Plasma- and chemical-induced graft polymerization on the surface of starch-based biomaterials aimed at improving cell adhesion and proliferation

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Plasma and chemical induced graft polymerization of acrylic monomers on starch-based biomaterials has been performed with the aim to improve cell adhesion and proliferation on the surface of the polymers, in order to adequate their properties for bone tissue engineering scaffolding applications. Plasma and chemical surface activation was aimed to induce the polymerization of acrylic polar monomers being carried out by applying a radio frequency plasma and expose the samples to a mixture of Ar/O_2, or by immersion in a $H_2O_2/(NH_4)_2S_2O_8$ solution with UV radiation, respectively. Both procedures were followed by the graft polymerization of the corresponding monomers. Polymer grafting was analyzed by Fourier transformed infrared spectroscopy (FTIR) and X-ray photoelectron spectroscopy (XPS) and by contact angle measurements. Properties such as mechanical performance, swelling degree, and degradation behavior, as well as bioactivity, have been studied and compared for the different activation methods. Finally, preliminary cell adhesion and proliferation tests were performed, using goat bone marrow cells, showing a remarkable improvement with respect to original non-surface modified starch-based biomaterials.

1. Introduction

It is well known that the surface of polymeric biomaterials governs the initial cellular response when they are implanted in the human body [1, 2]. In this sense, some polymers have generated great interest in tissue engineering applications in tissue replacement or regeneration, in which the cell proliferation and adhesion are intimately dependent on the surface chemistry of the corresponding polymers [3, 4]. One important requirement of polymers for tissue engineering applications is a controlled biodegradable character, an aspect exhibited by the starch-based blends which are also inexpensive materials (compared to other biodegradable polymers) and easily processable systems. The properties, applications, and processing procedures of biodegradable starch-based thermoplastic blends, like starch/cellulose acetate and starch/(ethylene-co-vinyl alcohol) have been reported in the literature [4–7]. Their applications include drug delivery systems [9, 10], hydrogels [11], bone cements [9, 12] and porous degradable biomaterials to be used as scaffolds in bone tissue engineering [10, 13].

In this work, and in order to improve cell adhesion and proliferation (aiming at bone tissue engineering scaffolding applications), surface modifications of starch blends were performed by grafting acrylic polar monomers by plasma-induced and chemical-induced polymerization. Plasma-induced polymerization is a clean technique using radio-frequency (RF) produced by low-pressure glow-discharge and introducing gases, such as Ar and O_2, to create active species (free radicals or peroxides) to initiate free radical graft polymerization processes [14, 15].

This work reports the graft polymerization of acrylic polar monomers on the surface of starch-based blends by plasma induced, and chemical surface activation by using $H_2O_2/(NH_4)_2S_2O_8$ and UV radiation. Both grafting techniques are characterized by Fourier trans-

formed infrared spectroscopy (FTIR) and X-ray photoelectron spectroscopy (XPS) and by contact angle measurements. In addition, mechanical properties, swelling degree, degradation behavior, and bioactivity tests are presented and compared in terms of the surface activation method. Finally, preliminary cell adhesion and proliferations tests were performed by means of seeding goat bone marrow cells on the modified starch-based biomaterials surfaces.

2. Materials and methods

The surface modifications were performed on the following starch-based thermoplastic blends supplied by Novamont, Novara, Italy: (i) a corn starch/(ethylene-co-vinyl alcohol) (50/50 wt %, SEVA-C) and (ii) corn starch/cellulose acetate (50/50 wt %, SCA). Modifications were carried out on films prepared by compression molding at 120–130 °C and on ASTM dumb-bell tensile test specimens prepared by injection molding with a cross-section of $2 \times 4\,mm^2$. Acrylic acid (AA) (Merck, Germany), ethylene glycol methacrylic phosphate (EMP) (Aldrich, USA) and N-(hydroxymethyl)-acrylamide (HMA) (Merck, Germany) were used as received to be grafted on the starch substrates (see scheme on Fig. 1). Hydrogen peroxide 33% (w/v) (Panreac, Spain) and ammonium persulfate (Aldrich, Germany) were also used as received.

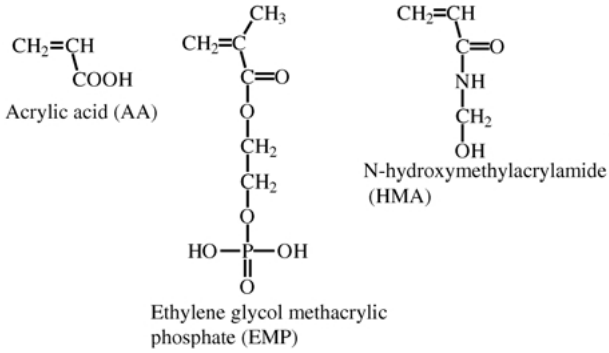

Figure 1 Chemical structure of the acrylic monomers grafted on the starch-based blends.

2.1. Surface modifications

Two methods, plasma and chemical, were applied to activate the starch biomaterials surfaces and to promote the formation of free radicals for the initiation of the graft polymerization of the acrylic monomers. The plasma treatment was performed in an apparatus as shown in Fig. 2 by applying a 13.56 MHz (radio frequency) at 20 °C and controlling the pressure of the reactor by adjusting the flow rate of the mixture of Ar and O_2 (20%). Samples were first placed in a pre-chamber and then transferred into the plasma reactor automatically when the pressure was 8×10^{-2} mbar, and located under the electrodes as shown in Fig. 2. When the pressure was reduced to 1×10^{-6} mbar, the Ar/O_2 mixture was introduced in the reactor and samples were exposed to the plasma for 30 min. After the treatment, samples were kept in the gas chamber for 5 min before being removed from the reactor.

The chemical activation consisted in immersing the starch blends samples in a $H_2O_2/(NH_4)_2S_2O_8$ solution (50 mL/2 g), maintained for 30 min under a UV lamp (Hanovia Uvitron) at 254 nm for 30 min. After surface activation treatments (plasma and chemical), samples were exposed to air for 15 min and immersed in degassed aqueous monomer (23% wt) solutions (AA, EMP, HMA) and kept at 60 °C for 2 h, removed from the tubes and washed with distilled water overnight to remove homopolymers and non-reacted monomers, and finally vacuum dried over phosphorus pentoxide.

2.2. FTIR analysis

The substrates were analyzed by FTIR spectroscopy in a Perkin Elmer System 1600 FTIR with an attenuated total reflectance (ATR) device, from Specac, aiming at detecting the grafting of acrylic polymers on the starch biomaterials.

2.3. X-ray photoelectron spectroscopy

The XPS measurements were performed in a V.G. Scientific apparatus using a Mgkα source (15 kV, 15 mA). The angle of incident X-rays to the surface was fixed at 90°. XPS was mainly used to characterize the surface chemistry of controls and surface modified samples.

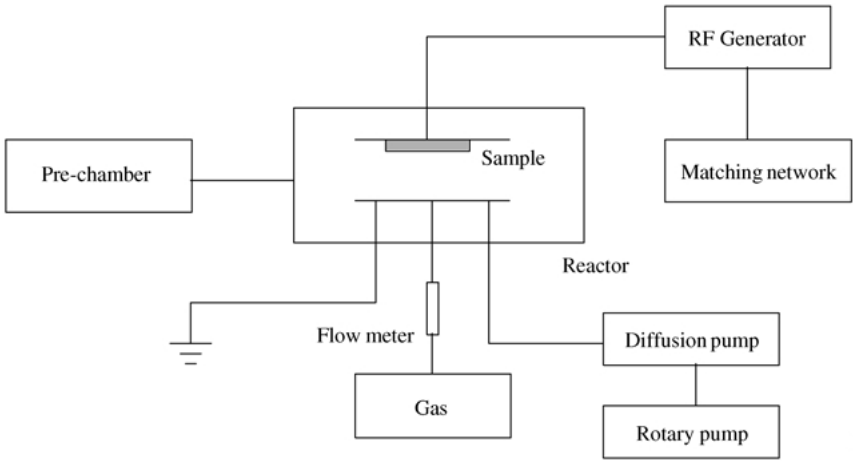

Figure 2 Scheme of the plasma reactor.

2.4. Contact angle measurements

The contact angle measurements were obtained by the sessile drop method using a G10 model with an image processor G1041 model from Kruss by depositing a drop of distilled water and methylene iodide on the surfaces to study. The aim was to evaluate the polar and dispersive components of the surface energy. The quantity of liquid was measured with a syringe monitored by a micrometric screw. The results correspond to the average value of at least ten measurements.

2.5. Mechanical properties

Tensile tests were performed on an Instron 4505 machine, fitted with resistive extensometer (gauge length 10 mm) in a controlled environment (23 °C, 55% RH). The crosshead velocity was 5 mm/min 8.3×10^{-5} m/s until 1% strain (to determine the secant modulus with higher precision) and then increased to 50 mm/min (8.3×10^{-4} m/s) until fracture. Samples were stored under controlled temperature and moisture conditions until testing, and at least six specimens were used.

2.6. Swelling and degradation behaviors

The water up-take and the degradation of the surface modified materials (tensile specimens) were studied over a period of 30 days by conditioning samples to minimum weight at 37 °C in an oven with desiccant prior to being immersed in 100 mL of a simulated physiological isotonic solution (0.154 M NaCl aqueous solution at pH = 7.4). Specimens were removed at regular intervals of 3, 7, 14, and 30 days, being taken out of the solution, blotted on filter paper to remove surface solution and further rinsed with distilled water and weight. After being removed from the solution, the specimens were dried in an oven at 60 °C to constant weight in order to determine the eventual weight loss. Equilibrium hydration degree was considered when no weight change ($\pm$ 0.001 g) was observed after 2–3 days of immersion.

2.7. Bioactivity tests

Surface modified tensile specimens were immersed in a simulated body fluid solution (SBF) for 3, 7, 15, and 30 days, maintained at 37 °C and renewing the SBF solution every 2 days. The SBF solution has been proposed by Kokubo *et al*. [16, 17] and has been widely used since in this type of tests. It has an ionic composition that mimics that of human blood plasma.

The surface of the samples was analyzed by scanning electron microscopy (SEM), in a Leica Cambridge S 360 microscope, after different SBF immersion periods. This observation was utilized for morphological characterisation of eventually deposited calcium-phosphate (Ca-P) layers. Energy dispersive spectroscopy (EDS) was used for elemental analysis of the eventually formed Ca-P bioactive layers.

2.8. Cell adhesion and proliferation tests

Surface modified samples grafted with acrylic polymers were sterilized with EtO and placed in distilled water to swell at 37 °C for 24 h. Then seventh passage goat bone marrow cells were seeded (at a constant initial concentration of 5000 cells/cm^2 and cultured on the surface of the different samples (controls and surface modified samples) for 7 days. For subsequent qualitative analysis cells present on the surface of the different samples were stained with methylene blue.

3. Results and discussion

The hypothesis being tested in this study is that the grafting of acrylic polar polymers with ionisable and hydrophilic groups will lead to significant changes in the surface chemistry of starch-based biomaterials. The changes might have two distinct positive effects. They may confer to the starch-based blends a bioactive behavior, or they will enhance cell adhesion and proliferation leading to an improvement of the materials adequability to be used on applications such as tissue engineering scaffolding. This type of surface modifications will influence properties such as hydration degree, degradation behavior, and mechanical performance. In this work the proposed modifications were also compared in terms of the activation method, plasma or chemical treatment, that initiate the grafting polymerization generating free radicals on the surface by peroxide formation and decomposition, as it has been previously described [3, 14, 18].

3.1. FTIR analysis

The grafting of acrylic polar polymers onto the starch-based blends was characterized by FTIR-ATR spectroscopy by analyzing the carbonyl region and the charac-teristic signal at about 1740 cm^{-1} corresponding to the stretching vibration of the carbonyl group the acrylic polymers, which was observed in all modified surfaces independently of the activation method used to initiate the graft polymerization. Fig. 3 shows the carbonyl region of SEVA-C, SEVA-C-g-pAA, and SEVA-C-g-pHMA activated chemically, being possible to observe the assignment of these signals on the spectra of grafted starch blends. Grafting can be intuitively performed on the starch hydroxyl groups activated by the chemical or plasma treatments as it has been described in the literature when grafting acrylic polymers onto starch [19, 20].

3.2. XPS analysis

XPS analysis was carried out to monitor the chemical composition of the grafted surfaces and compare them to non-treated samples. N1s were only present on the modified surfaces grafted with pHMA as in the case of P2p for those grafted with pEMP. Fig. 4(a) and (b) show the C1s peak fit of SEVA-C and SCA blends grafted with all the polymers by plasma induced polymerization. It can be observed that the introduction of new –CH and CO groups increases the atom percentage of the original chemical state of the surfaces (at 285 eV) which is also revealed by the binding energy shoulders of the main peak at 285.7 eV in the case of grafted pAA, assigned to carboxylic –C–COOH, at about 286.5 eV assigned to

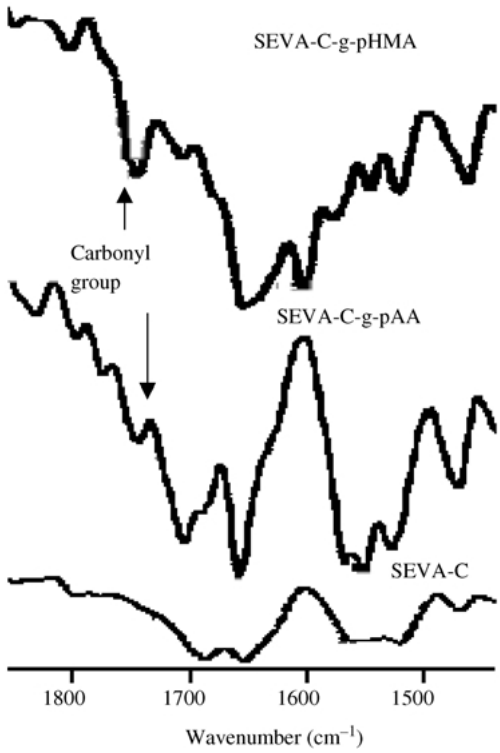

Figure 3 FTIR spectra of the carbonyl region of SEVA-C, SEVA-C-g-pAA, and SEVA-C-g-pHMA chemically activated.

hydroxylic –C–OH, and at 287.7 eV assigned to carbonyl –C=O, in all cases [21]. It was also observed that peaks were more intense when the chemical activation method was performed in comparison to the plasma induced process.

3.3. Contact angle measurements

Contact angle measurements were performed in the modified surfaces using water and methylene iodide, and the total surface energy, as well as the corresponding polar and dispersive components were calculated according to a method proposed by Owens and Went [22], where the surface tension (γ_s) of each phase can be resolved into a polar (γ_s^p) and a dispersive part (γ_s^d). Tables I and II show the values obtained for both plasma and chemical activation in the grafting of AA, EMP, and HMA polymers. It can be observed that there are great differences depending on the activation method used to create free radicals on the starch blend surfaces. In the case of SEVA-C when chemical activation is used, the total surface energy increases when grafting AA and HMA increasing also the polar component of the surface energy as a consequence of the incorporation of carboxylic (AA) and hydroxyl and amide groups (HMA). In the case EMP grafted both total and polar surface energy decreases as EMP has a long aliphatic side chain with a more hydrophobic character than AA and HMA. But in the case of plasma activation there is, in all cases, an increase of the total surface energy as well as in the influence of the polar component, being the dispersive component of the same order of magnitude, as in the case of chemical activation. It can be also observed that the surface energy values obtained by plasma activation are very similar both in SEVA-C and SCA when the same acrylic polymers are grafted, whereas in the case of chemical activation there is a greater dispersion of the results. This can be attributed to a more homogeneous grafting of the blend surfaces when plasma activation is used, as compared to the chemical activation.

3.4. Mechanical properties

The tensile mechanical properties of the starch-based blends SEVA-C and SCA grafted with the corresponding acrylic polymers by the plasma and chemical activation

TABLE I Contact angle values at room temperature in air and surface energy γ_s, with its dispersive (d) and polar (p) contributions of the starch blends grafted with acrylic polymers activated chemically

Material	H_2O C. Angle(°)	CH_2I_2 C. Angle (°)	γ_s (mN/m)	γ_s^d (mN/m)	γ_s^p (mN/m)
SEVA-C	**65.1 (1.3)**	**52.0 (1.1)**	**45.1**	**32.3**	**12.8**
SCA	49.2 (0.9)	44.4 (1.7)	58.3	37.9	20.4
SEVA-C-g-pAA	**58.4 (3.4)**	**43.9 (3.4)**	**51.7**	**37.5**	**14.2**
SCA-g-pAA	39.7 (3.2)	49.2 (2.0)	61.4	34.7	26.7
SEVA-C-g-pEMP	**68.8 (3.7)**	**54.5 (2.4)**	**42.3**	**31.7**	**10.6**
SCA-g-pEMP	40.8 (3.8)	51.8 (2.3)	60.1	33.3	26.9
SEVA-C-g-pHMA	**53.0 (3.5)**	**47.4 (3.4)**	**53.8**	**35.7**	**18.1**
SCA-g-pHMA	56.8 (3.5)	46.8 (3.3)	51.7	36.0	15.7

TABLE II Contact angle values at room temperature in air and surface energy γ_s, with its dispersive (d) and polar (p) contributions of the starch blends grafted with acrylic polymers activated by plasma

Material	H_2O C. Angle(°)	CH_2I_2 C. Angle (°)	γ_s (mN/m)	γ_s^d (mN/m)	γ_s^p (mN/m)
SEVA-C	**65.1 (1.3)**	**52.0 (1.1)**	**45.1**	**32.3**	**12.8**
SCA	49.3 (0.9)	44.1 (1.7)	58.3	37.9	20.4
SEVA-C-g-pAA	**47.9 (1.8)**	**37.0 (1.3)**	**54.0**	**28.7**	**25.3**
SCA-g-pAA	47.5 (1.8)	37.6 (2.5)	54.1	28.3	28.8
SEVA-C-g-pEMP	**48.6 (1.7)**	**33.9 (1.8)**	**54.2**	**30.2**	**24.0**
SCA-g-pEMP	50.2 (0.7)	32.8 (1.6)	53.5	31.1	22.5
SEVA-C-g-pHMA	**48.7 (0.4)**	**32.6 (0.8)**	**54.4**	**30.8**	**23.6**
SCA-g-pHMA	45.9 (2.2)	30.0 (0.4)	56.4	31.4	25.0

TABLE III Tensile mechanical properties of starch blends grafted with acrylic polymers by chemical and plasma activation treatments.

Sample	Treatment	SEV AC-g-pAA	SCA-g-pAA	SEV AC-g-pEMP	SCA-g-pEMP	SEVAC-g-pHMA	SC A-g-pHMA
UTS (MPa)	Chemical PLASMA	34.0 ± 1.5	20.3 ± 0.7	32.7 ± 1.8	47.5 ± 1.2	30.2 ± 1.2	24.2 ± 0.3
		39.2 ± 3.3	28.8 ± 0.4	34.9 ± 2.8	28.8 ± 0.5	39.2 ± 0.9	28.2 ± 0.8
$E_{1\%}$ (GPa)	Chemical PLASMA	1.2 ± 0.2	0.9 ± 0.2	1.4 ± 0.4	1.7 ± 0.1	1.7 ± 0.2	1.0 ± 0.1
		2.0 ± 0.1	2.2 ± 0.3	2.3 ± 0.2	2.2 ± 0.1	2.2 ± 0.1	2.3 ± 0.2

UTS, ultimate tensile strength. E, secant modulus at 1% strain.

methods were evaluated and compare to the original materials. Table III shows the ultimate tensile strength (UTS) and secant modulus ($E_{1\%}$) values of all surface modifications performed. Taking into consideration that SEVA-C has a UTS (MPa) $= 27.5 \pm 0.2$ and $E_{1\%}$ (GPa) $= 2.2 \pm 0.5$ and SCA has a UTS (MPa) $= 28.8 \pm 0.7$ and $E_{1\%}$ (GPa) $= 2.2 \pm 0.3$, it can be observed that in the case of SCA modified with plasma treatment activation both values UTS and $E_{1\%}$ remain constant with respect to the original blend, whereas in the case of chemical surface activation UTS is lower when grafting pAA and pHMA and higher in the case of pEMP, being $E_{1\%}$ lower in all grafting cases. On the other hand, SEVA-C grafted samples exhibit an increase in UTS for both activation treatments, but $E_{1\%}$ values become lower for the chemical activation samples and remain constant in the case of plasma treated samples. The dispersion of the results obtained by the chemical activation surface modifications can be attributed to a grafting not only on the surface, but also in some extent of the bulk of the material, whereas in the case of plasma induced polymerization the results indicate that the polymer grafting is more homogeneous in terms of surface modification as the mechanical properties remain similar to the original material. Variations on the mechanical properties may be due to some degradation, cross-linking effect or changes on the equilibrium moisture content of the samples. The effects of the different influences are being studied on ongoing works.

3.5. Swelling and degradation behaviors

Both swelling and degradation behaviors were studied to determine the effect of grafting acrylic hydrophilic polymers on the biodegradable starch-based materials in terms of equilibrium hydration degree and weight loss percentages. As it could be expected, the equilibrium hydration degree was increased in most of the cases. This can be observed in Figs. 5(a) and 6(a) where are shown the swelling isotherms of SEVA-C grafted with the acrylic polymers by plasma induced polymerization, and the equilibrium hydration degree of SCA with polymers grafted by both treatments. Taking into consideration the kind of grafted polymers the highest hydration degree was observed for pAA (about 60%) in the case of SEVA-C and for pHMA (about 60%) in SCA. When comparing the type of grafting activation method (see Fig. 6(a)) it can be observed that the equilibrium hydration degree is higher when the grafting polymerization was activated chemically (5–10% higher). This is in agreement with the grafting of polymers in some extent of the bulk when polymerization was chemically activated.

In terms of degradation behavior (see Figs. 5(b) and 6(b)) it was observed that in most of the cases the weight

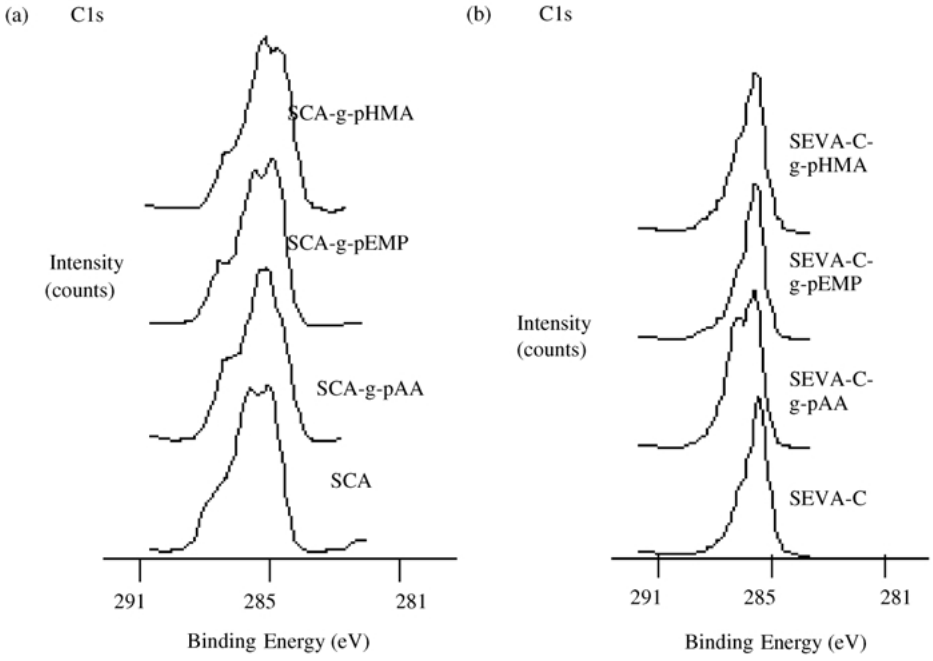

Figure 4 C1s peak fit of (a) SCA and SCA grafted with pAA, pEMP, and pHMA (plasma activated) and (b) SEVA-C grafted with pAA, pEMP, and pHMA (plasma activated).

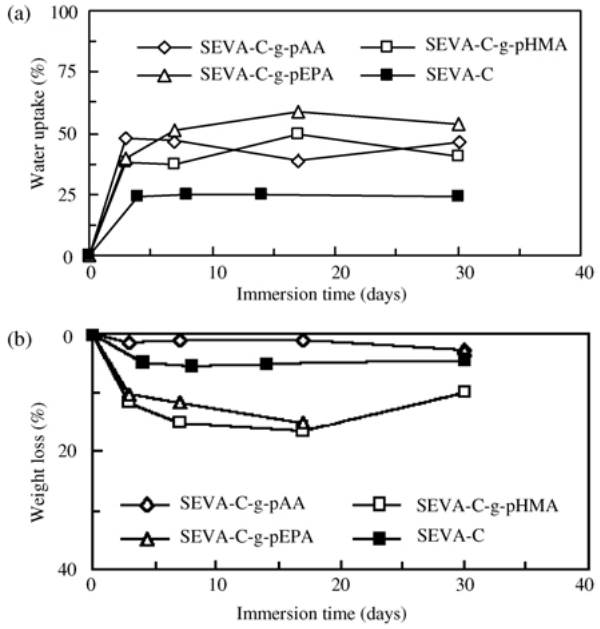

Figure 5 (a) Swelling isotherms in isotonic saline solution at 37 °C of SEVA-C, SEVA-C-g-pAA, SEVA-C-g-pEMP, and SEVA-C-g-pHMA (plasma activated). (b) Weight loss of SEVA-C, SEVA-C-g-pAA, SEVA-C-g-pEMP, and SEVA-C-g-pHMA (plasma activated) as a function of immersion time in isotonic saline solution at 37 °C.

loss percentage is higher than that of non-treated samples as the hydration degree was increased, with a remarkable exception when pAA was grafted in SEVA-C showing a lower weight loss with respect to the original samples. This effect can be explained in terms of the cross-linked nature of the grafted pAA as samples were found to be insoluble in organic solvents as DMSO whereas non-treated SEVA-C samples and SCA-g-pAA are completely soluble. The weight loss percentage was also found to be higher in the samples chemically activated to graft polymerization, as their hydration degree was also higher.

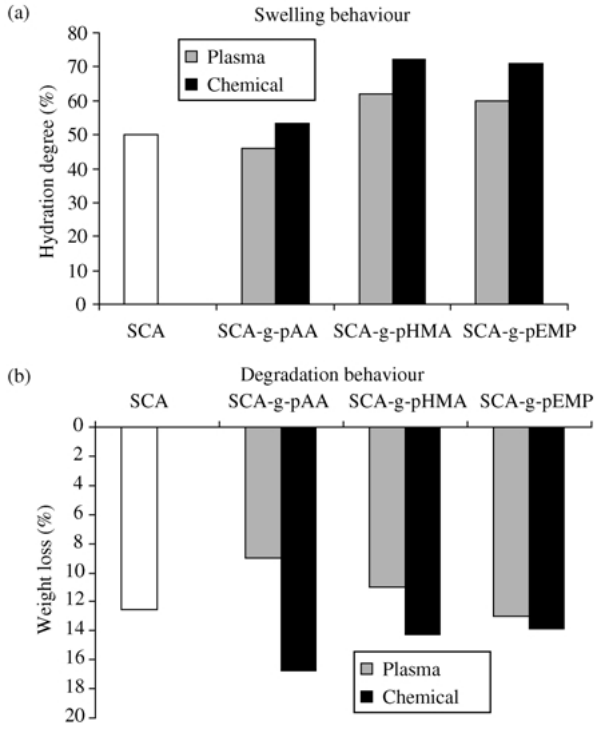

Figure 6 (a) Hydration equilibrium degree of SCA, SCA-g-pAA, SCA-g-pEMP, and SCA-g-pHMA (chemically activated) in isotonic saline solution at 37 °C. (b) Weight loss percentage of SCA, SCA-g-pAA, SCA-g-pEMP, and SCA-g-pHMA (chemically activated) in isotonic saline solution at 37 °C after one month of immersion.

3.6. Bioactivity tests

The influence of the surface modification over the induction and growing of a calcium-phosphate layer was also studied. Both SEVA-C and SCA are not intrinsically bioactive and are not able by themselves to nucleate a Ca-P layer on their surfaces if not modified. The aim of the herein reported tests was to establish whether or not after surface modification some samples would exhibit a bioactive character. In fact, the eventual formation of a Ca-P layer will indicate that the material may present a bone bonding behavior *in vivo*. Fig. 7(A) and (B) show the SEM micrographs of plasma activated SCA-g-pHMA and SEVA-C-g-pHMA, respectively, where it can be observed the formation of a calcium phosphate layer formed at different nucleation fronts. This type of nucleation was observed in all modified starch blends with the grafted acrylic polymers and confirmed by EDS. Fig. 8(a) and (b) exhibit the EDS spectra of chemically activated SCA-g-pAA and SCA-g-pEMP where can be observed typical peaks associated to the formation of a Ca-P layer.

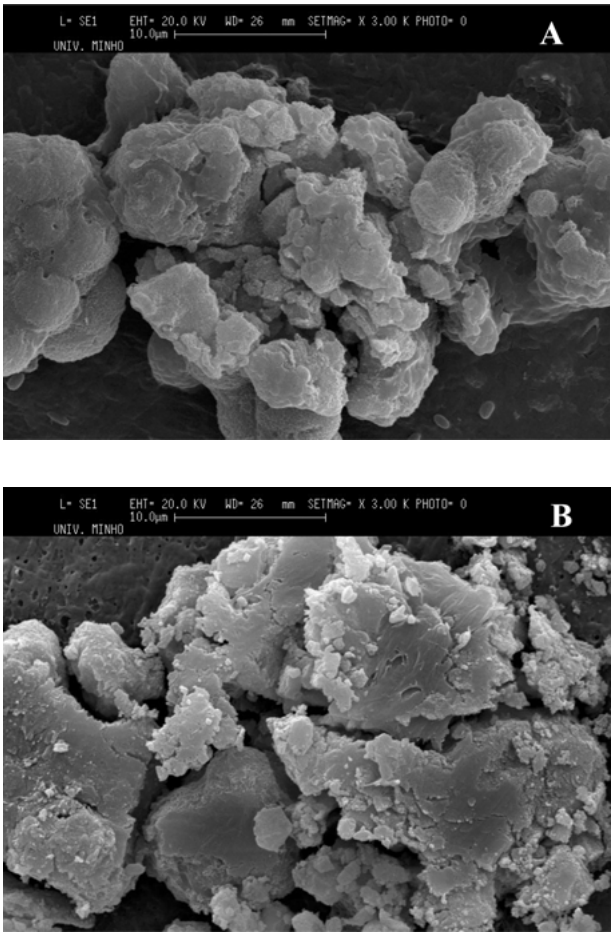

Figure 7 SEM micrographs of plasma activated (A) SCA-g-pAA and (B) SEVA-C-g-pHMA immersed in the SBF solution for 15 days.

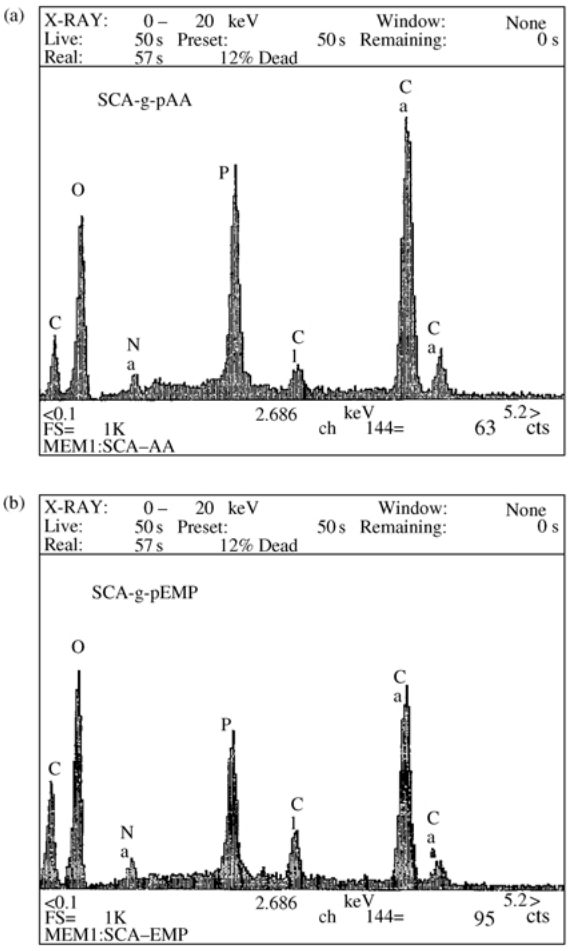

Figure 8 EDS spectra of chemically activated (a) SCA-g-pAA and (b) SCA-g-pEMP showing the formation of Ca-P layers.

3.7. Cell adhesion and proliferation tests

Preliminary *in vitro* cell adhesion and proliferation tests were performed in some of the starch-based biomaterials grafted with the polar acrylic polymers in order to evaluate the possibilities of applying these surface modified biomaterials in bone tissue scaffolding. All studies were still performed using compact samples in order to allow for an easier evaluation of the obtained results. These polymers have proved before that they are not bad for cell adhesion and proliferation, especially when compared with other biodegradable polymers, but it is believed that this performance can be strongly enhanced by developing adequate surface modification routes. Fig. 9(a)–(c) show the optical photographs of culture of goat bone marrow cells on tissue plates (control) after 5 days of culture as well as on original SEVA-C and on chemically activated SEVA-C-g-pAA after one week of culture, respectively. In the last two cases the cells were stained with methylene blue. It can be observed that a confluent monolayer can be observed on the tissue culture plates (control). However, in the case of untreated SEVA-C only some isolated cells (or clusters of cells) can be observed on the surface. The most remarkable results were detected when pAA was grafted (chemically or by plasma activation) on the starch blends, as goat bone marrow cells showed a very good (and strongly enhanced) adhesion and proliferation, growing into an almost confluent mono-layer, which is the most desirable proliferation in scaffolds used for bone tissue engineering.

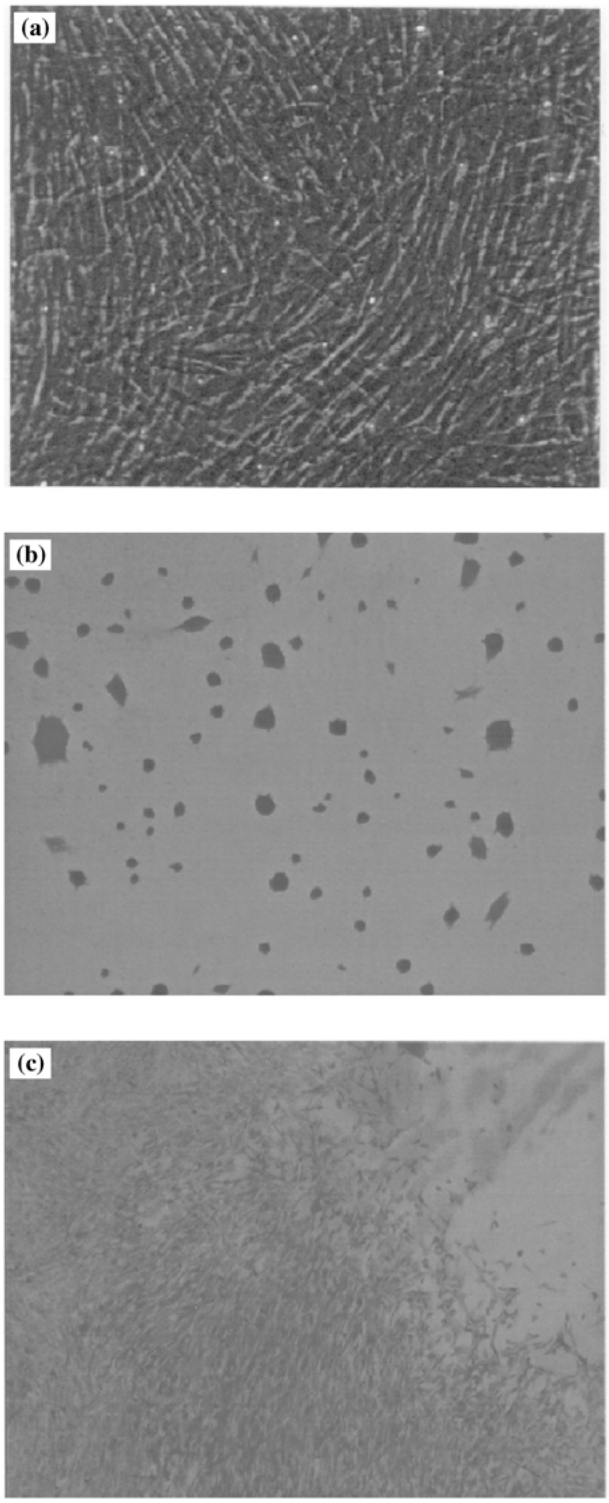

Figure 9 Optical photographs of (a) control tissue culture plates, (b) cultured bone marrow cells on untreated SEVA-C, and (c) cultured bone marrow cells on SEVA-C-g-pAA chemically activated. Cells on (b) and (c) were stained with methylene blue.

4. Conclusions

Acrylic polar polymers have been grafted on the starch blends by plasma induced and chemical induced radical polymerization in order to modify the surface properties of these materials to improve cell adhesion and proliferation and bioactivity in order to make them

even better candidates for bone tissue engineering scaffolding applications. The grafting was characterized by FTIR and XPS spectroscopic techniques and contact angle measurements. The studied properties, mechanical, swelling, and degradation, indicate that the plasma induced graft polymerization was more homogeneous in terms of surface modification than the chemical induced treatment that also modified to some extent the bulk of the materials. All surface modifications lead to the formation of Ca-P layers when incubated in SBF solution as it was determined by SEM and EDS. This seems to indicate that the grafting of these groups confers a bioactive behavior to the starch-based polymers. Finally cell adhesion and proliferation tests with goat bone marrow cells exhibited a remarkable improvement with respect to the original starch blends when pAA was grafted, as cells grow into an almost confluent monolayer at a much faster rate over the modified surfaces. These modifications may be very useful to tailor the cell adhesion and proliferation on starch-based tissue engineering scaffolds.

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