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Polymeric active coatings with functionality in vascular applications

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Copolymers containing functional groups with activity as antiaggregating agents for platelets, based on random chains of metacryloyloxyethyl [2-(acetyloxy)-4-(trifluoromethyl)]benzoate, TH, and 2-acrylamido-2-metylpropanesulfonic acid, AMPS, with AMPS molar fractions ranging from 0.1 to 0.4, have been prepared. The spectroscopical characterization and the *in vitro* swelling behavior have been studied, as well as the surface free energy, showing the copolymers an appropriate surface properties from a haemocompatible point of view. Preliminary *in vitro* tests using human blood have shown a promising antiaggregating behavior.

Introduction

Thrombosis is one of the major problems associated with the use of biomaterials in blood transport applications [1]. Development of improved blood contacting devices requires design of non-thrombogenic materials with high reliability [2]. In this sense, several approaches have been proposed to improve the long-term efficacy of vascular substitutes, specially in small-caliber arterial or venous systems. An important strategy involves the modification of blood contact surfaces to obviate the thrombogenicity of synthetic grafts by chemical modification, coating with polymers bearing specific functional groups, or by adsorbing biologically active or biomimetic molecules [3].

One of the most relevant approaches considers the immobilization of sulfonic groups in the surface of blood-contact devices as they are expected to repel protein adsorption and cell adhesion as well as suppress the procoagulant factors, possibly by electrostatic repulsion because of the heparin-like anticoagulant activity of the surface bound sulfonate groups [4–9]. Heparin itself has potentially severe effects such as bleeding and heparin-induced thrombocytopenia [10], and therefore, many efforts are devoted to develop new polymeric coatings that simulates heparin activity and avoids all these side effects. Fig. 1 shows the chemical structure of heparin and that of the copolymer chains presented in this article.

On the other hand, several agents that are known to inhibit thrombosis or promote anticoagulation, such as heparin, prostaglandin E_1, hirudin, or aspirin, have been bound to the lumen of synthetic vessels. It has been demonstrated by our group that the application of coatings of polymeric derivatives of salicylic acid and Triflusal (Disgren®, [2-(acetyloxy)-4-(trifluoromethyl)]-benzoic acid) on the inner surface of small diameter vascular grafts, improve the prevention of adhesion and aggregation of platelets on the surface [11–13]. In these systems, the active agent is covalently attached to the backbone of a bioresorbable polymer by direct polymerization of the functionalized drug (2-methacryloyloxybenzoic acid or methacryloyloxyethyl [2-(acetyloxy)-4-(trifluoromethyl)] benzoate with other biocompatible monomers. Modification or attachment of drugs to synthetic polymers can provide materials not only with intrinsic pharmacological activity, but also with behavior as drug delivery systems, able in this case to maintain a controled drug plasma level for long periods without reaching a toxic level or dropping below the effective level.

The aim of this work is to conjugate both strategies, that is, to prepare a system that simultaneously bears a heparine-like sulfonic derivative and an antiaggregant drug as Triflusal. We propose the use of random copolymers of poly(metacryloyloxyethyl [2-(acetyloxy)-4-(trifluoromethyl)]benzoate-*co*-2-acrylamido-2-metylpropane sulfonic acid), poly(TH-*co*-AMPS), at selected monomer ratios, to study their physico-chemical properties, as well as the platelet aggregation in static conditions using human plasma as experimental model.

Materials and methods

Reagents

HEMA: 2-hydroxyethyl methacrylate (Fluka) was exhaustively purified as described in the literature [14];

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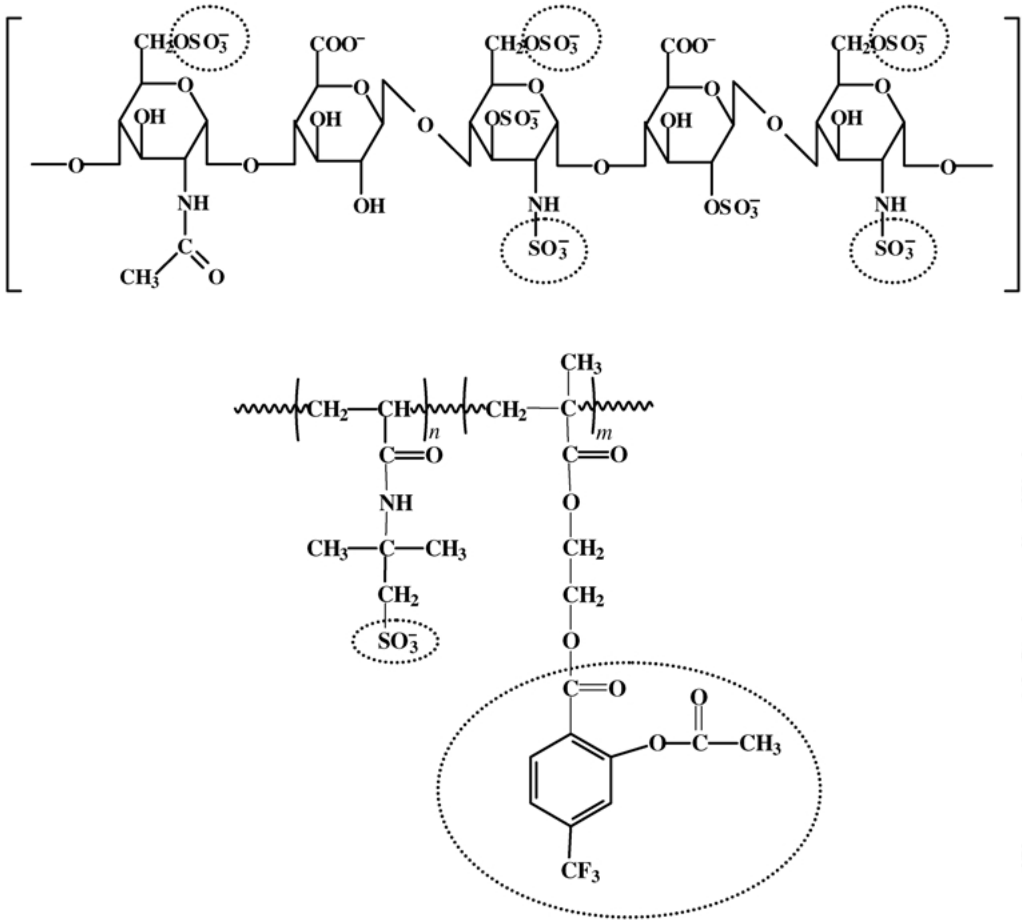

Figure 1 Schematic chemical structure of heparin and the copolymer system components (TH : AMPS).

AMPS: 2-acrylamido-2-methylpropanesulfonic acid (Avocado), TRIFLUSAL (TRF): 2-(acetyloxy)-4-(trifluoromethyl)]benzoate (Uriach), Dioxane (Panreac) and Diethyl ether (SDS) were used without further purification. AIBN: azo*bis*(isobutyronitrile) (Merck) was recrystalized twice from ethanol. DMSO-d_6 (Merck). TH: metacryloyloxyethyl [2-(acetyloxy)-4-(trifluoromethyl)]benzoate was synthesized in our laboratory as described elsewhere [11].

Copolymer synthesis

Polimerization was carried out by free radical polymerization in water–dioxane (10 : 90) as solvent. Both monomers were mixed in suitable proportions in order to obtain a final concentration of 0.3 M. After deoxygenation with gaseous N_2 for 30 min, the reaction was induced thermally at 50 °C by using AIBN (1.5×10^{-2} M) as free-radical initiator. After 24 h (total conversion) the solvent was removed by evaporation and the isolated solid redissolved in chloroform and precipitated in diethyl ether. The copolymers were filtered and dried under reduce vacuum to a constant weight. The synthesized products were labeled as THA90, THA80, THA70 and THA60 depending on the feed molar composition of TH (see Table I).

Polymer characterization

^{1}H-NMR experiments were carried out in a Varian 300 XL, working at 300 MHz. The IR spectra of the materials were recorded on a Perkin-Elmer 1720 FTIR spectrophotometer using a KBr pellet technique. The IR spectra showed that the copolymer chains are built from AMPS units (υ_{NH} at 3318, $\upsilon_{C=O}$ (amid I) at 1687, υ_{CN} and δ_{CNH} (amid II) 1633 , δ_{NH} at 1544, δ_{CH_3} at 1455 cm^{-1}, υ_{SO_2} at 1214, υ_{SO_3H} at 1041, υ_{S-O} at 624) and TH units ($\upsilon_{C=O}$ at 1777 and 1731, comb$_{ar}$ 1700–1550 (1630 and 1584),

TABLE I Feed TH weight (W) and molar fractions (f), contact angles (θ) and critical surface tensions of the copolymers (σ_c)

	Composition		Contact angle			σ_c (mN/m)	SES (mN/m)		
	W_{TH}	f_{TH}	θ_{water}	$\theta_{glycerine}$	$\theta_{diiodomethane}$		γ_s^p	γ_s^d	γ_s
Poly(AMPS)	0	0	0.00 ± 1.74	44.39 ± 3.31	46.67 ± 1.83	194.05	36.2	28.4	64.6
THA60	0.72	0.60	68.85 ± 4.25	73.78 ± 3.91	54.48 ± 2.26	33.17	11.7	26.6	38.3
THA70	0.80	0.70	74.77 ± 5.31	73.20 ± 8.13	54.82 ± 2.17	32.11	7.4	29.6	36.9
THA80	0.87	0.80	77.29 ± 2.86	70.34 ± 4.50	54.96 ± 3.72	28.23	6.9	28.0	34.9
THA90	0.94	0.90	73.08 ± 5.24	70.48 ± 1.97	55.63 ± 5.30	26.60	9.4	25.1	34.5
Poly(THEMA)	1	1	81.18 ± 2.36	72.06 ± 4.95	54.39 ± 2.76	31.54	4.8	30.6	35.4

$\delta_{CH_3\ (methylcetones)}$ at 1418, $\upsilon_{C-O\ (acetates)}$ 1257, υ_{CF_3} and $\delta_{oop\ arC-H}$ 785, 750, and 704 cm^{-1}).

Preparation of polymer films

Clear transparent films (0.4–0.5 mm thick) were prepared by the casting method. A 30 wt % dissolution of the appropriate poly(TH-*co*-AMPS) in THF for THA80 and THA90 or THF : ethanol (80 : 20) for THA60 and THA70 was spread on a cylindrical mold of Teflon (15 mm diameter and 5 mm deep) and dried at room temperature for 24 h and finally dried overnight under reduced pressure to a constant weight.

Swelling behavior

The swelling behavior of copolymers in buffered solutions at 7.4 (1.5 M ionic strength) at 37 °C, was monitored gravimetricaly. The water uptake, $W = (\text{WEIGHTwet} - \text{WEIGHTdry})/\text{WEIGHTdry}$, was calculated by measuring the weight gain of the sample at different times after carefully wiping the surface with a filter paper. The experiments were done by triplicate.

Contact angle

Contact angles were measured on polymer-coated glass slides. Polymer solutions (10 wt %) prepared in the same conditions as described for swelling experiments were used for casting. A few drops of polymer solution were allowed to sit on the glass slide for 24 h at room temperature and then dried under vacuum for at least 24 h. The contact angles were measured by using the sessile drop technique and employing liquids with known surface tension: water ($\gamma_L = 72.8$ mJ/m^2) and methylene iodide $\gamma_L = 51.8$ mJ/m^2). γ_L refers to the total surface free energy of the liquid. The surface free energy of homopolymers and copolymers was calculated by the Owens' method [15].

Coating of the prosthesis

Gore-Tex® vascular grafts were coated by addition of 100 µl of a 20 wt % solution of the four poly(TH-*co*-AMPS) copolymers in THF on their surface. The solvent was evaporated at room temperature for 24 h. This process was repeated twice to ensure that the prosthesis was covered homogeneously. The thickness and the weight gain of the coatings were determined measuring the weight difference between the coated specimen respect to the original uncoated sample. Homogeneous thin coatings (see Fig. 5) with a weight gain of 11.4 ± 0.5% were obtained.

Platelet adhesion

Coated and uncoated prosthesis were used in the *in vitro* experiments by using the basal contact method. All experiments were carried out in static conditions, by fixing the open prostheses to the bottom of a cylindrical container of 8 mm diameter and 10 mm depth. Platelet-rich plasma from arterial blood (PRP) was isolated by centrifugation of 40 ml of volunteer's human blood at 1500 r.p.m. during 4 min and collecting the supernatant. The centrifuged plasma-rich medium (100 µl) was added to the cylindrical containers and allowed to contact with the surface of the vascular graft during 1 h, 1 day, and 3 days. All experiments were carried out in a 5% CO_2 chamber at 37 °C.

After the treatment time, the prostheses were washed three times with minimal essential medium and fixed with 3% buffered glutaraldehyde solution (pH 7.4) during at least 1 h. The adhered platelets were visualized by using an environmental scanning electronic microscopy (ESEM- Philips XL-300).

Results and discussion

Four copolymers with an AMPS molar content of 0.10, 0.20, 0.30, and 0.40 (which is the range of water-insolubility and therefore the range of compositions below the application limit) have been synthesized. The presence of AMPS units contributes to the design of the new antiaggregating coatings not only by including the ionized sulfonic groups, biologically active as it has been commented in Introduction, to the structure (Fig. 1), but also by improving adhesion (these systems have actually shown a good stability as inner coatings of commercial vascular grafts) and increasing hydrophilia (wettability), being both properties critical in their biological performance. The hydrophilic/hydrophobic balance (and consequently the interfacial free energy in an aqueous solution) will strongly depend on the monomer composition since TH is relatively hydrophobic and AMPS is an anionic molecule.

Copolymerization was carried out until 100% conversion and the isolated copolymers characterized by FTIR and ^{1}H-NMR. Chemical structure and ^{1}H-NMR spectrum of THA70, taken as an example, are shown in Fig. 2. These analysis confirmed the incorporation of both comonomers to the polymer chains and the achieving of high conversion because of the agreement between feed and copolymer compositions.

The reactivity of both monomers during the copolymerization has been described previously [16], being the reactivity ratios $r_{TH} = 4.54$ and $r_{AMPS} = 0.32$. Basically, TH is much more reactive than AMPS and therefore, there is a tendency towards the formation of TH blocks at the beginning of the reaction (because is more reactive) and AMPS blocks at the end of the reaction, as it has been deeply described elsewhere [16]. These particular microstructural arrangements will influence the physical properties and can be biologically relevant.

The surface free energy of a material is a key parameter in the interaction with proteins and cells and therefore, in its haemocompatibility. The surface free energy components of the four copolymers have been determined from the contact angle data of three liquids by using the Owen's methodology [15]. Fig. 3 shows the variation of the dispersion and the polar components as well as the total surface tension (as calculated from the contact angle data quoted in Table I), as a function of the TH molar composition. As it can be seen in Fig. 3, the polar component decreases with the TH content, in agreement with the higher hydrophilicity of the ionic AMPS, while the dispersive one keeps almost constant.

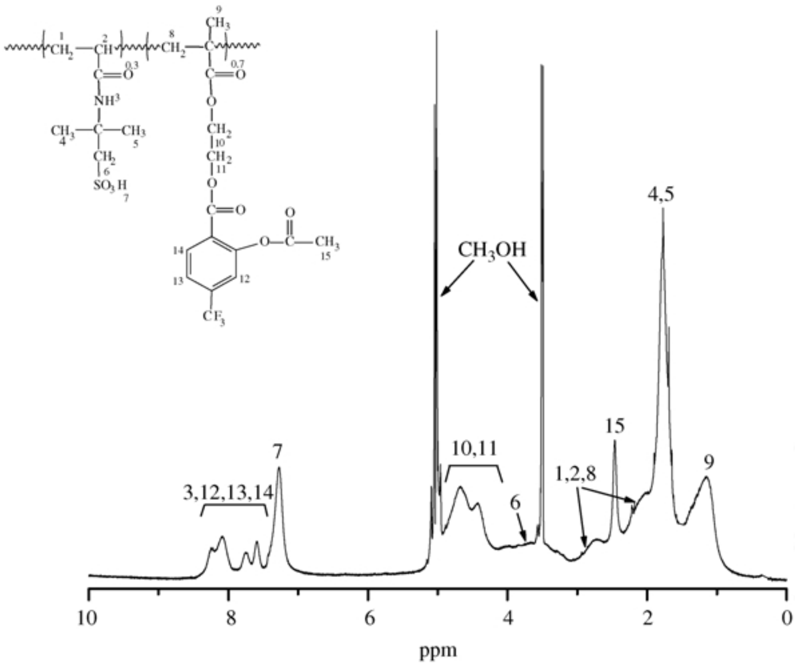

Figure 2 ^{1}H-NMR spectra (300 MHz) of copolymer sample of THA with a molar composition 70 : 30. Deuterated methanol was used as solvent.

The result is a decreasing of the surface free energy with the increasing TH content, that is, with the increasing content in the more hydrophobic comonomer. It has to be noted the deviation (toward lower values) of the experimental points from the additive molar rules for the polar component and the surface energy (Fig. 3). This behavior could be related with some particular molecular rearrangements, which could lead to TH rich surface domains. The tendency to the block formation during the polymerization could allow this rearrangements.

Critical surface tension (σ_c) is a valuable parameter used in the literature (among others) to estimate and predict haemocompatibility of a given material [17, 18]. This parameter has been calculated according to Zisman method [19] and is quoted in Table I for each material. The σ_c of the copolymers studied in this work ranges from 26.6 to 33.1 mN/m, which are in the interval of the Baier window of minimum bioadhesion and maximum haemocompatibility.

Besides, the copolymers swell to equilibrium in aqueous media in few hours reaching the water uptake values depicted in Fig. 4. The swelling degree increases obviously with the AMPS content (up to 200%) because of the higher hydrophilic character of this ionic component compared with the TH active unit. It is difficult to follow the swelling behavior or the water uptake for AMPS-rich copolymers because they become partially soluble and the gravimetrical assay gives higher dispersion in the measurements associated to the solubilization. This aspect has been taken into consideration in Fig. 4, with the error bars introduced by the samples with 60% and 70% of TH.

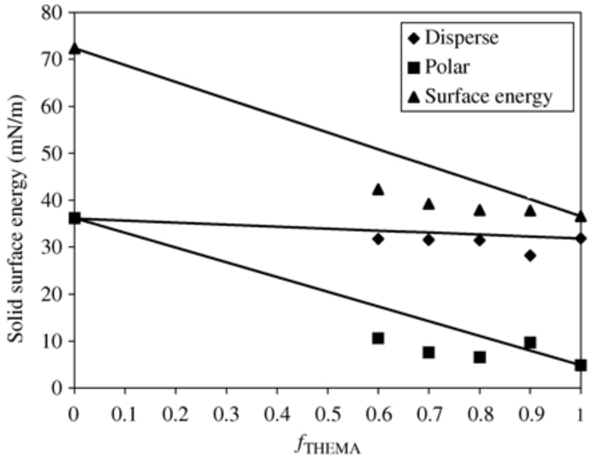

Figure 3 Solid surface energy of copolymer films prepared by casting from solutions of THA in a mixture of tetrahydrofuran-ethanol.

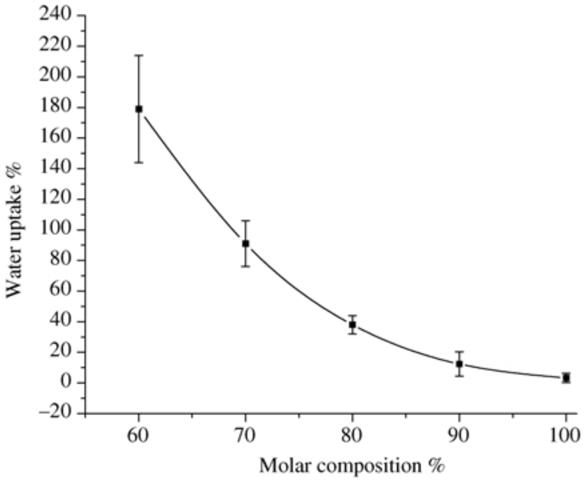

Figure 4 Swelling behavior of THA copolymers at pH 7.4 and 37 °C.

Platelet adhesion experiment

The extent of platelet adhesion and surface induced activation is considered to be an early indicator of the thrombogenic potential of blood-coating biomaterials [6]. Platelet adhesion and activation were determined

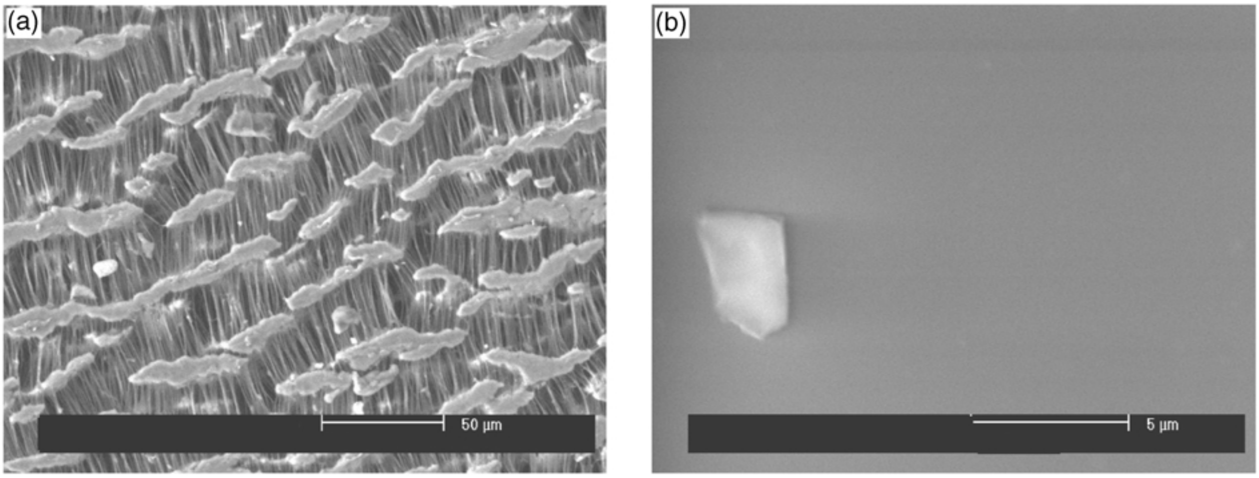

Figure 5 ESEM micrographs of Gore-Tex surface: (a) uncoated and (b) coated with THA80.

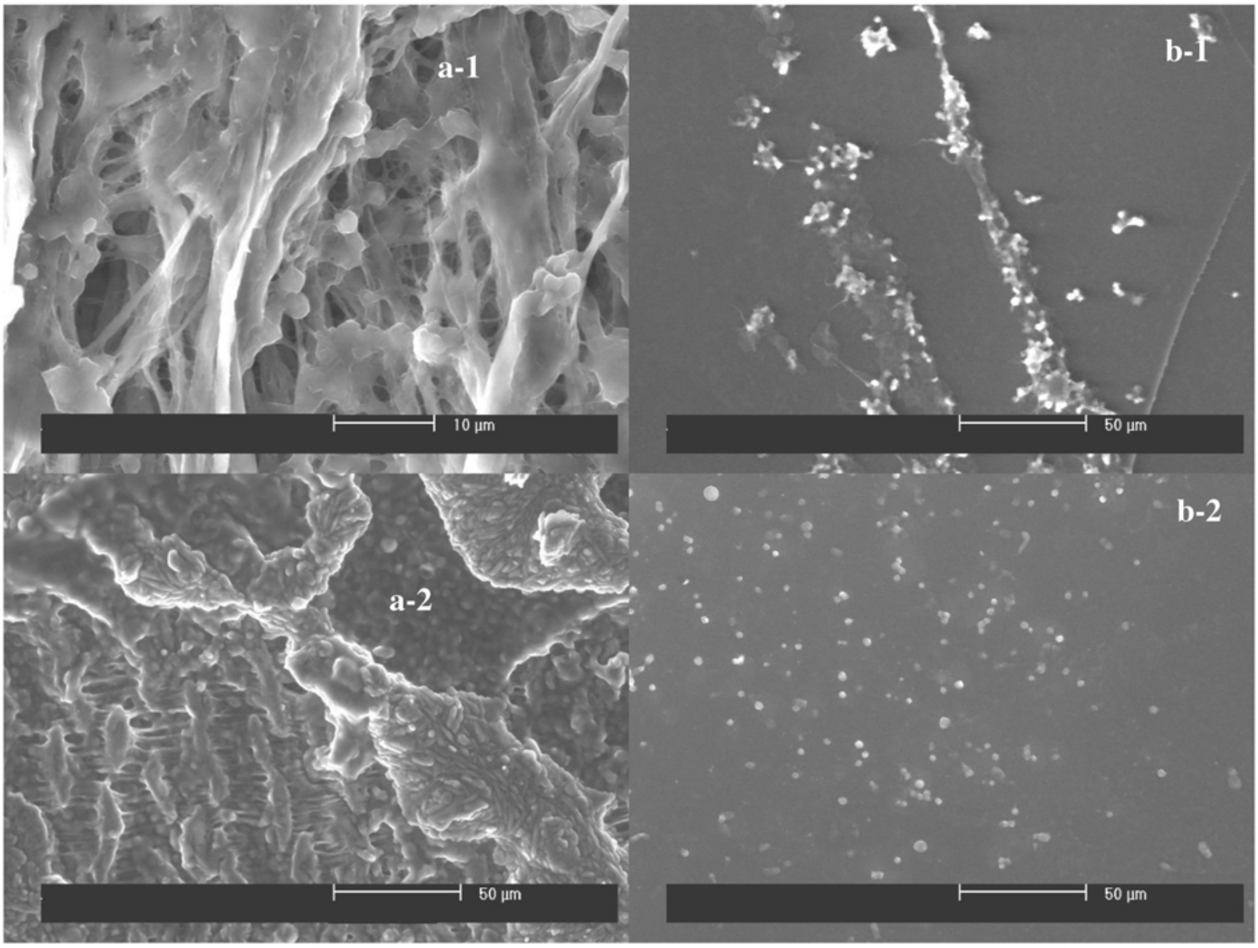

Figure 6 ESEM micrographs of platelets adhering: (a) uncoated surface of Gore-Tex; *a-1*: 60 min of treatment and *a-2*: 24 h of treatment and (b) coated with THA80, *b-1*: 60 min of treatment and coated with THA90, *b-2*: 24 h of treatment.

qualitatively from ESEM images. The spreading behavior of the adherent platelets is strongly related to their activation stage on the polymeric surfaces. Initially, they retain their discoid shape present in the resting state (spread area 10–15 μm^2). Upon activation, platelets extent their pseudopods and initiate the release of granular contents. This is considered the partial activation stage (spread area 35 μm^2). When platelets are fully activated they retract their pseudopods to form circular or ''pan-cake'' shape (spread area: 45–50 μm^2). Lin *et al*. [20] described five activation stages based on the spreading profiles of activated platelets.

Homogeneous thin coatings (see ESEM photographs, Fig. 5) with a weight gain of 11.4 ± 0.5% were obtained using the four active copolymers described in this work. The ESEM analysis confirmed that the presence of the sulfonic groups in the copolymer chains provides a higher adhesion to the Gore-Tex® surface and the coatings were stable for longer periods of time than the homopolymer Poly(TH). Besides, the aggregation of platelets in contact with the surface of the prosthesis can be noticeably reduced by the coatings. Fig. 6 shows some pictures of platelet retention on uncoated and coated surfaces of Gore-Tex® prosthesis from preliminary aggregation tests using volunteer's human plasma. The platelets are clearly less aggregated in the case of coated surfaces, and, moreover, the absence of pseudopods that indicates the activation of platelets is evident from the ESEM micrographs (Fig. 6).

As a conclusion, these preliminary studies have shown that the active polymeric coatings proposed in this work bearing Triflusal as well as sulfonic groups prevent adhesion and activation of platelets [6].

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