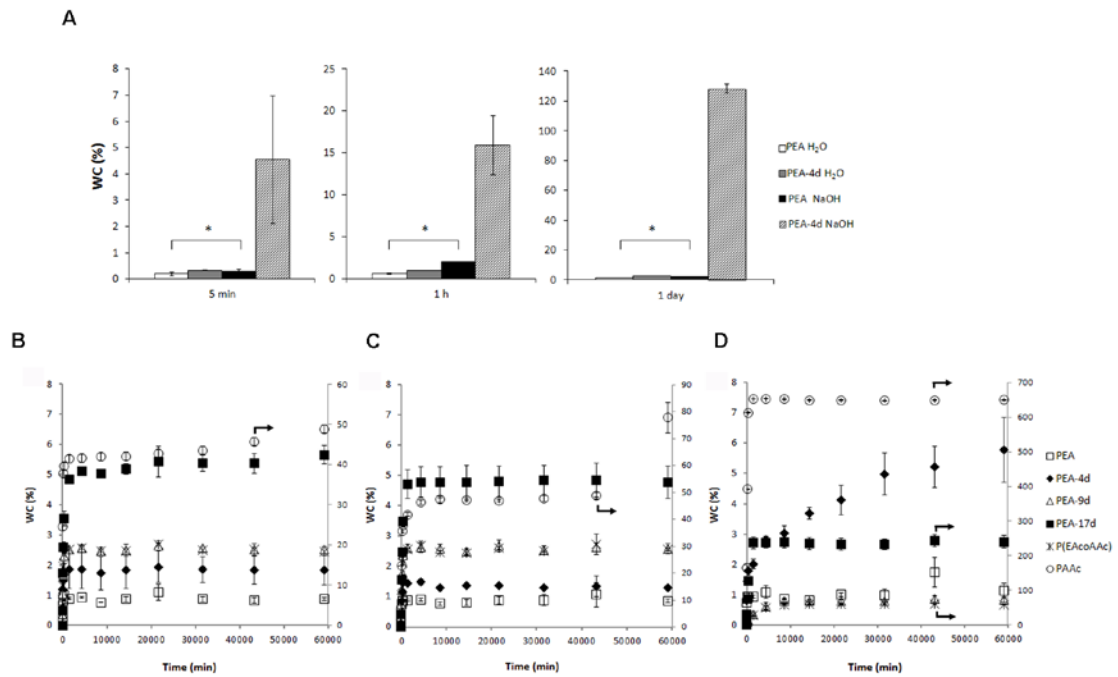


Figure 3. (A) Water content of the untreated and 4 days acid-treated PEA samples as a function of time of immersion in water or in a NaOH 0.2 M aqueous solution. (*) No significant differences were observed between PEA swollen in water or NaOH (aq) for all time points. (B-D) Water content WC of acid-treated PEA samples and of P(EA-co-AAc) and PAAc as a function of time of immersion in solutions with pH equal to 2 (B), 4.6 (C) and 8 (D). Arrows indicate the data series to be read with the right-hand scale on the plots. For PEA, no significant differences were observed at different pH values. For the PEA-4d, PEA-9d, PEA-17d and P(EA-co-AAc) no significant differences were observed between the *EWC* attained at pH = 2 and pH = 4.6.

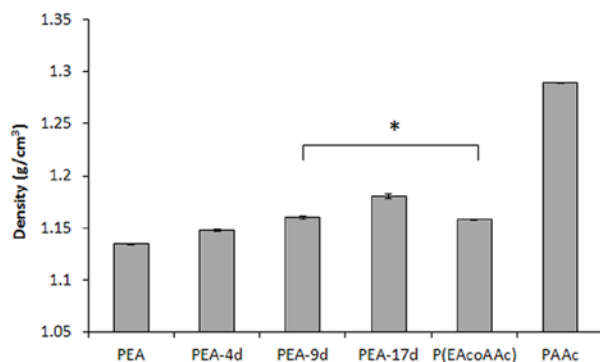
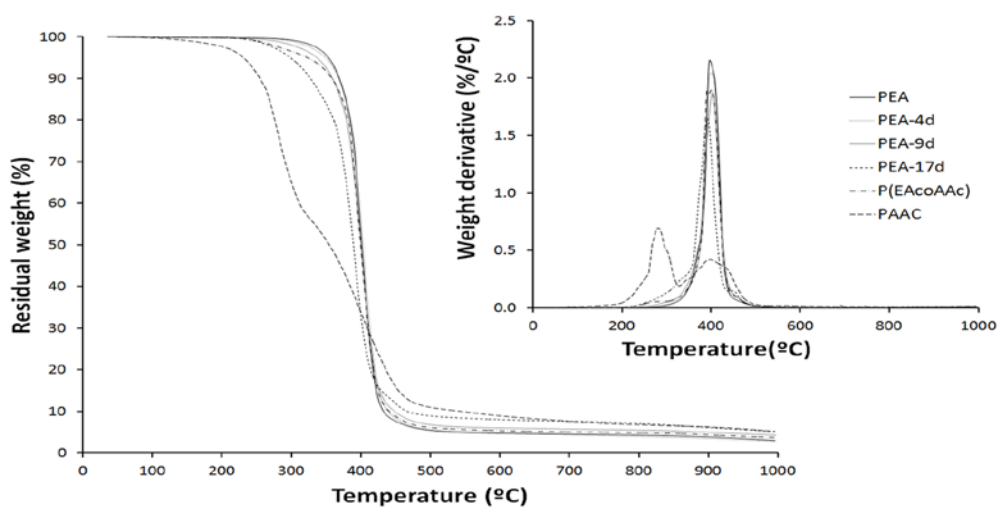
A**B**

Figure 4. (A) Densities of acid-treated PEA samples and P(EA-co-AAc) and PAAc references. (*) No significant differences were observed between the PEA-9d and P(EA-co-AAc) densities. (B) Thermograms for acid-treated PEA, P(EA-co-AAc) and PAAc samples, plotting the residual mass fraction as a function of temperature, and the first derivative of the residual mass fraction as a function of temperature (inset).

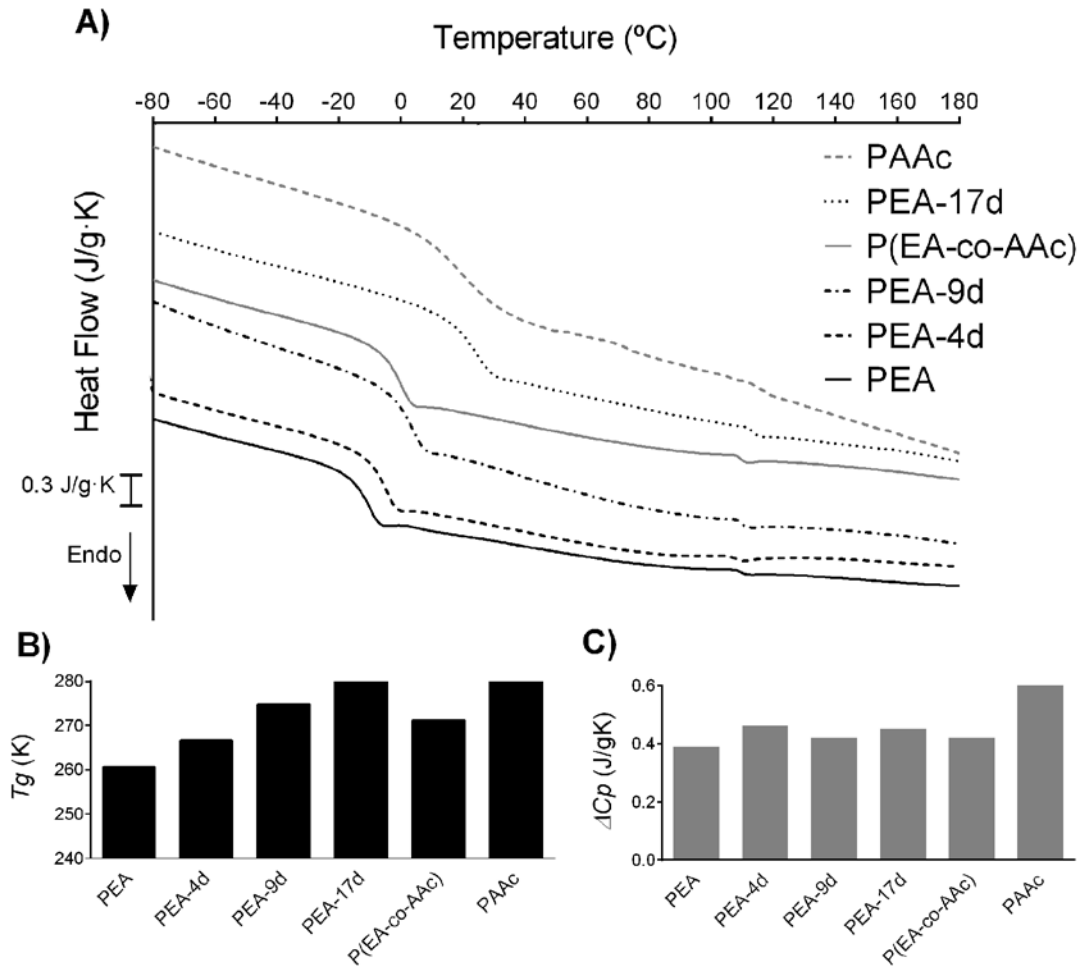


Figure 5. (A) DSC thermograms plotting the heat flux per unit mass and unit temperature as a function of temperature of acid-treated PEA samples and the P(EA-co-AAc) and PAAc references. (B) Glass transition temperature and (C) specific heat capacity jump at the glass transition for the different materials.

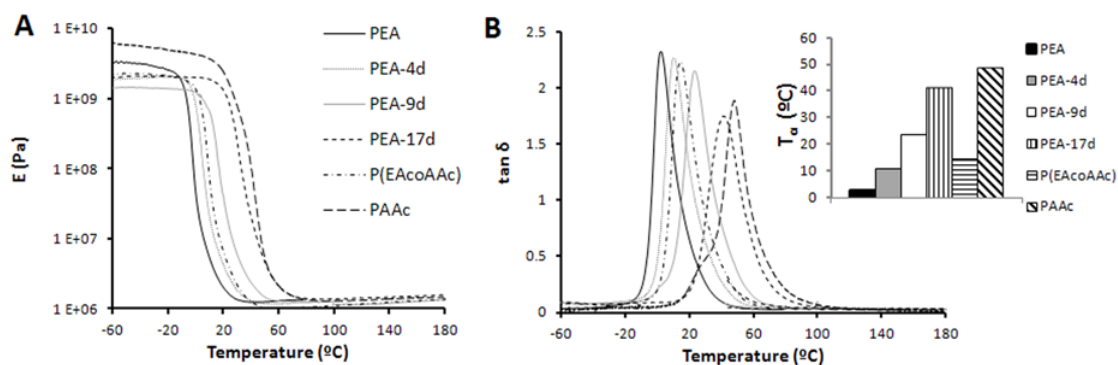


Figure 6. Elastic modulus variation with temperature (A) for acid-treated PEA samples and P(EA-co-AAc) and PAAc references, $\tan \delta$ as a function of temperature (B), and T_g (inset).

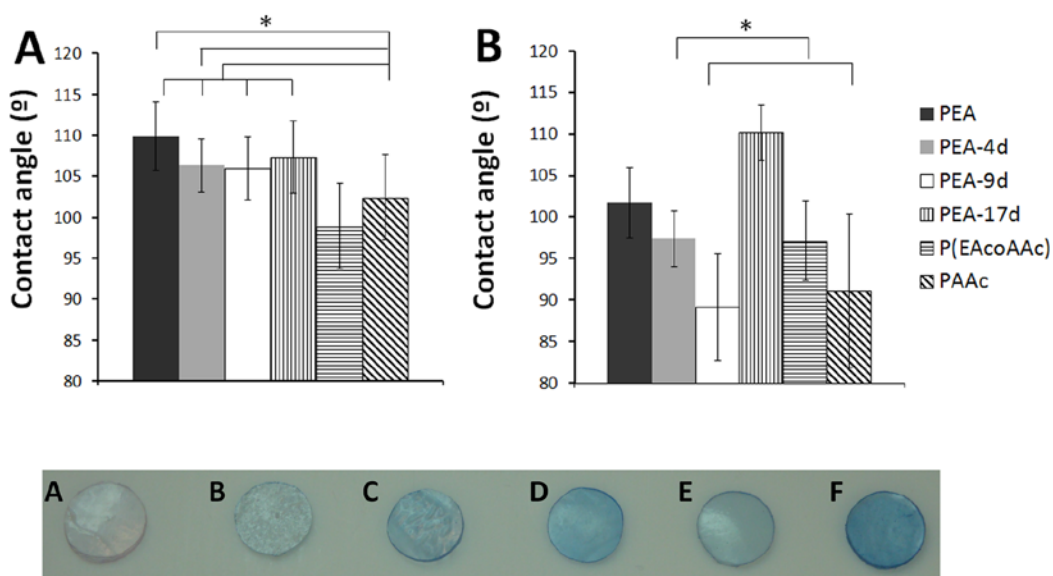


Figure 7. Water contact angle measurements of dry (A) and swollen (B) acid-treated PEA, P(EA-co-AAc) and PAAc samples. (*) No statistically significant differences were observed between the labelled materials. (A-F) Toluidine staining for PEA (A), PEA-4d (B), PEA-9d (C) PEA-17d (D), P(EA-co-AAc) (E), and PAAc (F).

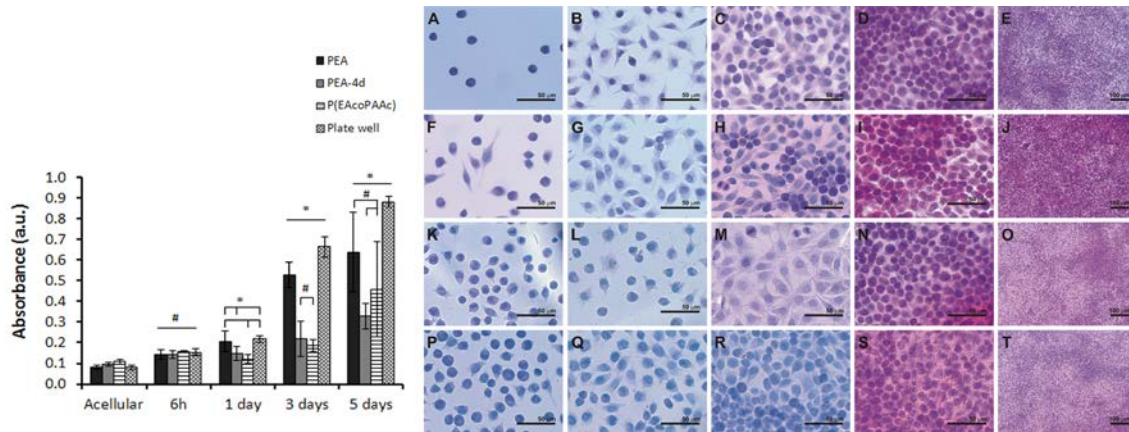


Figure 8. MTS results after 6h, 1, 3 and 5 days culture of L929 fibroblasts on untreated PEA, PEA-4d, P(EA-co-AAc) and plate wells. Bright field images of samples stained with haematoxylin-eosin after (from left to right) 6h, 1, 3 and 5 days at 40X magnification, and 5 days at 10X magnification, after culture of L929 fibroblasts on covers (A,B,C,D,E), PEA (F,G,H,I,J), PEA-4d (K,L,M,N,O) and P(EA-co-AAc) (P,Q,R,S,T). (*) Differences are statistically significant. (#) Differences are not statistically significant.

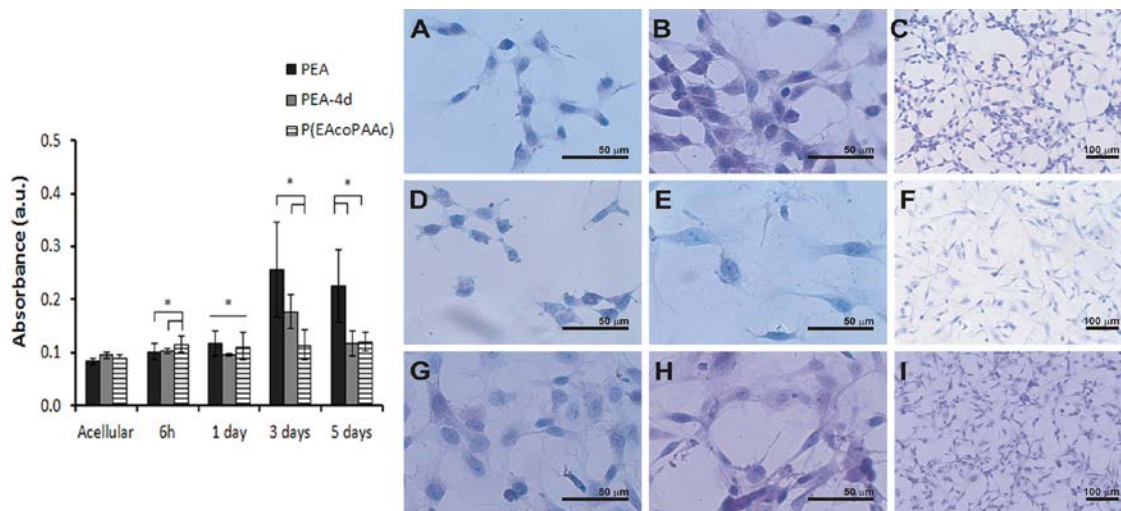


Figure 9. MTS results after 6h, 1, 3 and 5 days culture of HUVECs on untreated PEA, PEA-4d, P(EA-co-AAc) and plate wells. Bright field images of samples stained with haematoxylin-eosin after (from left to right) 6h, and 5 days at 40X magnification and after 5 days at 10X magnification of HUVECs culture on PEA (A,B,C), PEA-4d

(D,E,F) and P(EA-co-AAc) (G,H,I). (*) Differences are statistically significant. (#)

Differences are not statistically significant.