New resorbable polymeric systems with antithrombogenic activity

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The synthesis and application as resorbable coatings of vascular grafts of a new polyacrylic derivative of Triflusal (2-acetyloxy-4-trifluoromethyl)benzoic acid, a commercial drug with antithrombogenic properties, are described. The high-molecular-weight polyacrylic system is rather stable in physiological conditions and provides a chemical support for the slow release of the pharmacologically active compound, Triflusal, or its main metabolite (2-hydroxy-4-trifluoromethyl)benzoic acid (HTB). Experiments of deposition and retention of platelets in static basal conditions using plasma-rich medium from blood of sheep, seem to indicate that the polymeric coating of the polyacrylic derivative of Triflusal improves the antiaggregating character for platelets of the surface of small-diameter vascular grafts without the application of other antithrombogenic drugs.

1. Introduction

The biological response of the human body to the surface of foreign implants and devices in contact with blood is characterized by the activation of the coagulation cascade, the aggregation of platelets and the formation of thrombus [1, 2]. The most generalized method to prevent this phenomenon has been the administration of heparin solution as well as polymeric derivatives of heparin [3–5]. However, in addition to its potent anticoagulant activity, heparin presents side effects such as the promotion of platelet aggregation and blood lipid clearance [6], which has promoted the direction of research towards the preparation of biocompatible polymeric systems with antithrombogenic properties by the anchorage of residues with intrinsic antithrombogenic activity, like thrombine inhibitors [7–9] or compounds with antiaggregating effects for platelets [10, 11]. In this sense, the antiaggregating properties of aspirin and other derivatives of salicylic acid [12–14] are well known and we have demonstrated recently that the application of coatings of polymeric derivatives of salicylic acid on the inner surface of small diameter Dacron or Gore-Tex® vascular grafts, improved the prevention of adhesion and aggregation of platelets on the surface of the vascular graft under dynamic conditions [15, 16].

The derivatives of salicylic acid studied were constituted by high-molecular-weight polyacrylic chains ($M_n > 40\,000$ Da) bearing the salicylic residue as side substituents bound to the polymeric chain by weak carboxylic ester functional groups, which are easily hydrolyzed in the physiological medium. In this way, this kind of coating presents an intrinsic antiaggregating character, but also acts as controlled delivery system of salicylic acid. In addition, after the release of the active residue, the main polymeric chain becomes totally soluble in the physiological fluids, since they are constituted by the sodium salt of polymethacrylic acid. The hydrolytical process does not produce the biodegradation of the polyacrylic chains, but changes the solubility of the support which is cleared readily from the body by the classic metabolic pathway.

Triflusal, 2-acetoxy-4-trifluoromethyl benzoic acid, is a commercial platelet inhibitor with a chemical structure closely related to aspirin and a characteristic pharmacological profile [17, 18]. In view of the previous results obtained with polymeric derivatives of salicylic acid, we considered the interest of the preparation and application of polyacrylic derivatives of Triflusal, which in addition to the structure of salicylic acid, has acetyl groups, which are considered to be related with the irreversible deactivation of platelets in the aggregation process and

therefore, in the inhibition of celular thrombus [1, 17, 18].

The present work describes the preparation of polyacrylic derivatives of Triflusal with the appropiate molecular weight, as well as the preliminary results of the application of coating of the polymeric material on the surface of commercial prostheses of polytetra fluoroethylene (PTFE) or Gore-Tex, in static experiments of aggregation of platelets using sheep blood as experimental model.

2. Materials and methods

2.1. Reagents

Trifusal (TRF) was kindly supplied by Laboratorios Uriach. HEMA (purchased from Fluka) was purified according to the literature [19]. Other reagents were of extra-pure grade and used as purchased.

2.2. Monomer synthesis

The acrylic derivative of TRF, methacryloyloxyethyl [2-(acetyloxy)-4-(trifluoromethyl)] benzoate (HTRF) was prepared by a two-step route involving well-known organic reactions, according to Fig. 1. The first step was the preparation of the acid chloride derivative of TRF (CITRF) by reaction with thionyl chloride. In a typical experiment 0.1 mol of TRF was added to 70 ml of thionyl chloride and the mixture was stirred under reflux for 4 h. The thionyl chloride excess was removed by distillation. The acid chloride derivative was isolated by distillation under high vacuum. The yield was 64%. The second step was an esterification reaction of HEMA with the acid chloride derivative. HEMA (0.025 mol) and triethylamine were dissolved in diethyl ether. To this solution the acid chloride derivative dissolved in diethyl ether (0.025 mol) was added dropwise. The reactions, under nitrogen flux and at room temperature, were kept for 24 h under magnetic stirring. The precipitated triethylamine chlorhydrate was removed by filtration. The filtrate was washed with an aqueous solution and the residual organic phase was dried over $MgSO_4$. The solvent was removed under vacuum until constant weight was reached. The yield was 52%.

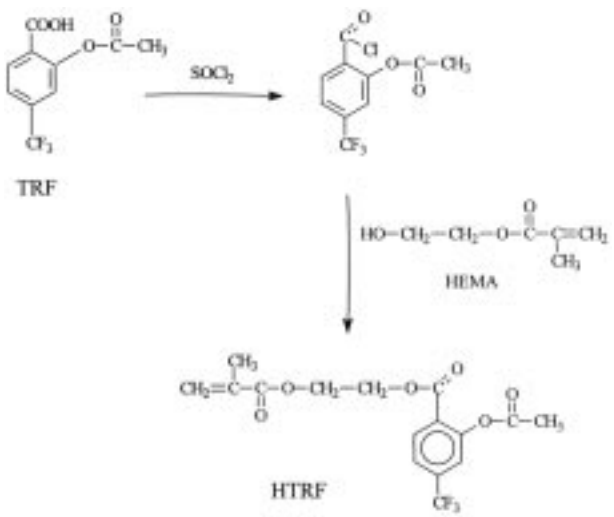


Figure 1 Synthetic scheme of the acrylic derivative of trifusal, HTRF.

2.3. ^{1}H nuclear magnetic resonance (NMR)

CITRF (dimethyl sulfoxide (DMSO) d_6, 300 MHz, 20 °C); $\delta = 8.1$ p.p.m. (d, 1H arom.), $\delta = 7.7$ p.p.m. (d, 1H arom), $\delta = 7.6$ p.p.m. (s, 1H arom), $\delta = 2.3$ p.p.m. (s, CH_3—COO—).

HTRF (DMSO d_6, 300 MHz, 20 °C); $\delta = 8.1$ p.p.m. (d, 1H arom.), $\delta = 7.8$ p.p.m. (d, 1H arom.), $\delta = 7.7$ p.p.m. (s, 1H arom), $\delta = 6.1 - 5.7$ p.p.m. (d,m, CH_2=C—); $\delta = 4.6 - 4.4$ p.p.m. (t,t, —CH_2—CH_2—), $\delta = 2.3$ p.p.m. (s, CH_3—COO—), $\delta = 1.9$ p.p.m. (s, CH_3—C).

2.4. Polymerization

Poly(methacryloyloxyethyl [(2-(acetyloxy-4-trifluoromethyl)] benzoate) (poly-HTRF) was prepared by the free radical polymerization of HTRF in dioxane/acetone (4 : 1) at 60 °C in Pyrex glass ampoules. Reactions were carried out in the absence of oxygen by bubbling nitrogen twice for 30 min before sealing the system. The monomer and initiator concentrations were 0.5 and 1.5×10^{-2} mol l^{-1}, respectively. The sealed ampoules were immersed in a water bath maintained at the polymerization temperature. After 24 h the solution was poured into an excess of ethanol and dried in an oven under vacuum until constant weight was attained.

2.5. Characterization

The intermediate product, monomer and polymer obtained were analyzed by ^{1}H and ^{13}C NMR spectroscopy with a Varian XL-300 spectrometer working at 300 and 75.5 MHz respectively. The spectra were recorded at room temperature and 70 °C using 5 and 15% (w/v) deuterated DMSO solutions for the proton and carbon spectra, respectively.

Gel permation chromatography (GPC) measurements were performed in a Perkin Elmer apparatus equipped with a isocratic LC pump 250 and a refractive index detector series 200 to determine molecular weight distributions. Data were acquired with a PL-DCU (Polymer Laboratories). A set of 10^3 nm, 10^2 nm and 50 nm PL-gel columns conditioned at 25 °C were used to elute the samples of 10 mg ml^{-1} concentration at 1 ml min^{-1} high pressure liquid chromatography (HPLC)-grade chloroform flow rate. Polystyrene standards were used for calibration. The average molecular weight of the acrylic polymer obtained was 48 000 Da with a polydispersity index of 1.8.

2.6. Swelling and hydrolytic behavior

The absorption of water was followed gravimetrically measuring the weight uptake of dry thin films immersed in a buffered solution (pH = 2, 7.4 and 10) at 37 °C. Films were obtained by compress molding.

In the release experiments, 200 mg of polymer powder were immersed in 10 ml of buffered solutions at pH 2, 7.4 and 10. The systems were introduced in a thermostatic bath (37 °C) and kept at constant magnetic stirring. Portions (0.3 ml) of the supernatant were periodically collected for analysis and replaced by fresh medium (the same volume). The hydrolytical behavior was followed

by the analysis of the active metabolite 2-(hydroxy)-4-(trifluoromethyl) benzoic acid (HTB) because of the well-known unstability of TRF in aqueous media [20]. The release was monitored by HPLC. These measurements were performed using methanol/aqueous solution of PIC A (60 : 40) as the mobile phase and a flow rate of 1 ml min^{-1}. The system consisted of a Perkin Elmer LC-250 pump, a u.v./VIS detector Perkin Elmer LC-95 and a Waters μBoundapak C-18 column of 3.9 × 300 mm.

2.7. Methods

Commercial thin-walled vascular grafts of expanded polytetrafluorethylene, Gore-Tex, of 4 mm inner diameter, were used in two series of experiments *in vitro* by using the basal contact method. Series A corresponded to control (uncoated) whereas series B was assigned to prosthesis coated with the polyacrylic systems derived from Triflusal. All experiments were carried out in static conditions, i.e. by fixing the open prosthesis to the bottom of a cylindrical container of 8 mm diameter and 10 mm depth. Plasma rich in platelets from sheep arterial blood was isolated by centrifugation of 40 ml blood at 1500 r.p.m. during 10 min and the supernatant discarded. The content of platelets in the prepared medium was determined with a haematologic counter (Serono 3000). The centrifuged plasma-rich medium (100 μl) was added to the cylindrical containers and the contact with the surface of the vascular graft was kept during different periods of time, i.e. 10, 30, 60 min; 3, 24, 48 h; 3, 4 and 7 days. All the experiments were carried out in a 5% CO_2 chamber at 37 °C.

After the treatment time, the fragments of the prostheses were washed three times with minimal essential medium and the number of platelets retained in the prostheses was determined by comparison with the control.

2.8. Coating of vascular grafts

The coating of commercial Gore-Tex vascular grafts was carried out by immersion of the prostheses into a solution of the polymeric derivative of Triflusal (2.0 wt %) for 30 min. The wet segments of the prostheses were dried at room temperature in a controlled atmosphere of nitrogen until constant weight. The thickness and quantity of the coating was determined by measuring the weight gain of the coated specimen with respect to the original uncoated samples. Homogeneous thin coatings of 3–5 μm with a weight gain around 10 wt% were obtained. The stability of the adhesion of the coating was qualitatively tested by following the changes of the surface or the detachment of the coating by scanning electron microscopy (SEM) and by gravimetry. In all cases, the coating was well adhered to the surface of the prostheses at least during the first 4 or 5 days of treatment. It is necessary to take into consideration that the experiments were carried out in static conditions, without the application of flow or hydrostatic pressure. These conditions do not simulate the normal dynamic situation and therefore the results obtained can be extrapolated to *in vivo* applications as a first approximation, exclusively.

2.9. Morphological studies

After the treatment, all the samples were fixed with glutaraldehyde, washed with buffered solution (pH = 7.4) and dehydrated in a graded acetone series reaching a critical point in CO_2 polaron E-300, metallized with palladium gold and examined through a Zeiss 950 differential scanning microscope (DSM) for SEM. Statistical studies were assessed by non-parametric analysis of variance (ANOVA) and the Mann–Whitney *U* test.

3. Results and discussion

Knitted or woven poly(ethylene terephthalate) Dacron as well as expanded poly(tetrafluorethylene) Gore-Tex fabrics are the most traditional biomaterials used clinically in cardiovascular devices and vascular grafts. The hemocompatibility of devices of relatively high diameter (> 10 mm) is considered to be acceptable or good for long implantation time if heparin is administered to the patients. However, in the case of small diameter vascular grafts the situation is more compromised and therefore several attempts to improve their biocompatibility have been considered by recognized schools [5, 10]. The combination of synthetic polymers with pharmacologically active substances seems to be promising, and one of the most interesting approaches consider the attachment of antithrombogenic agents to the polymeric systems through weak and reversible covalent bounds, which could be hydrolyzed in physiological conditions [21–26].

This work describes the behavior of a new biocompatible and bioresorbable polyacrylic derivative of Triflusal applied as a thin coating into the surface of porous Gore-Tex vascular grafts of 4 mm diameter. The scheme of the synthetic route followed to obtain the precursor

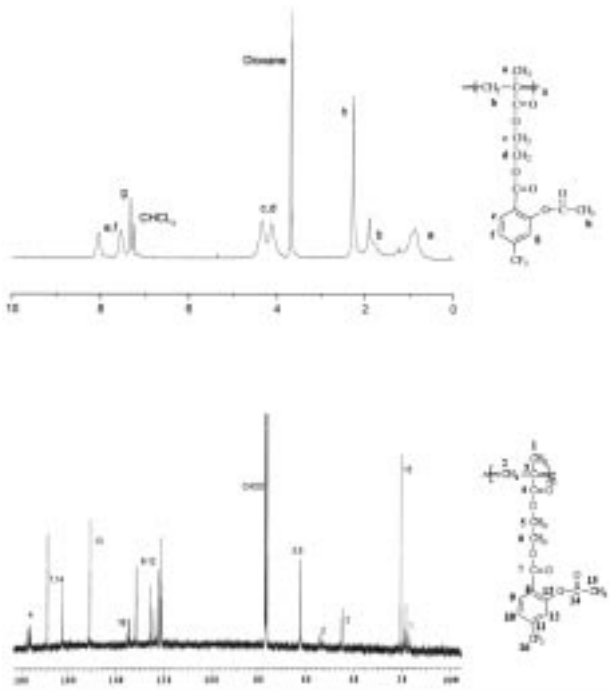


Figure 2 ^{1}H and decoupled ^{13}C NMR spectra of poly-HTRF recorded at 300 MHz (^{1}H NMR) and 75.5 MHz (^{13}C NMR) in deuterated chloroform solutions at 50 °C.

methacrylic monomeric compound is shown in Fig. 1 and the chemical structure and the corresponding ^{1}H and ^{13}C NMR spectra of the high molecular weight polymeric system are collected in Fig. 2. It is interesting to stress that whereas the acetyl group of the pharmacologically active compound triflusal is easily released from the molecule, in the case of the prepared polymeric systems, the chemical stability increases noticeably. This is probably due to the relatively strong hydrophobic character of the polymeric derivative of triflusal, which gives rise to more hydrolytically stable systems in contact with hydrated media.

Fig. 3 shows the gravimetric studies after the immersion of the films (80–100 μm thickness) in buffered solutions at pH 2, 7.4 and 10. Films exhibit a slow swelling rising value of water uptake (measured as [wet wt − dry wt]/ wet wt) below 4.0% in all the experiments because of their low global hydrophilicity. At pH 2 and 7.4 the films absorb 1.7 and 3.3% in 10–20 days, respectively. However, at pH 10 there is an intermediate behavior: the film takes 2.3% of water in about 10 days but at higher times it exhibits a linear increase in the water uptake. This behavior has to be ascribed to the slow increase in hydrophilicity as a consequence of the hydrolysis of the ester side group and the formation of the more polar hydroxy end group in the side chain of the macromolecule. The difference between the values obtained at pH 7.4 and 10.0 (i.e. in alkaline medium) is in the interval of the experimental error of the technique considering the low absolute values of water uptake. Fig. 4 shows the cumulative release of the active component as a function of time for the same conditions as the swelling experiments, which is in agreement with the gravimetrical behavior discussed previously. These results demonstrate the relatively high chemical stability of the polymeric drug derived from Triflusal as a consequence of the hydrophobic character of the polymeric systems. Some experiments with more hydrophilic polymeric formulations prepared by copolymerization of the acrylic monomer of Triflusal HTRF (Fig. 1) with other hydrophilic monomers have shown that the hydrolytical stability and therefore the rate of release of Triflusal, can be easily controlled by preparing polymeric systems with the appropriate hydrophilic character. It is necessary to take into consideration that the hydrolytic results refer to data obtained in buffered solution, but in the human body the strong catalytic effect of esterases in the breaking of ester bonds has to be

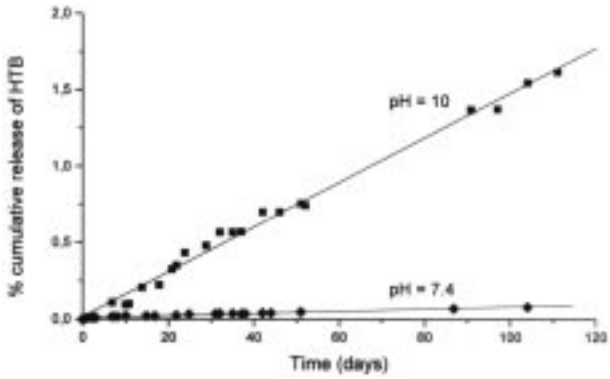


Figure 4 Cumulative release of the active metabolite HTB as a function of time.

considered. This fact noticeably activates the hydrolytic process, which has been verified by the following experiments using plasma rich in esterases from rats, instead of buffered solutions. New results obtained will be given in a forthcoming article dedicated to studies *in vivo*.

The hydrolytical experiments show a slow but remarkable linear slope, higher in the case of the experiments at pH 10 because the breaking of the ester is favored at basic pH (at pH 2 the release is very small). This zero-order in the release kinetic has to be related to the increase in hydrophilicity when the hydrolysis advances as shown schematically in Fig. 5. The occurrence of the hydrolysis gives rise to the formation of the more polar hydroxy end group. Therefore, there is an increasing accessibility of more water molecules (as was confirmed by the gravimetric experiments), which in fact is the hydrolytic agent, leading to a continuous process that shows a zero-order release kinetic.

From a biomedical point of view, the system releases the antithrombogenic drug in a constant rate for several weeks despite its probable activity in its macromolecular form, as has been described for similar systems [27], and the final residue which can be considered as poly-HEMA has a good haemocompatibility, as described in the literature [28] because of its hydrophilic character. Moreover, these poly-HEMA macromolecules formed after the hydrolysis of the acrylic chains and the delivery of free acetyl groups and HTB residues, are slowly cleared from the body as solution in the physiological fluids or as highly swollen gels, as happens with non-crosslinked fractions of collagen. This is an important

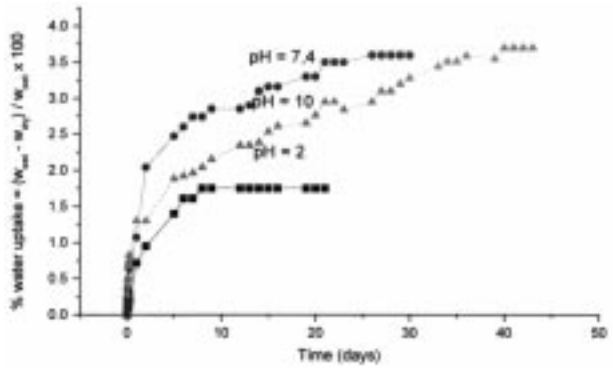


Figure 3 Water uptake of films immersed at 37 °C in buffered solutions at pH 2, 7.4 and 10.

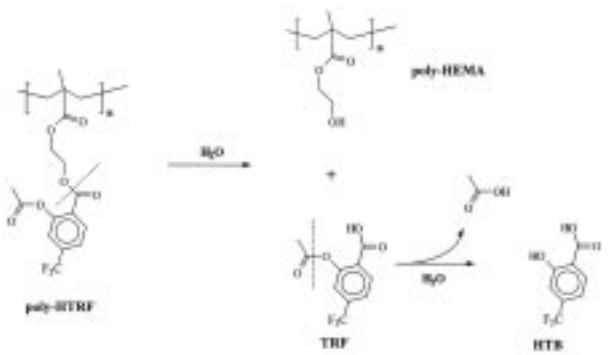


Figure 5 Schematic mechanism of the hydrolytical process overcome by poly-HTRF in aqueous media.

factor in the application of these systems as surface coatings of vascular grafts, because of the possibility of endothelization of the inner surface and the anchorage of a neointima into the regenerated porous structure of Gore-Tex after the clearance of the acrylic polymer support.

The aggregation experiments of platelets were carried out in static conditions using plasma rich in platelets collected from sheep blood, as described in Section 2. This assay has to be considered as a preliminary comparative screening respect to the control, uncoated prostheses. In static conditions, the deposition of platelets on the surface of the prosthesis is always produced in a more or less long period of time. This is a physical phenomenon of sedimentation of cells into the surface, but the aggregation of platelets on the sedimented layer in contact with the surface of the prosthesis can be controlled by the characteristics of the coatings. In this sense, the aggregation behavior at the level of the surface was analyzed by counting labeled platelets with ^{111}indium–oxine complex as well as by the examination of the treated prostheses by SEM. Fig. 6 shows the platelet retention with respect to the content of platelets in the applied medium, as a function of the contact time Although the level of the percentage of platelets counted is relatively high, it seems that the surface of Gore-Tex coated with a thin film of the polyacrylic systems of Triflusal presents lower retention of platelets than the uncoated controls, and the variation with time seems to be less affected during at least one week of treatment. Fig. 7 shows the SEM photographs of control (Fig. 7a) and coated (Fig. 7b) surfaces of Gore-Tex vascular grafts after 7 days of treatment. It is clear from the pictures that the platelets are less aggregated in the case of coated surfaces where the layer of coatings disappear partially and several fragments of the polymeric coatings can be found. However, the control presents coagulated domains of aggregated platelets with a strong adhesion to the porous structure of the surface of Gore-Tex.

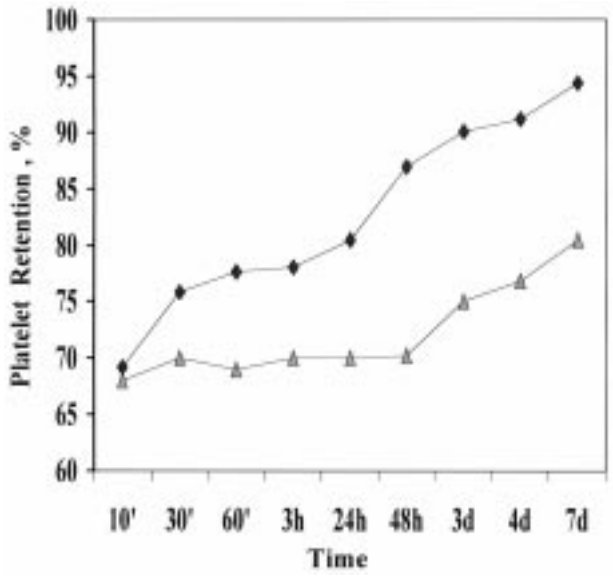
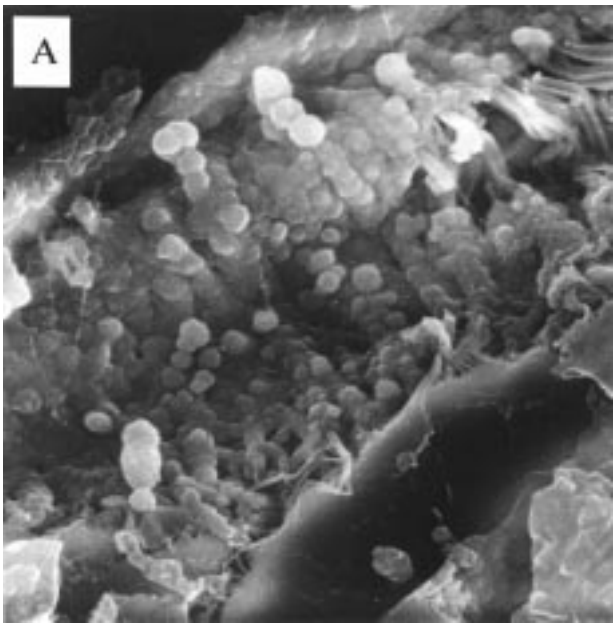


Figure 6 Retention of platelets of plasma from sheep blood in segments of Gore-Tex vascular grafts, in static basal deposition tests.



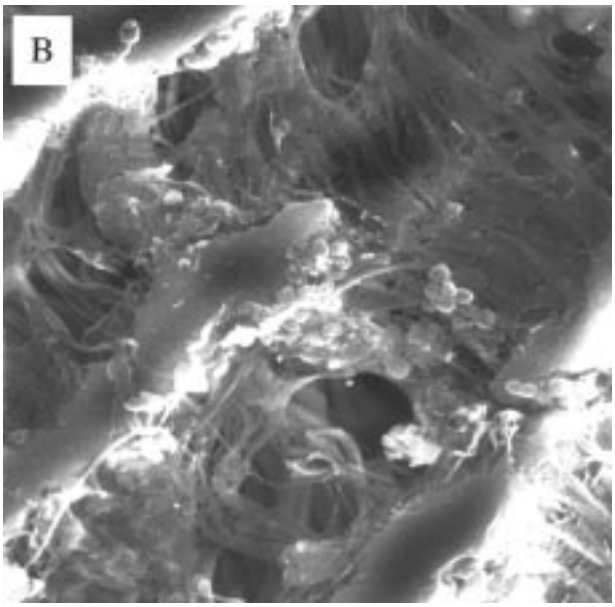


Figure 7 Scanning electron microscopy micrographs (× 2000) of (A) uncoated surface of Gore-Tex vascular grafts and (B) vascular grafts coated with poly-HTRF. In both cases the time of treatment correspond to a period of 7 days.

In conclusion, the preliminary results of aggregation found in static conditions seem to indicate that the coating of the surface of vascular grafts of Gore-Tex improves the antithrombogenic character of the prostheses and provides a resorbable system which allows the re-endothelization of the prosthesis after implantation in a moderate interval of time. In addition, the new polyacrylic systems derived from Triflusal can be considered as controlled delivery systems of the antithrombotic drug Triflusal, which could be interesting for other applications not considered in the present work.

Acknowledgments

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