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Immobilization of a nonsteroidal antiinflammatory drug onto commercial segmented polyurethane surface to improve haemocompatibility properties

Gustavo A. Abraham[a,c], Alvaro A.A. de Queiroz[b,*], Julio San Román[c]

[a] *Instituto de Investigaciones en Ciencia y Tecnología de Materiales (INTEMA) (UNMdP-CONICET), Av. J.B. Justo 4302, 7600, Mar del Plata, Argentina*

[b] *Instituto de Ciencias, Departamento de Física e Química—Escola Federal de Engenharia de Itajubá, Av. BPS, 1303, 37500-000 Itajubá-MG, Brazil*

[c] *Instituto de Ciencia y Tecnología de Polímeros, CSIC, Juan de la Cierva 3, 28006, Madrid, Spain*

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Abstract

A method has been developed in which a layer of *p*-aminosalicylic acid (4-amino-2-hydroxybenzoic acid) (PAS), a water soluble pharmaceutical compound of the nonsteroidal anti-inflammatory drug (NSAID) class with antiaggregant platelet activity, is covalently immobilized onto a segmented polyurethane, Biospan^{TM} (SPU) surface. Thus, SPU surfaces were modified by grafting of hexamethylenediisocyanate, and the free isocyanate remaining on the SPU surface were then coupled through a condensation reaction to amine groups of *p*-aminosalicylic acid. The bonding of PAS from aqueous solution onto SPU surface was studied by ATR–FTIR, UV and fluorescence spectroscopy. Plateau levels of coupled PAS were reached within 1.2 μg/cm^2 using PAS solution concentrations of 1 mg/ml. The surface wettability of the polymeric films measured by contact angle indicate that the introduction of the PAS turns the surface more hydrophilic ($\theta_{water} = 43.1° \pm 2.1°$) relatively to the original SPU films ($\theta_{water} = 70.3° \pm 1.9°$). The in vitro albumin (BSA) adsorption shows that the PAS–SPU films adsorb more BSA (250/μg mm^2) than the original SPU (112 μg/mm^2). Thrombogenicity was assessed by measuring the thrombus formation and platelet adhesion of the SPU containing PAS relatively to nonmodified SPU surfaces. The polymeric surfaces with immobilized PAS had better nonthrombogenic characteristics as indicated by the low platelet adhesion, high adsorption of albumin relatively to fibrinogen and low thrombus formation, making them potentially good candidates for biomedical applications.

Keywords: Segmented polyurethanes; Biospan^{TM}; Albumin; *p*-aminobenzoic acid; Thrombogenicity

1. Introduction

Segmented polyurethanes (SPU) have already been established as one of the leading polymers used to make biomedical devices due to have lower amounts of bacterial adhesion [1,2], good physico-mechanical properties [3,4] and relatively good biocompatibility, biostability and antithrombogenicity characteristics [5,6].

These materials became the choice for a wide variety of biomedical devices such as catheters [7], cardiac assist devices [8], artificial heart [9] and a number of other current blood-contacting applications.

Although many successful results have been obtained in the use of polyurethanes in different biomedical devices, microscopic thrombi and evidence of microemboli have been observed in some case [10]. It is well known that the thrombus formation on polymeric surfaces can lead to occlusion of vascular graft or catheters and the embolization of these trombi may result in tissue damage or a stroke, making necessary the improvement of the blood compatibility for this kind of applications.

A wide variety of methods for polyurethane surface modification as an effective approach for developing clinically applicable polyurethane have been described by various research groups. Thus, several strategies have been proposed to improve the thrombogeneicity of biomaterials such as incorporation of ionic groups onto the polymeric surface [11], alteration of the surface properties by grafting techniques [12–14] and immobi-

*Corresponding author.

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lization of heparin, functionalized dextrans or biological compounds [15–17].

The known antiplatelet action of specific compounds such as prostaglandin [18], heparin [19] and thrombomodulin [20] have been utilized in attempts to prepare polyurethane surfaces with better thrombogenic properties either by chemically binding such materials to surfaces or by physically incorporating them in the bulk polymers.

In past decade there has been an explosive increase in the interest of the pharmacology of the blood platelet, motivated largely by the belief that a drug that controls the platelet function in vivo may be able to prevent at least some forms of thrombosis in man [21]. Thus, a number of clinical studies have examined the effect of nonsteroidal antiinflammatory drugs (NSAIDs) such as salicylic acid and their derivatives on the prevention of platelet aggregation and activation due to surface stimuli [22–25].

Currently, much interest is shown in the attachment of salicylic derivatives to hydrogels as a means of prevents the thrombus formation and platelet aggregation [26–29]. The obtained polymeric drugs exhibited a lesser platelet activation and deposition as well as thrombus formation with an additional antiaggregating effect for platelets in both, in vitro and in vivo experiments.

Various segmented polyurethanes have been used as polymeric drug reservoir [30–33]. However, the literature describing the salicylic derivatives covalently coupled to segmented polyurethanes and study of the antithrombogenic properties of the obtained material is scanty.

In order to prepare elastomers provided with better haemocompatibility with respect to the coagulation cascade and to platelet aggregation and activation we consider a low-cost alternative chemical treatments that consist of covalent coupling of *p*-aminosalicylic acid (PAS), a water soluble pharmaceutical compound of the nonsteroidal anti-inflammatory drug (NSAID) class, to commercially available medical-grade SPU, Biospan^{TM}.

2. Experimental

2.1. Materials

In this work Biospan^{TM}, a segmented poly(ether urethane urea) linear block copolymer of 4,4′-diphenylmethane diisocyanate (MDI) extended with mixture of ethylene diamine, 1,3-cyclohexanediamine, and poly(tetramethylene oxide) (PTMO) 75% w/w was purchased by The Polymer Technology Group, USA. This polymer contains as additive a linear copolymer of diisopropylaminoethylmethacrylate and decylmethacrylate 5% w/w based on dry polymer. Biospan^{TM} was provided in *N*,*N*-dimethylacetamide solution consisting of 25% w/v and was diluted to 12.5% w/v to reduce the viscosity. Hexamethylene diisocyanate (HMDI, Aldrich), toluene (Merck.) and *N*,*N*-dimethylacetamide (DMAc, Scharlau) were used without further purification. Triethylamine (Scharlau) was distilled and stored under potassium hydroxide. The *p*-aminosalicylic acid (4-amino-2-hydroxybenzoic acid, PAS) (Merck) was used as received.

2.2. SPU film preparation

Detailed procedures for the preparation of SPU films had been reported in a previous publication [34]. Briefly, SPU films were prepared by solvent casting technique from the DMAc solutions described above onto clean glass plates. The films were cast in one layer with evaporation of the solvent (24 h at 60°C). Final drying was made in a vacuum oven (3 mm Hg) at 60°C for 24 h.

2.3. HMDI grafting and PAS immobilization

Fig. 1 illustrates the reaction scheme of the HMDI grafting and PAS immobilization onto SPU surfaces. The reactive isocyanate (NCO) groups were introduced in the SPU surfaces by contacting films with hexamethylene diisocyanate (HMDI) in toluene solution (7.5% w/v), using triethylamine (2.5% w/v) as a mild catalyst. The reaction was carried out at 50°C for different time periods (0.5–18 h) under nitrogen atmosphere to find the optimum conditions to yield a maximum concentration of isocyanate groups on the polymeric surfaces. The films were subsequently rinsed in toluene to remove entrapped HMDI, followed by drying under vacuum at 25°C for 24 h.

Immobilization of PAS onto the NCO-grafted SPU films (SPU-g-HMDI) was carried out by immersing the SPU-g-HMDI films in PAS solution of pH 8.0 prepared by dissolving 1 mg de PAS in 1 ml of 0.2 M sodium bicarbonate. At given time intervals, the film was removed and washed successively with 0.1 M sodium acetate and distilled water. Amounts of the coupled PAS (SPU-g-PAS) were performed by UV differential spectroscopy (double-beam Perkin Elmer Lambda 16 spectrometer) [35]. Thus, the amount of immobilized PAS was determined by measuring the difference between the amount of *p*-aminosalicylic acid in the solution before and after contact with the SPU-g-HMDI films. The maximum wavelength absorption for PAS used in this work was 298 nm. The water interaction behavior of the drug (PAS) onto SPU surface was studied by surface fluorescence spectroscopy. Thus, fluorescence spectra at an excitation wavelength of 298 nm (PAS) were measured using a Model LS 30 luminescence spectrometer (Perkin Elmer). The relative fluorescence intensity (%) in an emission wavelength range of 300–475 nm was recorded.

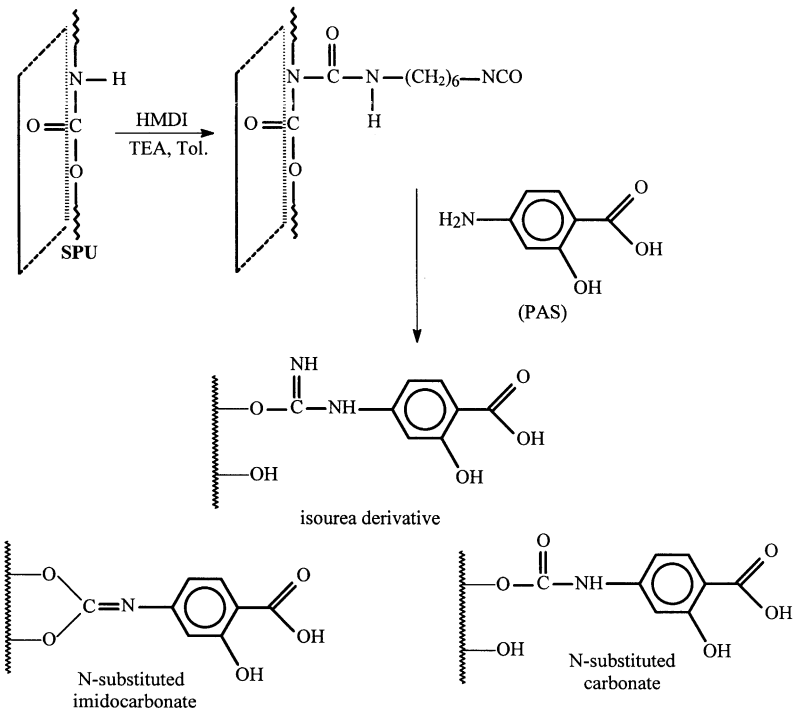

Fig. 1. Most probable reaction mechanism for the covalent attachment of PAS to modified SPU.

2.4. Characterization of SPU films

2.4.1. Attenuated total internal reflectance-FTIR (ATR–FTIR)

Infrared analyses were performed using Nicolet 520 infrared spectrometer equipped with a ATR accessory (Spectra-Tech Inc., Model 300). The spectra were recorded at an incident angle of 45° using a Ge crystal, giving an analysis depth of 1–2 μm.

2.4.2. Contact angle measurements

For the evaluation of surface wettability, the water contact angles of the modified SPU surfaces were measured at 25°C using a contact angle goniometer (Krüss, GmbH Hamburg, model G10) equipped with a video measuring system (model G1041). A droplet of water (10 μl) was put on the air-side surface of a film at 25°C and after 30 s the contact angle was measured. In this experiment five measurements were carried out for a single sample and the values obtained were averaged.

2.5. Water sorption and diffusion

The water sorption and diffusion measurements were obtained after known weights of the dry SPU and SPU-g-PAS films were immersed in phosphate buffered saline (0.1 M NaCl, 0.086 M KH_2PO_4, 0.041 M Na_2HPO_4, pH 7.4) (PBS) at 37°C until equilibrium was reached. Then the films were removed, blotted quickly with absorbent paper to remove the water attached on its surface and weighed.

The swelling behavior of the films were calculated from the following relation:

$$\text{Swelling}\,(\%) = [(W_t - W_0)/W_0] \times 100, \quad (1)$$

where W_t is the weight of swollen film at time t and W_0 is the initial weight of the dry film.

2.6. Biological properties of SPU surfaces

2.6.1. Molar ratio of fibrinogen to albumin adsorption

Bovine serum albumin (BSA) and bovine fibrinogen (Fbg) were purchased from Sigma Co. The fibrinogen was 95% clottable and the albumin was 99% pure and were used without further purification. In order to perform equilibrium experiments, preequilibrated modified and original SPU films with 16 cm^2 surface area were introduced into tubes which containing 4 ml phosphate buffered saline (0.1 M NaCl, 0.086 M KH_2PO_4, 0.041 M Na_2HPO_4, pH 7.4) (PBS) at 37°C, before being exposed to the protein solution. Any air bubbles which would adhere to the sample were removed by allowing the samples to cross the air/buffer interface several times. The concentrations of BSA and Fbg were 1.0 mg/ml, respectively, in PBS buffer solution. Then, aliquots (4 ml) of the BSA or fibrinogen solution were then introduced into the tubes. After the protein solution remained in contact with the samples for 2 h at

37°C, the adsorption was terminated by dilution of the protein solution into the tubes with PBS. The samples were further rinsed gently until the surface remained constant.

The amount of adsorbed protein was determined by measuring spectrophotometrically the difference between the amount of albumin or fibrinogen in the solution before and after contact with the polymer films. The spectroscopic analytical methods utilized in this work for protein dosage is based on the reaction of albumin or fibrinogen with Coomassie brilliant blue (Fluka) dyestuff to record the absorbance of the albumin–Coomassie brilliant blue complex in according to Bradford's method [36].

2.6.2. Factor XII assay

The evaluation of the influence of immobilized PAS on blood coagulation was made in according to the method of interaction between the test materials and coagulation factor XII (FXII). The FXII activation measurements were performed by chromogenic peptide substrate assay with the tetrapeptide Bz-lle-Glu-Gly-Arg *p*-nitrophenylanilide (S-2222) [37–39]. Plasma samples were obtained after centrifugation of citrated whole human blood (one volume of 100 mM sodium citrate dihydrate solution to nine volumes of blood) at 3000 g (20°C, 15 min). After dilution with EDTA:NaCl 5 mM the recently prepared citrated human plasma was mixed with acetone (0.40 ml) and maintained for 17 h at 20°C. After remotion of acetone by vacuum evaporation (rotoevaporator) and loss of water correction the acetone treated plasma (CPLA) preparations were kept on ice until used. SPU-g-PAS and SPU films with a total surface area of 0.16 cm^2 were put into 0.2 ml of acetone treated human plasma and incubated for 1 h at 0°C to prevent inactivation of the kallikrein by the inhibitors in plasma. A chromogenic peptide substrate solution S 2302 (Kabi-Vitrum, Stockholm, Sweden) of 300 μl was added to the 300 μl of CPLA control and incubated for 10 min at 37°C.

After the addition of 300 μl of acetic acid to stop the reaction, the absorbance of the reacted chromogenic substrate was measured spectrophotometrically at 405 nm.

2.6.3. Kinetics of thrombus formation

The kinetics of thrombus formation onto polymeric surfaces procedure was detailed previously [40,41]. 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, SPU and SPU-g-PAS. Clotting was initiated by adding aqueous $CaCl_2$ solution, and the weight of thrombus formed during 12 min was assayed. The relative weights of thrombus formed on different samples were determined, with that formed on a glass plate being taken as 100%.

2.6.4. Platelet adhesion to polymer surfaces

The platelet adhesion onto SPU and SPU-g-PAS films were evaluated by using the methodology of Park et al. [42]. A fresh platelet-rich plasma (PRP) was prepared by collecting human blood into plastic syringes containing 3.8% sodium citrate solution (final dilution 1 : 9) to prevent blood coagulation. Blood was centrifuged at 200 g for 10 min at 4°C and the PRP supernatant collected. The platelets were further centrifuged at 155 g for 20 min at 4°C and PPP supernatant was mixed with the PRP to give a PRP with a final platelet concentration of $7 \times 10^5/\mu l$. The SPU and SPU-g-PAS films were put in appropriate teflon tubes (0.5 ml) and equilibrated with 0.2 ml PBS buffer (pH 7.4, ionic strength = 0.01 M) at 37°C overnight.

Prior to adhesion studies, the buffer was removed from the tubes and PRP (0.5 ml) was introduced into the teflon tubes and incubate at 37°C for 1 h. A control sample of PRP incubated without the polymeric films was used as a reference. The platelets were counts by phase microscopy and the degree of platelet adhesion was determined by:

$$\%\,\text{Adhesion} = [C_{po} - C_{pt}]/C_{po} \quad (2)$$

where C_{po} and C_{pt} are the counts of the number of platelets before and after contact with SPU and SPU-g-PAS films, respectively.

3. Results and discussion

3.1. HMDI grafting

In order to introduce free isocyanate groups (NCO) onto the SPU films, current methods of allophanate reaction of diisocyanate (HMDI) with the –NH groups of SPU surface were performed [43,44]. Fig. 2 shows the reflectance (ATR–FTIR) spectra of pure SPU, SPU-g-HMDI and SPU-g-PAS. In addition to the absorption peaks associated with pure SPU, the activated isocyanate SPU films showed a absorption at 2270/cm corresponding to NCO groups of HMDI grafted onto SPU films. The disappearance of this absorption band after reaction with PAS (Fig. 2c) indicates that the pharmaceutical compound reacted with NCO on the surface to form the polymeric drug SPU–PAS. Fig. 2(c) shows the ATR–IR spectra of SPU after PAS coupling. The sharp characteristic band of –NCO at 2270 cm^{-1} of grafted HMDI and vibrational NH_2 groups of PAS disappeared indicating that the NH_2 of PAS formed covalent bonds with the previously introduced NCO groups of the SPU-g-HMDI surface.

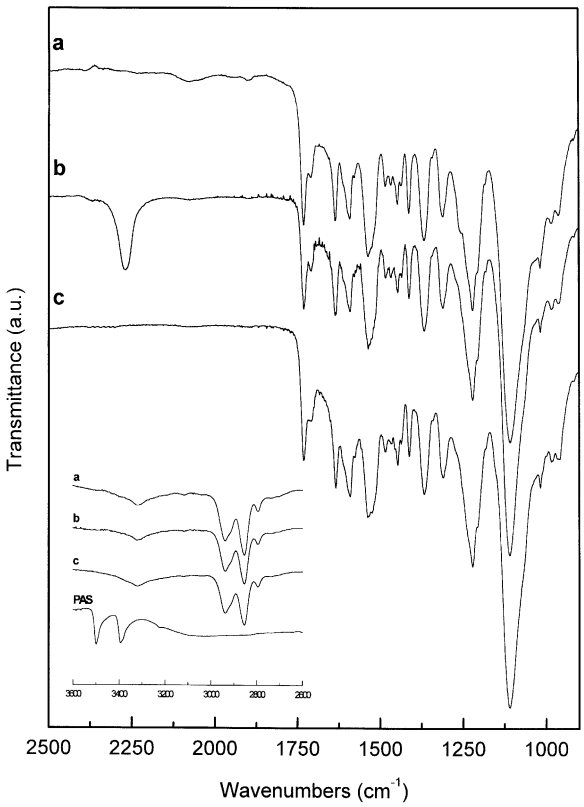

Fig. 2. ATR–FTIR spectra: (a) original SPU; (b) SPU-g-HMDI; (c) SPU-g-HMDI-g-PAS.

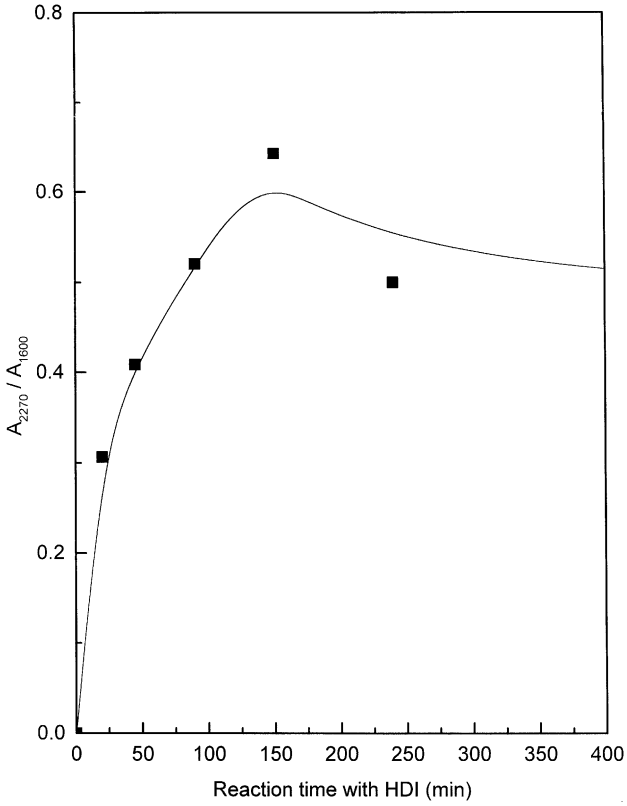

Fig. 3. Extent of the reaction with HMDI determined by ATR–FTIR.

The reaction with HMDI depends on various factors, such as the degree of swelling of the SPU film, solvent, reaction temperature and time, and the catalyst. Thus, Fig. 3 describes the reaction yield in relation to the reaction time. The reaction yield was estimated from the relative ratios of NCO absorbance at $2270\,cm^{-1}$ to aromatic rings (hard segments of SPU) absorbance at $1600\,cm^{-1}$ of the ATR–IR spectrum.

Thus, the yield decreased with a reaction time longer than 2 h. This fact may be due to isocyanate dimerization between the excess of HMDI remaining in the solution and NCO groups already introduced on the SPU surface [45].

3.2. PAS. immobilization

A large amount of work has been devoted during the past tree decades to the surface modification performed in order to immobilize anticoagulants, fibrinolytic enzymes or anti-platelet agents onto polymeric surfaces to attain better blood compatibility. The general idea involved was that the immobilization of substances known to inhibit in solution the development of a thrombus, or to lyse it, onto a surface could be the best way to obtain bioactive surfaces.

Clinicians are waiting for new drugs, which are capable of avoiding and therefore preventing the adherence and activation of platelets without adverse effects.

Due to the aminocarboxylic acid structure, PAS may be coupled to the free –NCO groups introduced on SPU surfaces through nucleophilic reactions with $-NH_2$ groups (Fig. 1). This coupling technique is simple, reliable and turned feasible the drug immobilization without their degradation.

Fig. 4 shows the result of PAS immobilization onto the SPU films activated by HMDI. The immobilization reaction time and the concentration of PAS solution were in all the cases kept constant to 24 h and 1.0 mg/ml, respectively. The amount of PAS immobilized gradually increases with the activation time and approaches a constant around $1.2\,\mu g/cm^2$, implying that the immobilization appears to be close to that of the monolayer covering of PAS molecules. We may assume that after saturation they are covalently immobilized on the outermost surface of SPU films, as a full monolayer, when the SPU film has previously been grafted with HMDI in toluene. However, very long grafting time (very long activation) is not good, because the film activated for longer than 90 min became turbid, probably due to a side reaction of HMDI, such as polymerization mediated by trace of water in the reaction mixture.

Although –NCO groups is sensitive to moisture and subject to decomposition into amine and CO_2 via an

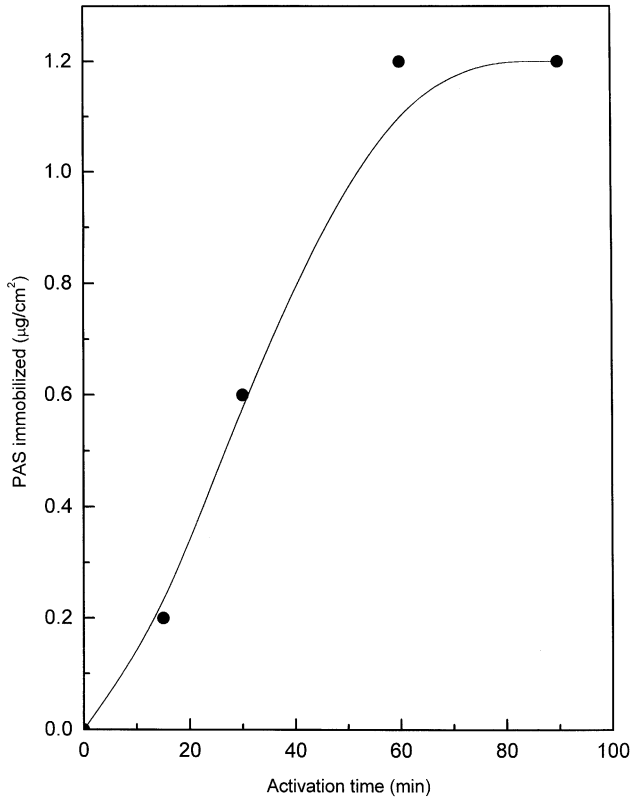

Fig. 4. Effect of the isocyanate activation time on PAS immobilization onto SPU films.

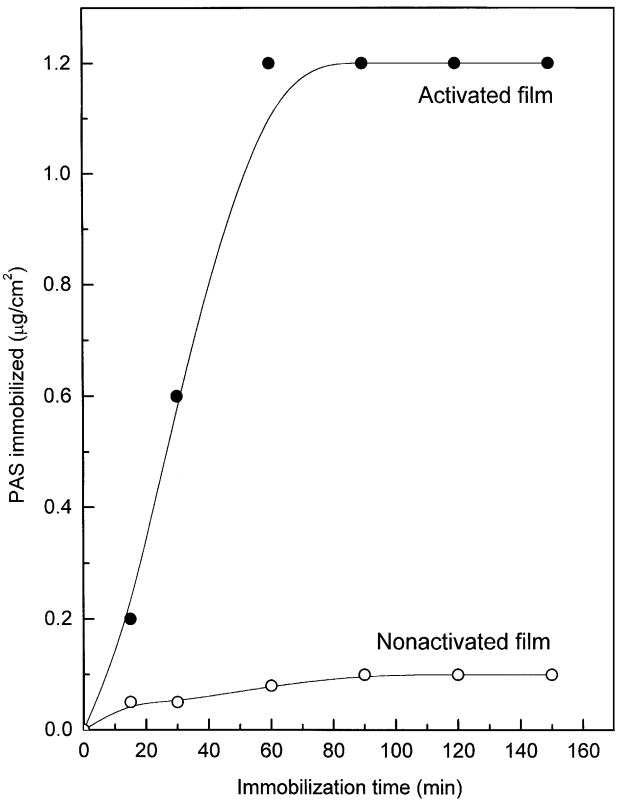

Fig. 5. Immobilization of PAS onto SPU films activated by HMDI (activation time: 150 min; PAS concentration: 1.0 mg/ml).

unstable carbamic acid, Fig. 5 shows clearly that the coupling of PAS is possible in the presence of water. This might be due to the higher reactivity of the NCO groups with $-NH_2$ than with water [46].

As shown in Fig. 5, the result obtained for the films with and without activation clearly demonstrates that the PAS immobilization is due to covalent binding, not to physical adsorption.

It was also confirmed that immobilized PAS was covalently bound onto SPU-g-HMDI surfaces. To examine the stability of immobilized PAS, the activated polyurethane film containing the immobilized drug and the nonactivated film containing the sorbed drug were immersed in a PBS (pH = 7.4) at 37°C for 48 h and the amount of PAS released from the substrate was measured by UV spectroscopy ($\lambda_{maxPAS} = 298$ nm).

Fig. 6 shows that the amount of physically adsorbed PAS on the nonactivated SPU surfaces was almost completely released within 10 hours. This finding clearly demonstrates that PAS is immobilized onto isocyanate activated polyurethane films by covalent bonding.

3.3. Contact angle measurements

Water plays an important role in determining the biocompatibility characteristic of the synthetic material. It is well known that high water levels on the surface of the biomaterial providing a low interfacial tension with blood, which would reduce fibrinogen adsorption and cell adhesion on the surface similarly to the biological tissues [47].

The hydrophilicity of the SPU films after the PAS immobilization may be indicated by use of contact angle measurements. Fig. 7 shows the relationship between the contact angle and the amount of PAS coupled to SPU films. As shown in this figure, the results revealed that the water contact angle of SPU ($70.3° \pm 1.9°$) was decreased by the introduction of PAS ($43.1° \pm 2.1°$), indicating the obtention of more hydrophilic surfaces after coupling of the nonsteroidal drug (PAS). This increase in hydrophilicity to the SPU surface is particularly desirable in biomedical applications of the SPU-g-PAS.

3.4. Water sorption and diffusion

It is well known that the biocompatibility of the polymeric materials may, in part, be due to their ability to swell in aqueous media. The sorption or water diffusion allows the diffusion of tissue metabolites within the polymer and probably markedly alters the morphology of the polymer surface which is in contact with blood or tissue in a living organism.

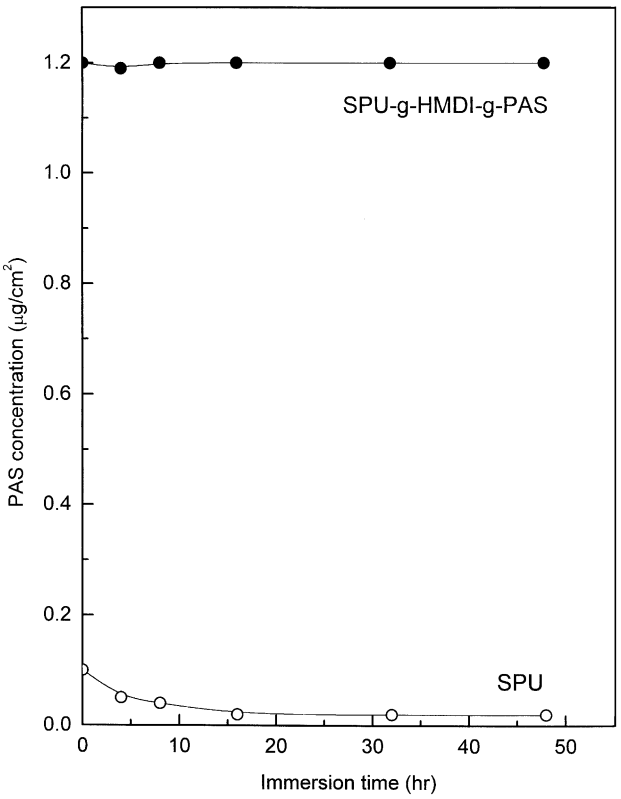

Fig. 6. PAS concentration bound to the SPU and SPU-g-HMDI-g-PAS surfaces as a function of immersion time in PBS (pH = 7.4) at 37°C for 48 h.

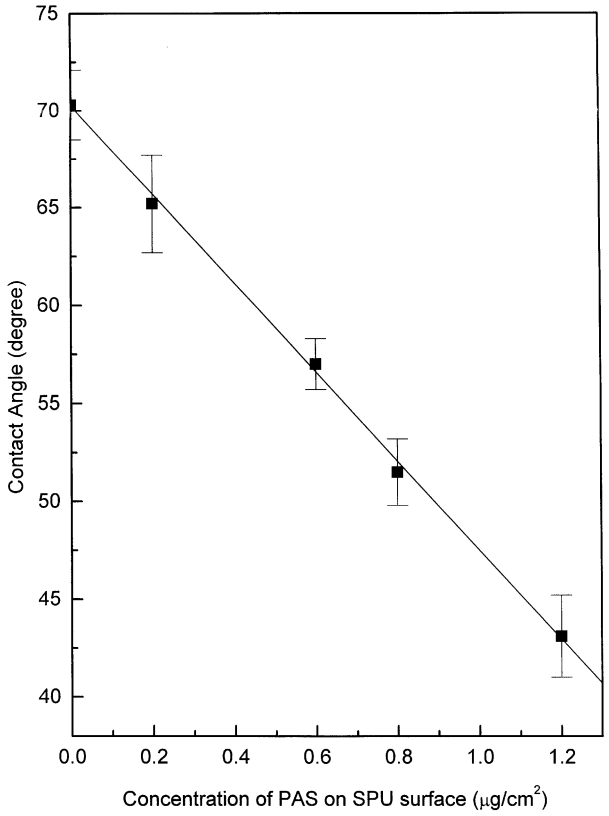

Fig. 7. Effect of PAS concentration on wettability of the SPU surfaces.

The sorption and diffusion of water into polymers has attracted much attention due to their potential applications in biomedical fields [48].

Water sorption results have been fitted to the empirical relationship [49]:

$$\frac{M_t}{M_\infty} = kt^n, \tag{3}$$

where k is a constant that is related to the structure of the network, the exponential n is a number to determine the type of diffusion and M_t and M_∞ are the mass uptake values at time t and at equilibrium, respectively.

From a least-square analysis of the log M_t/M_∞ vs. log t, the value of the exponential n was obtained. In the present investigation, the number necessary to determine the type of diffusion (n) was found to be 0.42 and 0.53 for SPU and SPU-g-PAS, respectively, that is suggestive a Fickian transport mechanism [50].

The amount of water sorbed by the polymeric systems is given by Eq. (3) [48]:

$$\frac{M_t}{M_\infty} = 1 - \frac{8}{\pi^2}\sum_{n=0}^{\infty}\frac{1}{(2n+1)^2}\mathrm{e}^{(-[D(2n+1)^2\pi^2/h^2]t)}. \tag{3'}$$

Thus, the water diffusion coefficient would be easily calculated from the late-time approximation of Eq. (3′), written as

$$\ln\left(1 - \frac{M_t}{M_\infty}\right) = \ln\left(\frac{8}{\pi^2}\right) - \frac{D\pi^2 t}{h^2}. \tag{4}$$

According to Eq. (4), the plot of $\ln[1 - M_t/M_\infty]$ vs. t should be linear at long diffusion times, and slopes of these lines are proportional to diffusion coefficients.

As shown in Fig. 8 the water diffusion coefficients linearly decreased with increasing PAS content on the SPU films indicating that the motional freedom of the water molecules becomes more restricted.

As is evident from the data in Fig. 9, the level of water uptake increases as the increasingly content of PAS on the SPU surface. Since PAS contains carbonyl and hydroxyl groups, the ionization of these groups in PBS solution may turn the polymeric surface more easily hydrated. Thus, the values of the diffusion coefficient at a low weight fraction of water on the polymeric films may indicate that the water molecules still possess a high degree of translational mobility and are not "tightly bound" or "immobilized" in the SPU-g-HMDI-g-PAS structures.

The activation energy, E_D, for the process of diffusion is estimated from the Arrhenius relation [51]:

$$D = D_0\mathrm{e}^{-E_D/RT} \tag{5}$$

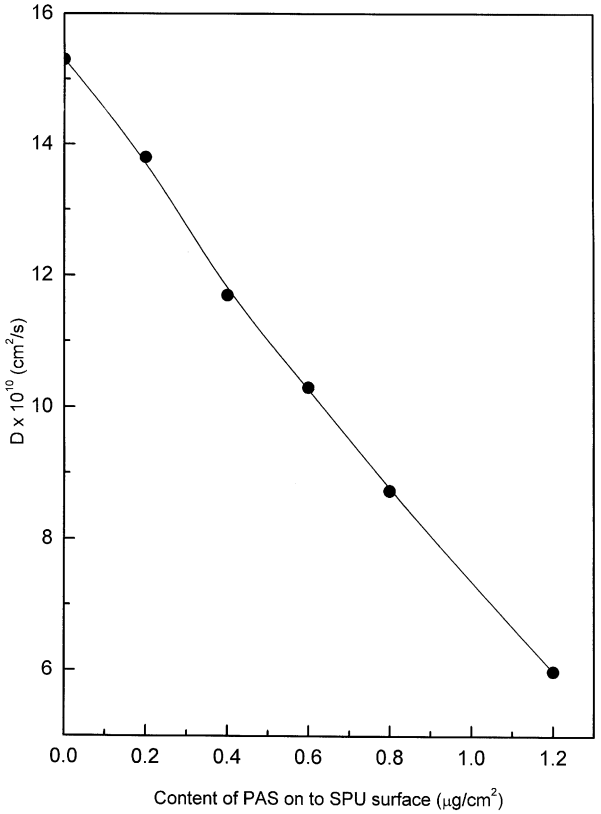

Fig. 8. Dependence of water diffusion coefficients of the PAS composition on SPU films ($T = 310$ K).

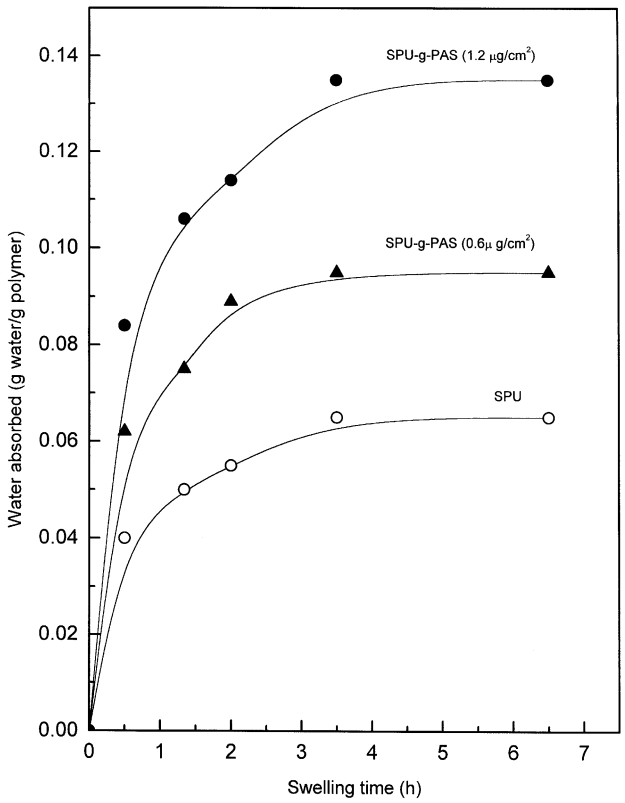

Fig. 9. Water uptake per dry polymer weight (g/g) for SPU and SPU-g-PAS.

where D_0 is the preexponential factor, R the molar gas constant, T the absolute temperature and E_D is the activation energy required to produce an opening between polymer chains segments large enough to allow the water to diffuse.

There is a systematic increase in the E_D values with the increasing of PAS on the PU surface as can be see in Fig. 10. These results could be explained on the basis of Eyring's hole theory according to which the energy required "to open a hole" in the polymer matrix to accommodate a diffusing molecule bears a direct relationship with E_D [52].

Thus, the higher E_D values with the increase of PAS in the SPU may be interpreted in terms of the difficulty associated with the movement of the aggregates of water molecules through the polymer matrix. This difficulty may be associated to both intra- and inter-chain forces between PAS and SPU that must be overcome in order to create space for a unit diffusional jump of the water molecules.

The heat of sorption, ΔH_s, is a parameter involving both Langmuir and Henry sorption mechanisms. Henry's law mode requires both, the formation of a site and dissolution of species into that site. Formation of a site involves an endothermic contribution to this process. In contrast, in the case of Langmuir mode, the site already exists within the polymer matrix so that sorption by a hole filling mechanism produces more exothermic heat of sorption.

The heat of sorption can be estimated by the van't Hoff relation. Thus, at longer sorption time the term $n \geqslant 1$, as well as $\ln 8/\pi^2$ in Eq. (4), may be ignored to give:

$$\ln \frac{M_\infty}{M_\infty - M_t} \cong \frac{\pi^2 D t}{h^2}, \tag{6}$$

where $k = \ln [M_\infty/(M_\infty - M_t)]/t$, is the first-order kinetic rate constants if the water sorption in the films follow first-order kinetics [53]. Thus, the temperature-dependent equilibrium sorption constant, k, values may be fitted to the vant'Hoff relation to estimate the heat of water sorption (ΔH_s).

It may be noted from Fig. 11 that the ΔH_s values are negative for all the systems, which suggests that the sorption is mainly dominated by a Langmuir mode given an exothermic contribution to the sorption process, that is, the amount of absorption at a particular relative pressure decreases with increasing temperature.

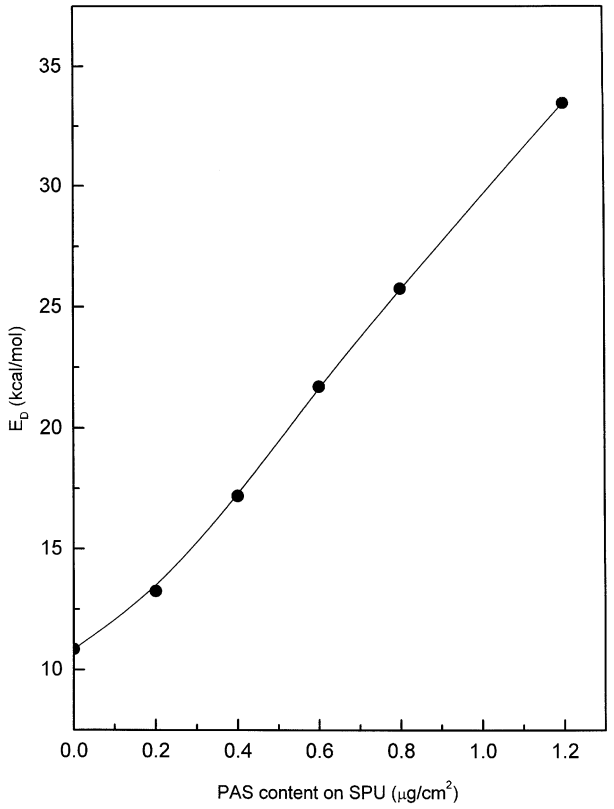

Fig. 10. Activation energy (E_D) for water diffusion (PBS pH = 7.4) on SPU films with different compositions of PAS.

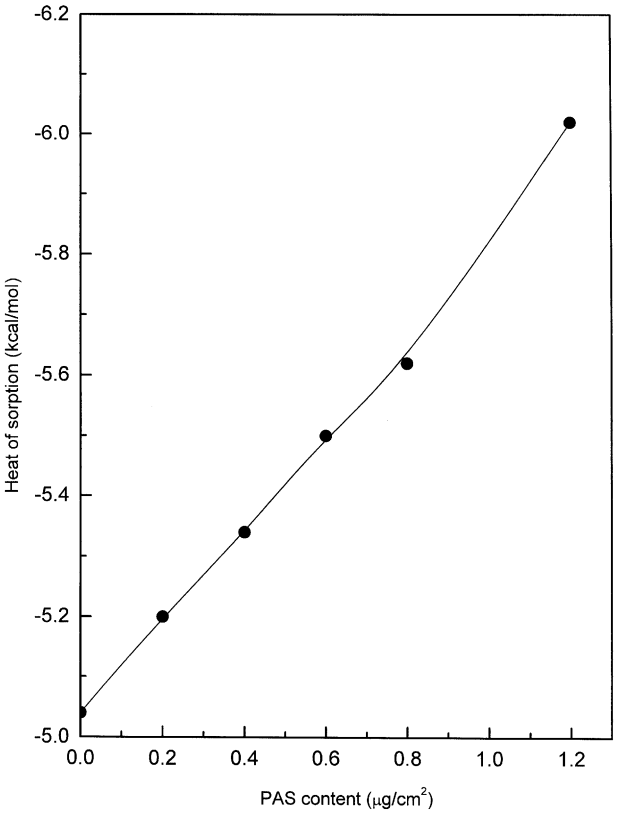

Fig. 11. Dependence of the water (PBS, pH = 7.4) heat sorption by SPU films in function of PAS content.

3.5. Fluorescence spectroscopy

Other evidence for introduction of PAS onto SPU surface was provided by fluorescence spectroscopy. Fig. 12 shows intrinsic surface fluorescence spectra of PAS (0.2 M sodium bicarbonate) and SPU control as well as the segmented polyurethane containing covalently coupled PAS (SPU-g-PAS). The observed strong fluorescence emission band with a λ_{max} is contributed mainly by aromatic ring of the PAS on the polymeric surface. The λ_{max} shows a red shift with the PAS coupling; for example, λ_{max} was 400 nm for the control PAS (in solution) but 412 nm for the coupled PAS onto SPU surface. The observed red shift in λ_{max} with PAS coupling (Fig. 12) suggest changes in the environment of PAS after reaction with NCO groups onto SPU surface.

The layer of immobilized PAS is stable in aqueous solution, on the other hand, the aromatic rings remain on the polymeric surface when the grafted SPU films are exposed to water solution. Thus, the interaction of the carboxylic and OH unreacted groups with water molecules may result in a red shift of λ_{max} [54]. Thus, the immobilization reaction between PAS and –NCO groups on SPU surfaces tend to orient the hydrophilic functional groups (e.g. carboxyl and hydroxyl) to a higher contact with polar molecules of water.

3.6. Plasma protein adsorption

Biomaterials in contact with blood are limited in their usefulness primarily because of thrombus formation at the blood–material interface, which is triggered by the preferential adsorption of some plasma proteins [55].

The nature of the proteinaceous film deposited on a biomaterial surface following implantation is a key determinant of the subsequent biological response. Two proteins were selected for the study: bovine serum albumin (BSA) and bovine plasma fibrinogen (Fbg). Albumin is the preponderant protein in blood, its abundance outweighing all the rest of plasma proteins [56]. Adsorption of this protein has a profound influence on the succeeding events of the blood coagulation cascade and a reduced platelet adhesion has been reported for polymers which adsorb relative amounts of albumin [57–59]. Fibrinogen plays a central role in hemostasis participating not only in the coagulation cascade, but it also promotes adhesion of platelets and activates then when adsorbed onto certain solid surfaces [60,61].

Fig. 13 shows the effect of the PAS content on BSA adsorption relatively to Fbg. It is seen that the ratio of albumin adsorption to fibrinogen increased with the

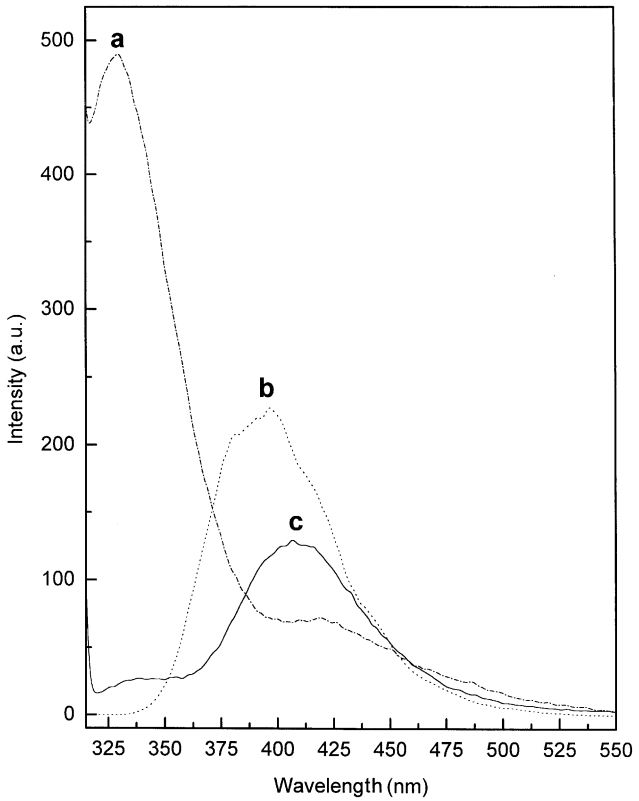

Fig. 12. Fluorescence spectra of (a) SPU; (b) PAS and (c) SPU-g-PAS.

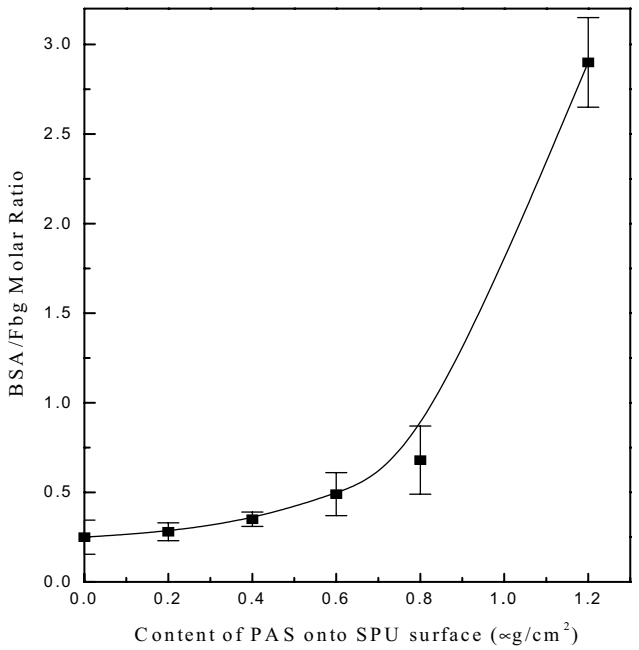

Fig. 13. Effect of PAS content on the BSA/Fbg molar ratio, $p<0.0001$. p: confidence limits. Each assay represents the average value of at least five single measurements.

PAS content on the SPU surface and suggesting that the selectivity of albumin relative to fibrinogen is greater for the materials containing PAS.

3.7. Assay of intrinsic coagulation activation (Factor XII)

The contact phase between blood and a polymeric surface comprises a large complex numbers of reactions which take place during the first times of initial blood contact and overlap with protein adsorption or displacement and boundary layer development [62].

In biomaterials, Factor XII is central to the reactions of the blood contact phase. A recent review on the theoretical understanding of the events which take place during the interaction of biomaterials with blood and the importance of Factor XII as blood contact phase has been reported by Basmadjian et al. [63].

Factor XII activation induces a series of intrinsic coagulation system chain reactions that are capable of triggering coagulation in the presence of a surface. Thus, in this study, the amount of activated Factor XII was evaluated from the kallikrein produced and Fig. 14 indicates kallikrein activity induced by plasma–SPU interaction. Less kallikrein was observed on the SPU-g-PAS films than on the nonmodified SPU films. Thus, these results indicate that intrinsic coagulation Factor XII is less active on the segmented polyurethane containing immobilized PAS.

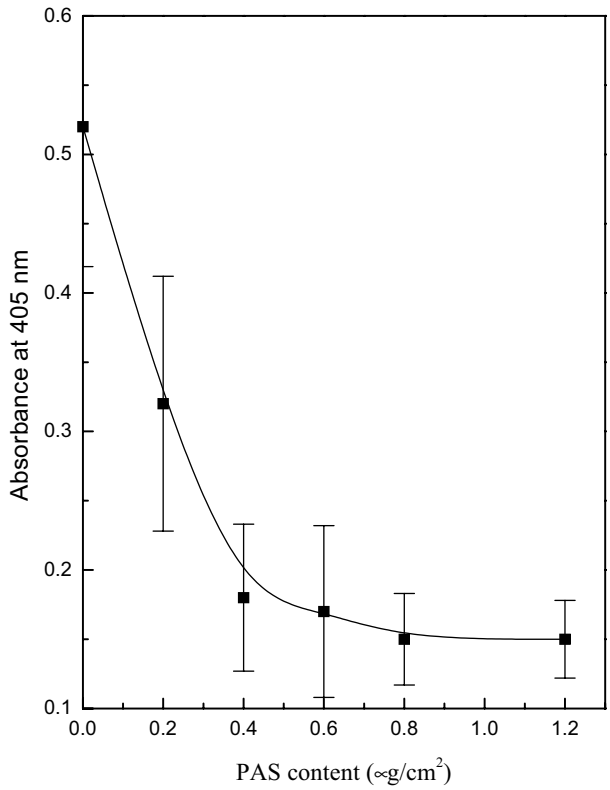

Fig. 14. Kallikrein activity induced by human plasma and PAS content on SPU films, $p<0.00035$. p: confidence limits. Each assay represents the average value of at least five single measurements.

3.8. Thrombus formation

A major complication in the use of blood-contacting materials is the formation of a thrombus at the blood–surface interface [64]. Thus, an important method to obtain more thromboresistant polymers is the modification of polymeric surfaces by covalent immobilization of antithrombotic agents that may reduce platelet adhesion and or coagulation [65]. Due to their pronounced effects in preventing platelet aggregation and possibly reduced activation with minor thrombus formation, PAS was covalently immobilized on the activated SPU surfaces of SPU-g-HMDI.

The thrombus formation decreased drastically with the PAS immobilization (Fig. 15) and this agreed with Figs. 13 and 14. As demonstrated in Fig. 13, the PAS immobilization onto SPU surfaces considerably reduces fibrinogen adsorption on the films. This indicates that the PAS immobilization procedures onto SPU surfaces delay the contact activation of intrinsic coagulation mechanism as shown in Fig. 14.

3.9. Platelet adhesion

It is well known that the surface chemistry is very important on the blood compatibility of the polymeric surfaces. Several functional groups have been studied with to the hemocompatibility or thromboresistance properties and was observed that those surfaces containing aromatic carboxylic acids presents higher blood clotting times as well as lower platelet interaction and activation. In this sense, the PAS with their structure of aromatic carboxylic acid may be a potential candidate to inhibit the trombogenicity of a synthetic surface.

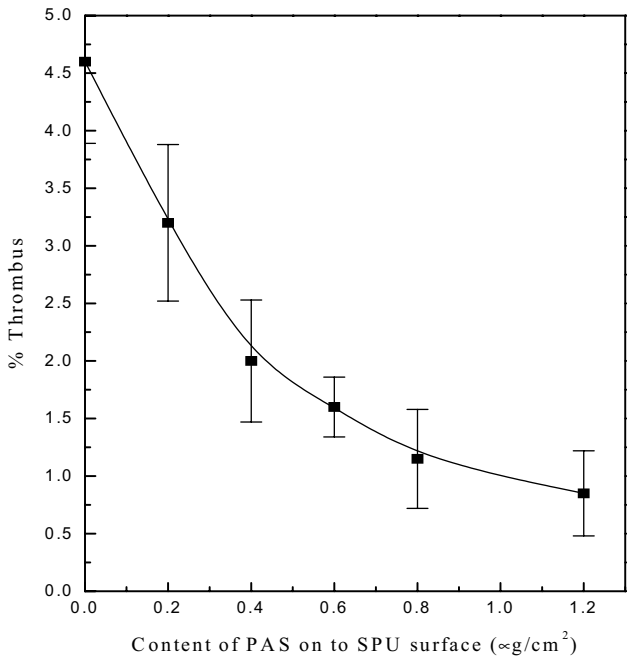

Fig. 15. Effect of PAS content on SPU films on blood coagulation kinetics, $p<0.00025$. p: confidence limits. Each assay represents the average value of at least 10 single measurements.

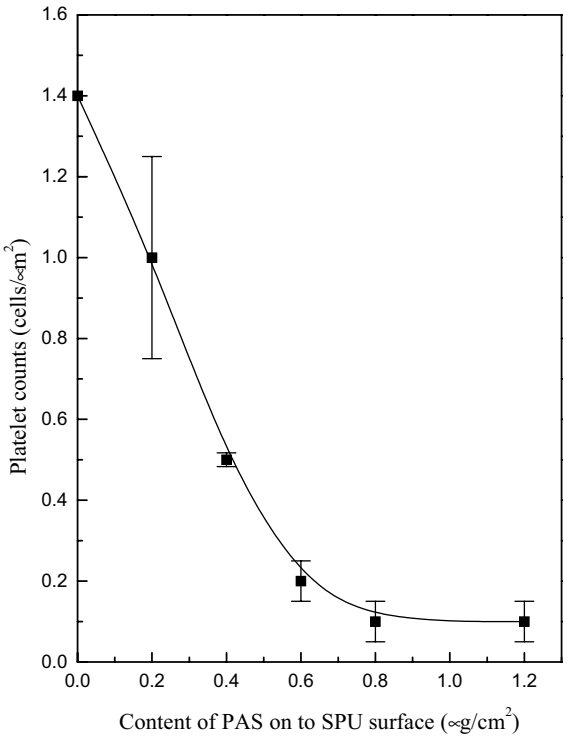

Fig. 16. Content PAS dependence of adherent platelet numbers onto PAS modified SPU films, $p<0.00015$. p: confidence limits. The average number of adhered platelets was obtained from five photographs of different surface areas of the same sample.

Relationships between platelet adhesion and amount of PAS on the SPU surfaces are shown in Fig. 16. SPU surfaces containing immobilized PAS shown significant effects on the platelet adhesion. The platelet adhesion for SPU-g-PAS surfaces decreased with increasing amounts of PAS on the polymeric surfaces and have therefore, better blood compatibility then SPU surfaces.

As cited above, when an artificial 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. Thus, surface-induced platelet activation is largely dictated by the type and the amount of blood proteins adsorbed at the biomaterial/blood interface. In this sense, preferentially adsorption of fibrinogen from the blood is known to accelerate platelet adhesion and activation onto foreign surfaces. On the other hand, albumin adsorption on the synthetic surfaces can inhibit platelet activation and, therefore, does not promote clot formation [66,67].

The observed reduction platelet adhesion with increase amounts of PAS on the SPU surfaces may be due to the fact that these polymeric surfaces adsorb

higher amounts of albumin relatively to the fibrinogen [68].

4. Conclusions

It was shown that the nature of polymer surface may be used to the design of synthetic surfaces with good biocompatibility properties. The PAS immobilization onto SPU surface was an important factor for the selectively adsorption of plasma proteins and consequently the platelet adhesion and activation processes. The higher albumin adsorption relatively to the fibrinogen by SPU-g-HMDI-PAS surfaces suggests a better blood compatibility properties in relation to SPU films. Both assays, Factor XII and the platelet adhesion characteristics of the modified SPU surfaces showed a significantly reduced platelet adhesion and a less activated Factor XII characteristics as compared with those of the unmodified SPU surface. These results suggest that the coupling PAS improves the antithrombogenicity of SPU surfaces. However, more detailed metabolic effects (active adhesion) of blood proteins and cells ex vivo or in vivo should be investigated to discuss real-time nonthrombogenic properties of SPU-g-PAS surfaces in whole blood.

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