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Surface studies of albumin immobilized onto PE and PVC films

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Abstract—The aim of this study is to evaluate the thrombogenic behaviour of the low density polyethylene and poly(vinyl chloride) modified by radiation-grafting technique. After copolymerization with acrylic acid by γ-rays from a ^{60}Co source, BSA was immobilized onto functionalized graft copolymers. The biological interaction between these materials and blood was studies by *in vitro* methods. The BSA immobilization effectively suppressed the adhesion and activation of platelets when it contacted whole blood.

Key words: Blood compatibility; protein adsorption; hydrogel; albumin immobilization; grafting; polyethylene; poly(vinyl chloride).

INTRODUCTION

Synthetic polymers of many types today play a very important role for practical application in medicine. The major application areas for polymeric materials are cardiovascular, ophthalmic, general soft tissue, dental, orthopedic, and drug delivery systems [1–3].

It is well known that one of the most serious problems usually encountered in the field of artificial prosthesis is the thrombogenic activity usually exerted by synthetic materials when they are in contact with blood [4]. The physical, chemical, or biological modification of polymeric surfaces to achieve blood compatibility has claimed the attention of a great number of scientific research groups [5–8].

Thus, many studies have been devoted to the preparation of blood-compatible hydrogels by radiation grafting of different hydrophilic monomers onto polymeric substrates [9–12]. The hydrogels supported on hydrophobic polymers seem to meet

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many of the requirements due to the fact that such grafting can induce hydrophilicity as well as good hemocompatible properties. The synthesis of polymeric surfaces with immobilized biocomponents is an alternative method for enhancing the blood compatibility of polymers [13–15].

In this paper, the polymeric surfaces of low density polyethylene and poly(vinyl chloride) were functionalized by radiation grafting of poly(acrylic acid) hydrogel. The obtained hydrogel layers were used to immobilize albumin, and their blood compatibility was studied.

EXPERIMENTAL

Materials

Low density polyethylene (PE) and poly(vinyl chloride) (PVC) films of 100 and 20 μm thickness, respectively, were washed thoroughly with tap water and subsequently rinsed with acetone and distilled water in a ultrasonic cleaner for 5 min. The films were then dried in vacuum at room temperature (25 C). Acrylic acid (AA) from Aldrich was used without further purification. The bovine serum albumin (BSA, crystallized) and fibrinogen purchased from Sigma Co., were used for adsorption experiments. The fibrinogen was 95% clottable and the albumin 99% pure and were both used without further purification. The other chemicals were reagent grade and used as-received.

Grafting procedure

Graft copolymers of PE-*g*-AA and PVC-*g*-AA were then prepared by simultaneous radiation-induced grafting technique. Thus, a weighed sample of PE and PVC films were placed in a glass ampoule containing a methanol/AA mixture of known bulk monomer concentration (30% w/w). The glass ampoules were then connected to a vacuum line system (10^{-4} mm Hg) and was evacuated by a freeze–thaw cycle which was repeated five times. After evacuation, the ampoule was irradiated with γ-rays from a ^{60}Co source at dose rate of 0.033–0.468 kGy h^{-1} and total dose of 6.0 kGy. The grafted films were removed and washed thoroughly with hot distilled water and soaked overnight in water to extract the residual monomer and homopolymer occluded in the films. The films were then dried in vacuum at room temperature for 48 h and weighed. The degree of grafting of PE and PVC films were gravimetrically determined.

The grafted films were removed and washed throughly with hot distilled water and then soaked overnight in distilled water to extract the residual monomer and homopolymer occluded in the films. The films were then dried in vacuum at room temperature for 48 h and weighed. The degree of grafting of PE and PVC films were determined by the percentage increase in weight as follows:

$$\text{degree of grafting } (\%) = \frac{W_g - W_0}{W_0} \times 100,$$

where W_0 and W_g represent the weights of initial and grafted films, respectively.

Activating carboxyl groups

The activation of the PE-*g*-AA and PVC-*g*-AA films was similar to that described in Coulet's paper [16]. The dried supports (PE-*g*-AA and PVC-*g*-AA) were esterified by refluxing the films with an excess of methanol (100 ml) containing 1% by weight of sulfuric acid as a catalyst for 24 h (step II — Fig. 1). The films were washed thoroughly with water and immersed in a 2% solution of hydrazine in a nitrogen atmosphere at 25 C for 24 h (step III — Fig. 1). The films were removed and washed with water. The azide formation was accomplished by immersing the films in 35 ml of cold, 4.3 M HCl, and 1 ml of 3.0 M aqueous $NaNO_2$ (step IV — Fig. 1). The reaction mixture was swirled for several seconds and the films were allowed to stand for 5 min. The PE-*g*-AA and PVC-*g*-AA activated films were removed and washed with cold 0.1 M NaCl solution.

Albumin immobilization

Bovine serum albumin was covalently immobilized on the activated surfaces of PE-*g*-AA and PVC-*g*-AA films. The coupling process is shown in Fig. 1 (step IV). The PE-*g*-AA and PVC-*g*-AA films with activated carboxyl groups were dipped into BSA solution (1.0 mg ml^{-1}) at 4 C, pH 7.4 (PBS buffer), for 24 h. This last step secures covalent binding of BSA through their free amino groups to poly(acrylic acid) grafted onto polymeric surfaces with activated carboxyl groups. The films were then washed with cold distilled water and stored in deionized water at 4 C. The measuring of protein content before and after the immobilization process were performed by using the Bradford method [17].

Surface characterization

Water sorption. Known weights of the clean and dry grafted films were immersed in distilled water at 25 C until equilibrium was reached (24 h in most cases). Then the films were removed, blotted quickly with absorbent paper to remove the water attached on its surface, and weighed. The water-uptake percent was calculated as follows:

$$\text{water uptake } (\%) = \frac{W_{\text{wet}} - W_{\text{dry}}}{W_{\text{wet}}} \times 100,$$

where W_{wet} and W_{dry} represent the weights of wet and grafted films, respectively.

Contact angle. The grafted and virgin films were conditioned at the equilibrium humidity of the instrument. The water contact angle was measured by putting a sessile drop of distilled and deionized water on the air-side surface of the polymeric films. Each value was taken as the average of the five readings.

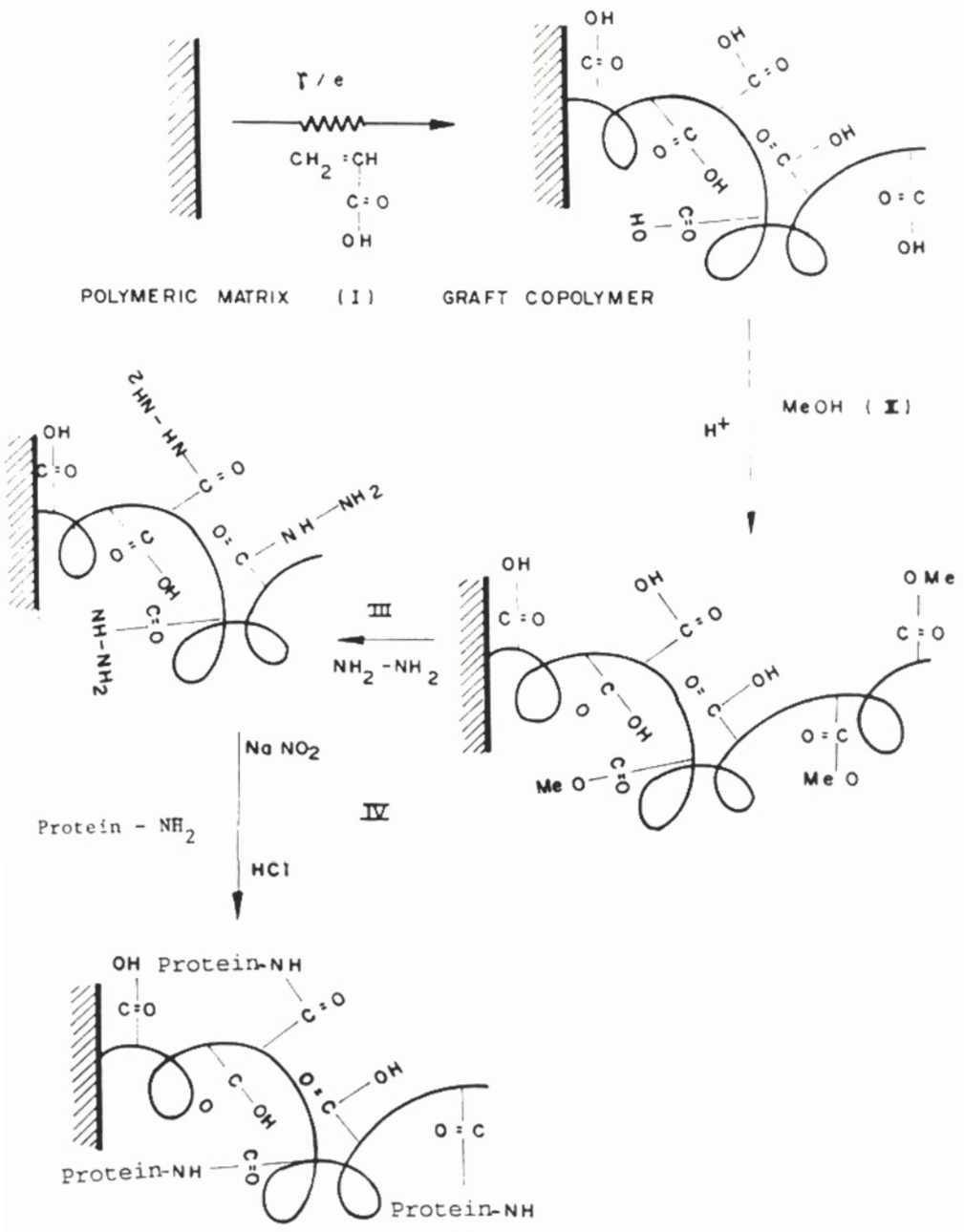


Figure 1. Experimental procedure for protein coupling on chemically activated PE-*g*-AA and PVC-*g*-AA films.

Infrared spectroscopy. The graft copolymers were analysed by Fourier transform infrared spectroscopy with multiple internal reflectance accessory (FTIR-MIR). The analyses were performed using a Perkin–Elmer 1730 spectrophotometer.

Thermal analysis. The surface free energy of polymeric materials was determined by the Hayes and Lee equation [18–19]:

$$\gamma_c^{0.86} = \frac{(0.03RT_g - 1.5) \cdot n\phi^2}{V_m^{0.71}},$$

where R is the gas constant (cal mol^{-1} deg^{-1}), T_g is the glass-transition temperature (K), n is a dimensionless number analogous to the degree of freedom, ϕ is the ratio between the reversible work of adhesion and the geometrical mean of the work of cohesion of the two components ($0.8 < \phi < 1$) and V_m is the molar volume (cm^3 mol^{-1}) of the polymeric system. The glass-transition temperature was obtained in a differential scanning calorimeter (DSC) and n, ϕ, and V_m are listed values. Differential scanning calorimetry (DSC) was performed using a Metler TA 4000 thermal analysis unit with a DSC cell. In each experiment 5 ± 0.1 mg of the polymer sample was heated from room temperature (25 C) to 200 C at a heating rate of 10 C min^{-1}.

In vitro *tests*

Protein adsorption. In order to quantify the surface concentrations of albumin (BSA) and fibrinogen adhering to the polymeric surfaces, the proteins were labelled with ^{125}I using the iodine monochloride method of McFarlane [20]. In this reaction iodine substitutes mainly in the aromatic rings of tyrosine residues [21], and the degree of substitution not exceeding one iodine atom/molecule of protein, it has been shown that biological activity is not altered [22]. Unincorporated iodine was removed by gel filtration on Sephadex G-250 (Pharmacia Fine Chemicals). The radioactivity of the labeled proteins was 30 μCi mg^{-1}.

The appropriate teflon tubes containing the polymeric surfaces were injected with sodium phosphate buffer (pH 7.4, ionic strength = 0.01 M) (PBS) to displace the air and then thermally equilibrated at 37 C. Any air bubbles which would adhere to the samples were removed by allowing the samples to cross the air–buffer interface several times. Aliquots (4 ml) of the labeled BSA or fibrinogen solution were then introduced into the tubes.

After the protein solution remained in contact with the samples for 2 h at room temperature, the adsorption was terminated by dilution of the labeled protein into the tubes with PBS (in order to avoid contact of the sample with the protein solution–air interface where some denaturation can take place). The samples were further rinsed gently until the radioactivity of the surface remainde constant. The amount of adsorbed proteins was determined by gamma radiation counting, using a Beckman Gamma 4000.

Thrombogenic behaviour. Blood compatibility of the PE-*g*-AA and PVC-*g*-AA films were evaluated by the open-static platelet adhesion test with whole human blood [23]. The tests were performed by depositing 2 ml of fresh blood onto each

of the five test surfaces. After contact times of 180 s, the surfaces were washed with saline (0.1 M NaCl) under carefully controlled conditions to remove all blood components that did not adhere. After fixation with glutaraldehyde (2%) and dehydrated in serial dilution of ethanol (25–100%), platelet counts were performed using SEM microphotographs. The average number of adhered platelets was obtained from five photographs of different surface areas (1 cm^2) of the same sample.

The kinetics of thrombus formation was performed by using the Imai and Nose technique [24]. Thus, whole human blood was added to one part of acid-citrate-dextrose (ACD) for nine parts of blood. The resultant ACD blood was placed on a glass plate, PE and PVC films, PE-*g*-AA, and PVC-*g*-AA with immobilized BSA (PE-*g*-AA-i-BSA and PVC-*g*-AA-i-BSA). Clotting was initiated by adding aqueous $CaCl_2$ solution, and the weight of thrombus formed during 12 min was weighed. The relative weights of thrombus formed on different samples were determined, with that formed on a glass plate being taken as 100%.

RESULTS AND DISCUSSION

The distribution of the grafted chains in the region near the surface was investigated by FTIR-MIR. Figure 2 shows the infrared absorption spectroscopy (FTIR-MIR) of PVC, PE, as well their graft copolymers PE-*g*-AA and PVC-*g*-AA. Such measurements show that the main change for grafted PVC and PE films is the development of a strong band at 1701 cm^{-1}. This is assigned to carbonyl groups (>C=O) of the grafted poly(acrylic acid) (PAA) chains [25].

Figure 3 shows the distribution of the grafted PAA chains in the region near the surface that was investigated by FTIR-MIR spectroscopy. As can be seen in Fig. 3, the values of the ratios of the CO/CH and CO/Cl peaks of different grafting degrees were calculated and plotted against the degree of grafting. It can be seen that ratios of the CO/CH and CO/Cl peaks increased with the degree of grafting and then level off. This means that the distribution of grafted chain in the surface increased with increasing degrees of grafting [26].

The contact angle and free energy for grafted and ungrafted polymeric systems are shown in Fig. 4. Both contact angle and surface free energy decreased with the grafting degree indicating the appearing hydrophilic region on PE and PVC.

Water plays an important role in determining the biocompatibility characteristic of the synthetic material. The potential advantage of high water levels within the surface of the biomaterial in respect of equivalence with normal tissues and especially for providing a low interfacial tension with blood, which would reduce protein adsorption and cell adhesion on the surface, has been recognized by Ratner and coworkers [27].

The swelling behaviour of the grafted films should be expected to play an important role in the biocompatibility of these synthetic materials. The swelling behaviour of grafted films is presented in Fig. 5. All samples show a degree of swelling which increases with an increase in percent grafting. The plateau attained for PVC

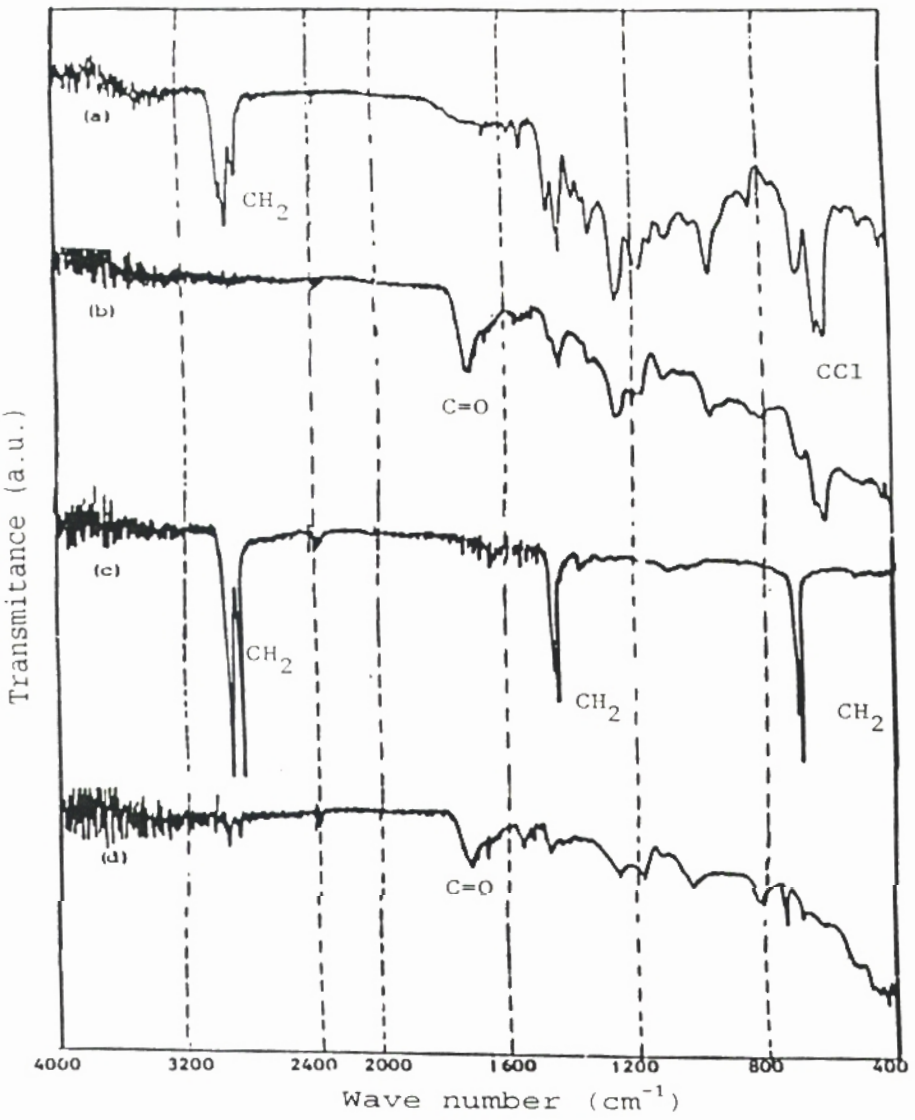


Figure 2. Infrared absorption spectroscopy (FTIR-MIR) of: (a) unmodified PVC; (b) PVC-*g*-AA (*G*: 20%); (c) unmodified PE; and (d) PE-*g*-AA (*G*: 154%). *G*: grafting degree (%).

at a higher grafting degree may indicate the cross-linking of the grafted polyAA chains.

When a material is placed in contact with blood, the first event to occur is the adsorption of proteins onto the surface, followed by platelet adhesion and activation [28, 29].

BSA and fibrinogen adsorptions onto PE-*g*-AA and PVC-*g*-AA graft copolymers were studied with the purpose of examining the extent of interaction of the surfaces with proteins in aqueous solution. The results are given in Figs 6 and 7. The graft copolymerization of AA onto PE and PVC films increase considerably the albumin and

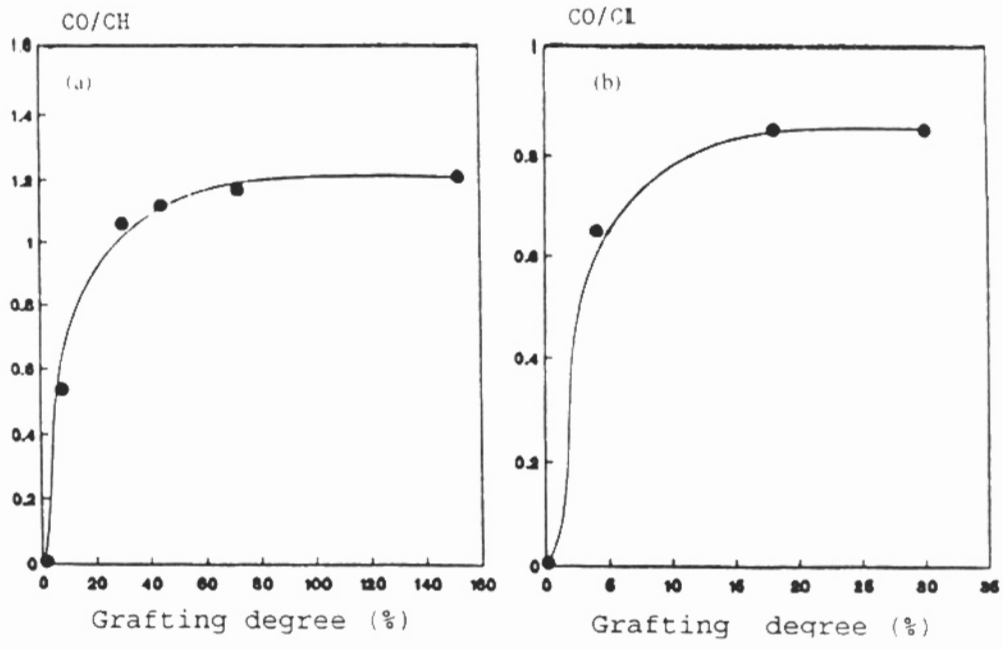


Figure 3. (a) CO/CH ratio for PE-g-AA and (b) CO/Cl ratio for PVC-g-AA, obtained from FTIR-MIR measurements.

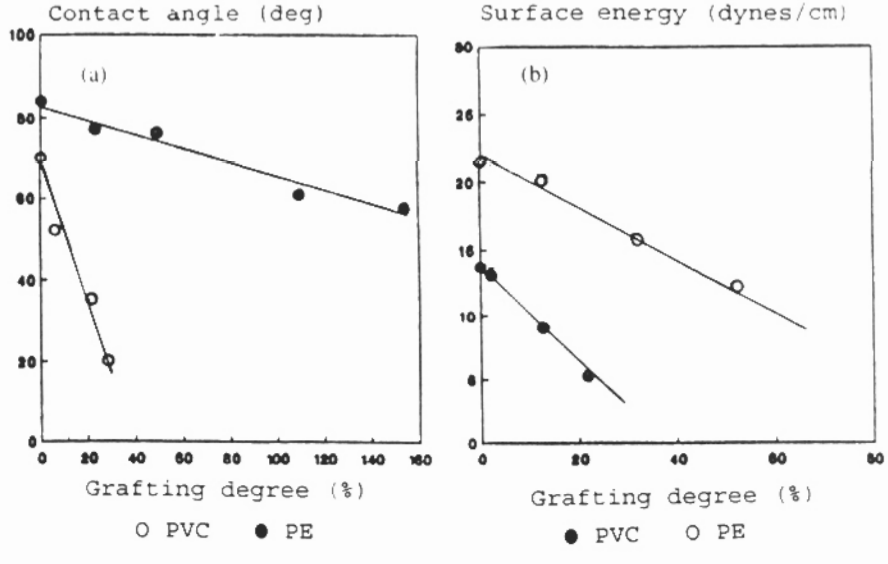


Figure 4. (a) Contact angle and (b) free energy for grafted and ungrafted PE and PVC films.

fibrinogen adsorptions. This increased proteins adsorption can be due to the increase of the swollen area caused by the anionic carboxylate groups in graft copolymers.

There is evidence that platelet adhesion is promoted when fibrinogen is adsorbed from the blood onto foreign surfaces [30, 31]. On the other hand, reduced platelet

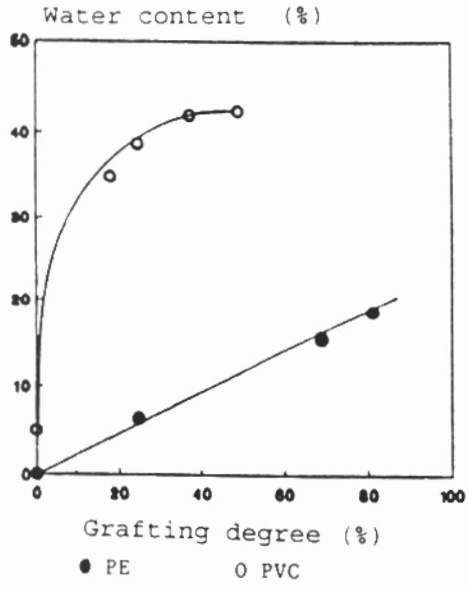


Figure 5. Effect of grafting degree on the water content of PE-*g*-AA and PVC-*g*-AA hydrogels.

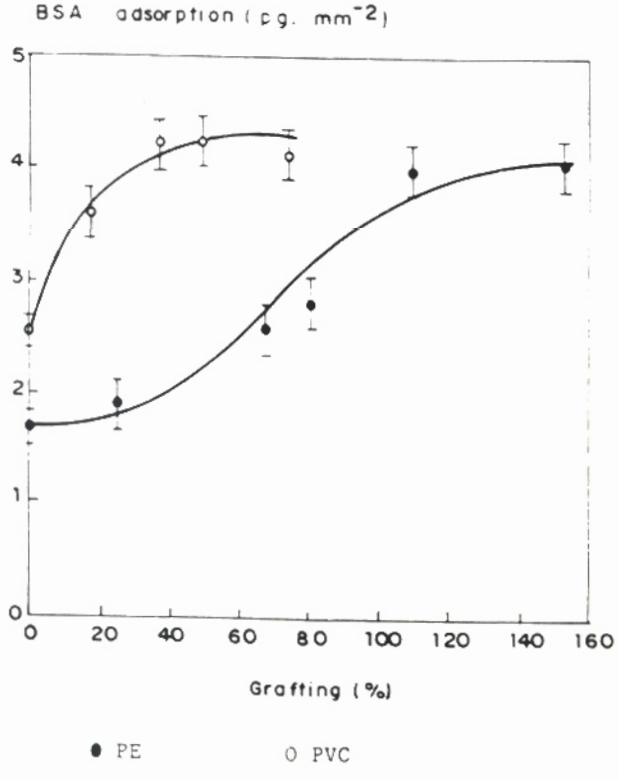


Figure 6. Surface concentration of BSA adsorbed as a function of grafting yield.

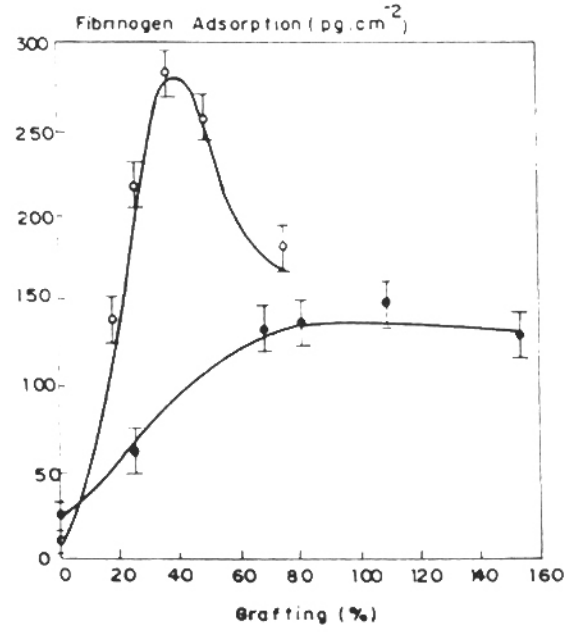


Figure 7. Surface concentration of fibrinogen adsorbed as a function of grafting yield.

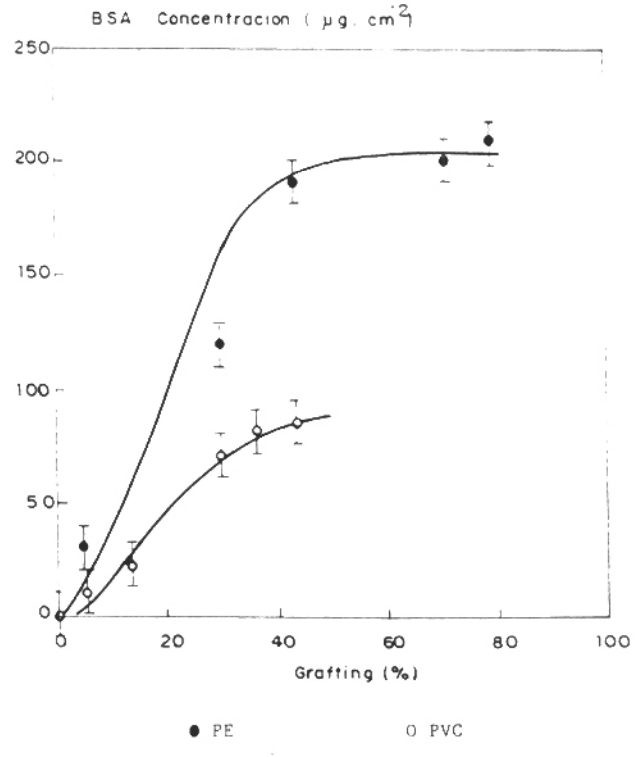


Figure 8. Amount of bound protein vs grafting yield of AA onto LDPE and PVC films

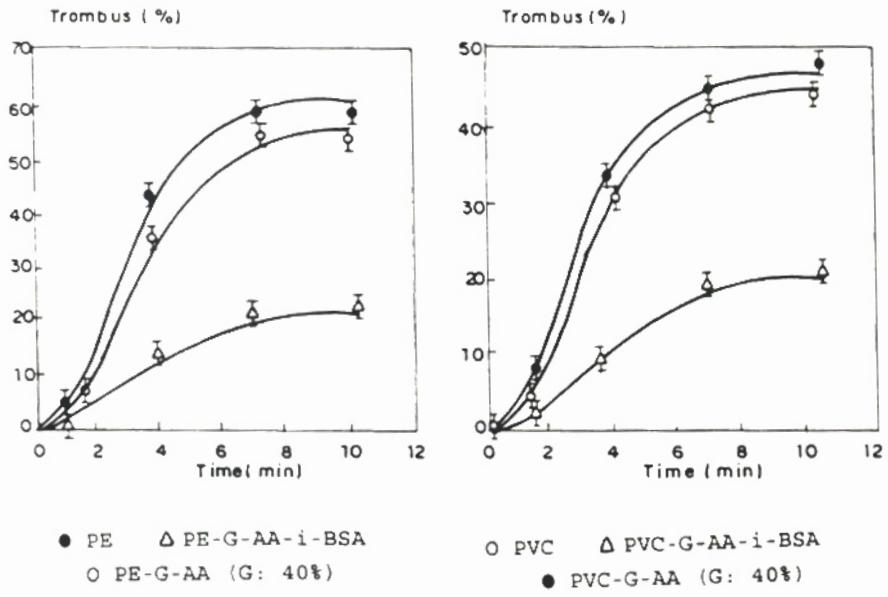


Figure 9. Amount of thrombus formed on films of BSA-immobilized polymers.

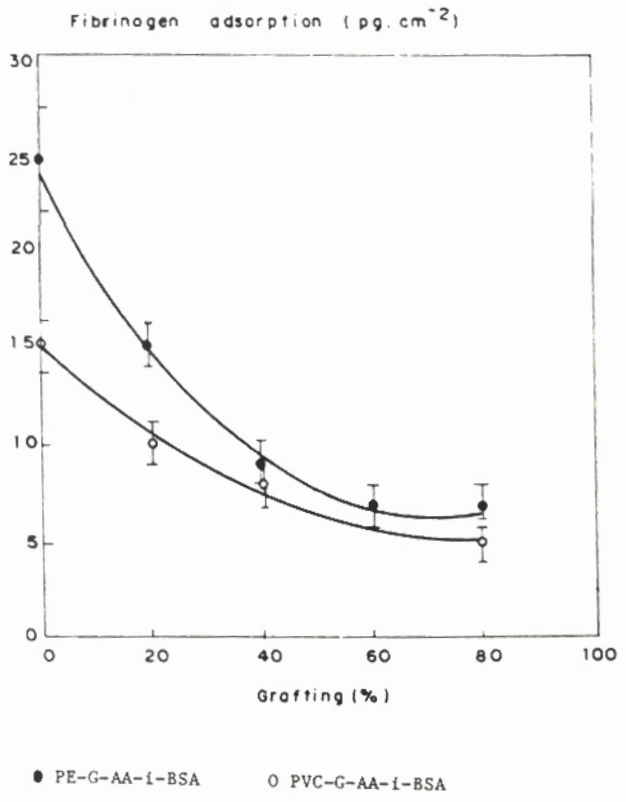
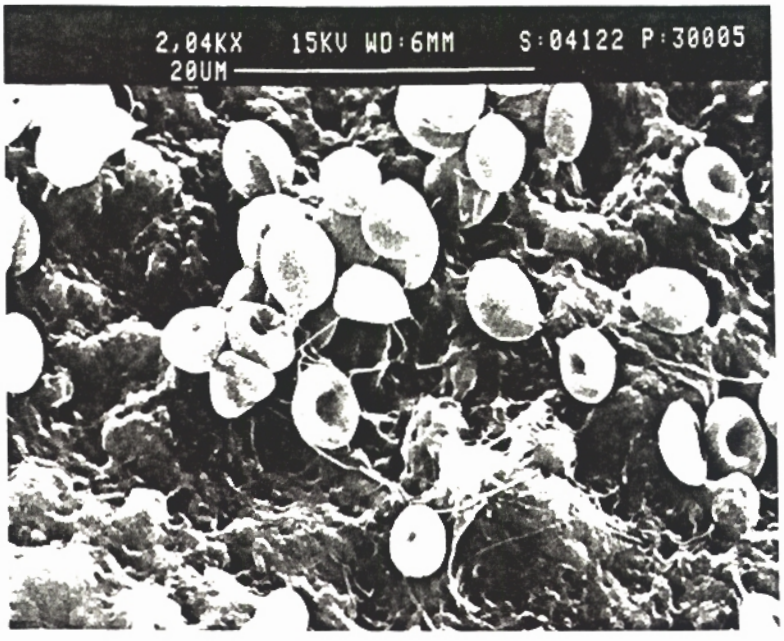
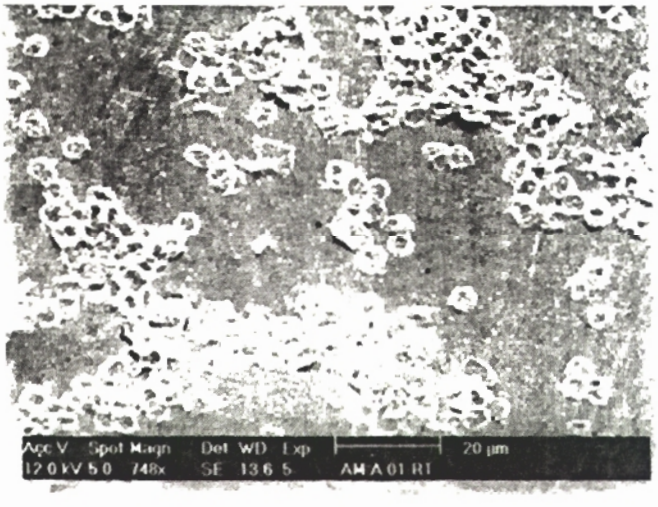


Figure 10. Fibrinogen adsorption onto PE-*g*-AA-i-BSA and PVC-*g*-AA-i-BSA films.



(a)



(b)

Figure 11. SEM micrographs of polymeric surfaces after incubation with human blood at 37 C for 3 min. (a) PVC-*g*-AA (*G*: 42%, magnification: × 1640), (b) PE-*g*-AA (*G*: 48%, magnification: × 748). *G*: grafting yield (%).

adhesion has been reported for polymers which adsorb relatively large amounts of albumin [32].

An important method to obtain more thromboresistant polymers is the modification of polymeric surfaces by coating them with biomolecules which reduce platelet adhesion and/or coagulation [33–35].

Due to their pronounced effects in preventing platelet adhesion and activation, BSA was covalently immobilized on the activated surfaces of PE-*g*-AA and PVC-*g*-AA

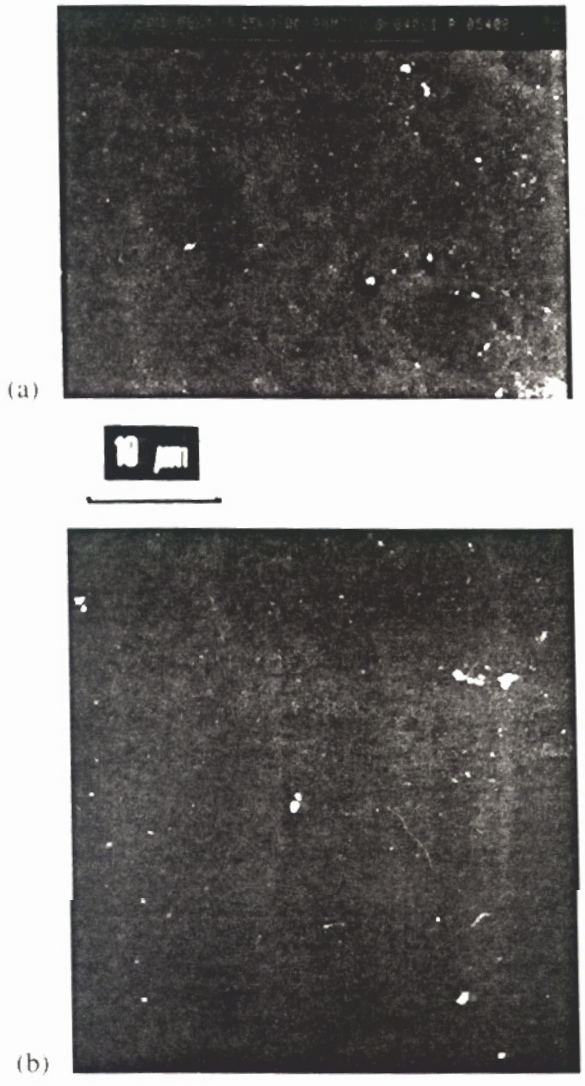


Figure 12. SEM micrographs of polymeric surfaces with immobilized BSA after incubation with human blood at 37 C for 3 min. (a) PVC-*g*-AA-i-BSA (G: 42%, i: 85 $\mu g\,cm^{-2}$) and (b) PE-*g*-AA-i-BSA (G: 48%, i: 192 $\mu g\,cm^{-2}$). G: grafting yield (%), i: amount of bound protein.

graft copolymers. The total amount of bound protein increased with increasing grafting yield (Fig. 8).

The thrombus formation decreased drastically with the protein immobilization (Fig. 9) and this agreed with Fig. 10. As demonstrated in Fig. 10, the BSA immobilization onto graft copolymers considerably reduces fibrinogen adsorption on the films. This indicates that BSA immobilized on polymers do not only reduce platelet adhesion but also delay the contact activation of intrinsic coagulation [36].

Scanning electron micrographs of the PE-*g*-AA, PE-*g*-AA-i-BSA, PVC-*g*-AA, and PVC-*g*-AA-i-BSA surfaces after contact with blood are shown in Figs 11 and 12.

The PE-g-AA and PVC-g-AA graft copolymers presented even more thrombogenic activity than on protein-immobilized surfaces.

CONCLUSION

The results obtained in this work demonstrate that BSA immobilization method offers several advantages with respect to others, such as chemical cross-linking of the absorbed albumin with glutaraldehyde: it does not require cross-linking agents or high temperature. These features allow avoid any damage or structural modifications of the biological macromolecule, avoiding the platelet adhesion and activation. Thus, PE-g-AA-i-BSA and PVC-g-AA-i-BSA effectively suppressed the adhesion and activation of platelets when it contacted whole blood. However, the BSA immobilization may involve a conformational change in the immobilized protein. The alterations on BSA conformations after immobilization process is still open to discussion. A more detailed study is now in progress and the results will be reported in the future.

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