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Biological performance of two materials based on sulfated hyaluronic acid and polyurethane

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Polyurethane bound with sulfated hyaluronic acid was synthesized by two different chemical routes. Both the materials obtained consist of a hydrophilic component, sulfated hyaluronic acid ($HyalS_{3.5}$), and a hydrophobic component, polyurethane (PU). In the material named Puhmdi, the $HyalS_{3.5}$ was cross-linked to the PU chains *via* hexamethylene diisocyanate (HMDI) while in that named Pubrac, the binding of $HyalS_{3.5}$ to the PU chains occurred only through a few carboxy groups *via* *N*,*N*′-dicyclohexylcarbodiimide (DCC) and bromoacetic acid.

The surface characteristics of the polymers were investigated by ATR FT-IR spectroscopy while the surface morphology was analyzed by scanning electron microscopy.

There was a significant difference between the surface characteristics of the films in dry and hydrated environments. In both materials the hydrophilic component ($HyalS_{3.5}$) migrates from the bulk to the surface, thus minimizing the surface free energy of the polymer when exposed to the hydrated environment. The different biological behavior of the two materials was demonstrated with the thrombin time test and platelet adhesion test. Pubrac inhibits the coagulation process while Puhmdi does not.

1 Introduction

Synthetic polymers have been widely used for a variety of biomedical applications, including the vascular replacement system. The physiochemical and mechanical characteristics of such polymers are generally designed to be appropriate for their proposed function, but the polymers do not necessarily possess optimal biocompatibility. Thus modification of the surface and/or the bulk of the material plays a very important role in biomaterials research.

Numerous alternatives for surface modification have been attempted in order to create materials with specific biological properties.[1,2] Surface modification has the advantage of generally maintaining the size, shape and mechanical properties of the original material. However the amount of bioactive compound bonded to the surface is generally low.

In the last few years, several investigations have been undertaken to develop new polymer materials containing biologically active residues. Through bulk modification, this procedure allows one to increase the amount of the bound bioactive molecule.

Several methods have been used to synthesize such new materials, which exhibit different chemical and physical properties. For instance, appropriate polymer matrices have been modified by the reaction with polysaccharide derivatives or by the introduction of specific functional groups.[3–9]

Several attempts have also been made to bind hydrophilic active compounds to hydrophobic polymers. A restructuring of the material's surface generally takes place as a function of the environment. In a hydrophilic environment (*e.g.* physiological media) the hydrophilic component of the material migrates to the surface, thus increasing its concentration at the biological system–material interface.[10]

Among the numerous biomedical polymers, polyurethanes have been widely used in blood-contacting applications.[11] Nevertheless their uses in dedicated applications, *i.e.* small-diameter vascular grafts, catheters, cardiac-assist devices and artificial hearts, require an improved blood compatibility. A commonly accepted method to improve the compatibility is to bind molecules with the required biological activity to the polyurethane (PU) chains.

Diisocyanates have been the most widely used cross-linking agents in polyurethane chemistry. They are able to cross-link the PU polymer chains both among themselves and with those of other polymers. For this reason, they have been used in the binding of several kinds of biomolecules to PU chains.[12–15]

In research on blood-contacting materials, attention has been particularly focused on the binding of heparin or heparin-like compounds to polyurethane matrices in order to create hemocompatible materials with excellent mechanical properties.[16] Recently a new heparin-like molecule has been synthesized by the sulfation of hyaluronic acid;[17] one of its properties is resistance to enzyme degradation.[18,19]

This paper deals with the synthesis of new materials in which the sulfated hyaluronic acid molecule is linked to PU chains *via* two different chemical routes. We studied the behavior of the materials in both dry and wet environments to assess whether surface restructuring occurs in physiological conditions. We also performed *in vitro* biological tests to assess the bioactivity of the sulfated hyaluronic acid linked to the PU.

2 Experimental

2.1 Materials

Tetrabutylammonium hyaluronate (Hyal-TBA, Mw ~ 180.000) was donated by Fab (Fidia Advanced Biopolymer, Abano Terme, Padua, Italy). Polyurethane (Pellethane2363-80AE) was purchased from Upjohn Polymer (Japan). All the other reagents were purchased from Fluka Chemie AG. (Switzerland).

2.2 Methods

The sulfation of hyaluronic acid was performed by the previously described procedure.[17] The sulfation degree of the compound used, determined by elemental analysis and HPLC,[20] was 3.5 per disaccharide unit ($HyalS_{3.5}$).

Sulfated Hyal-TBA ($HyalS_{3.5}TBA$) was obtained with an ion exchange resin (Dowex 50 WX 8) charged with TBA, by the following procedure.

250 mg of sulfated hyaluronate sodium salt was dissolved in the minimum amount of distilled water and then added to about 700 mg of resin at pH 9. The mixture was gently stirred at 5 °C for 3 h. The resin was then removed by filtration and the solution obtained was lyophilized and stocked in an anhydrous atmosphere.

2.2.1 Method 1: synthesis of Pubrac. The synthesis involved two reaction steps (Fig. 1).

1) 2 g of *N*,*N*′-dicyclohexylcarbodiimide (DCC) was added to 30 ml of polyurethane solution (10% w/w) in DMF. After the slow addition (drop by drop) of a solution of 2 g of bromoacetic acid in 5 ml of DMF, the mixture was stirred for 1 h at room temperature. At the end of the reaction, a white precipitate of *N*,*N*′-dicyclohexylurea (DHU) was removed by filtration.

2) 250 mg of $HyalS_{3.5}$ TBA solution and 2 g of $NaHCO_3$ were added to the DMF solution obtained in step 1. The mixture was stirred at 45 °C for 36 h.

The Pubrac material was obtained as a sheet by casting the solution onto a glass plate under vacuum (400 mbar) at 55 °C.

The material was then washed in acetone, in water and in a 1 M NaCl solution to remove non-reacted compounds.

The smoothness of the Pubrac material was improved by further dissolution of the material in tetrahydrofuran (THF) and subsequent casting.

2.2.2 Method 2: synthesis of Puhmdi. The synthesis of Puhmdi involved two reaction steps (Fig. 2).

1) 250 mg of $HyalS_{3.5}$ TBA was dissolved in the minimum amount of DMF and the solution was added to 200 μL of hexamethylene diisocyanate (HMDI) in a nitrogen atmosphere. The mixture was then stirred for 30 min at room temperature.

2) 30 ml of the polyurethane solution (10% w/w in DMF) was added to the previous solution in a nitrogen atmosphere and the mixture was stirred for 48 h at 50 °C.

The Puhmdi material was obtained as a sheet by casting the mixture onto a glass plate under vacuum (400 mbar) at 55 °C.

The material obtained was washed in acetone, in water and in a 1 M NaCl solution to remove non-reacted compounds.

3 Chemical characterization

3.1 Scanning electron microscope (SEM) analysis

The materials were dehydrated under vacuum and gold sputtered with an automatic sputter coater (BAL-TEC SCD 050, Balzers, Germany) at 10^{-1}–10^{-2} mbar of pressure in argon for 120 s. The scanning electron microscope (XL 20, Philips, The Netherlands) analysis was performed at 15 kV.

3.2 FTIR-ATR analysis

ATR spectra of the samples in the dry and hydrated form were recorded on a Perkin-Elmer M 1800 FTIR spectrometer between 4000 and 750 cm^{-1}. The hydrated sample was obtained by exposure of the material to an environment with controlled humidity for 24 h ($Na_2SO_3 \cdot 7H_2O$, relative humidity $\cong 95\%$).

An MCT detector was used and the apparatus was purged with nitrogen. Typically, 300 scans at a resolution of 2.0 cm^{-1} were averaged. The frequency scale was internally calibrated with a reference helium–neon laser to an accuracy of 0.01 cm^{-1}. A KRS5 crystal was used at an angle of 45°.

4 Biological characterization

4.1 Blood collection

Selected donors were normal, healthy people who had fasted for more than 8 hours and had not received any medication for at least 14 days. Blood samples were collected in 3.8% (w/v)

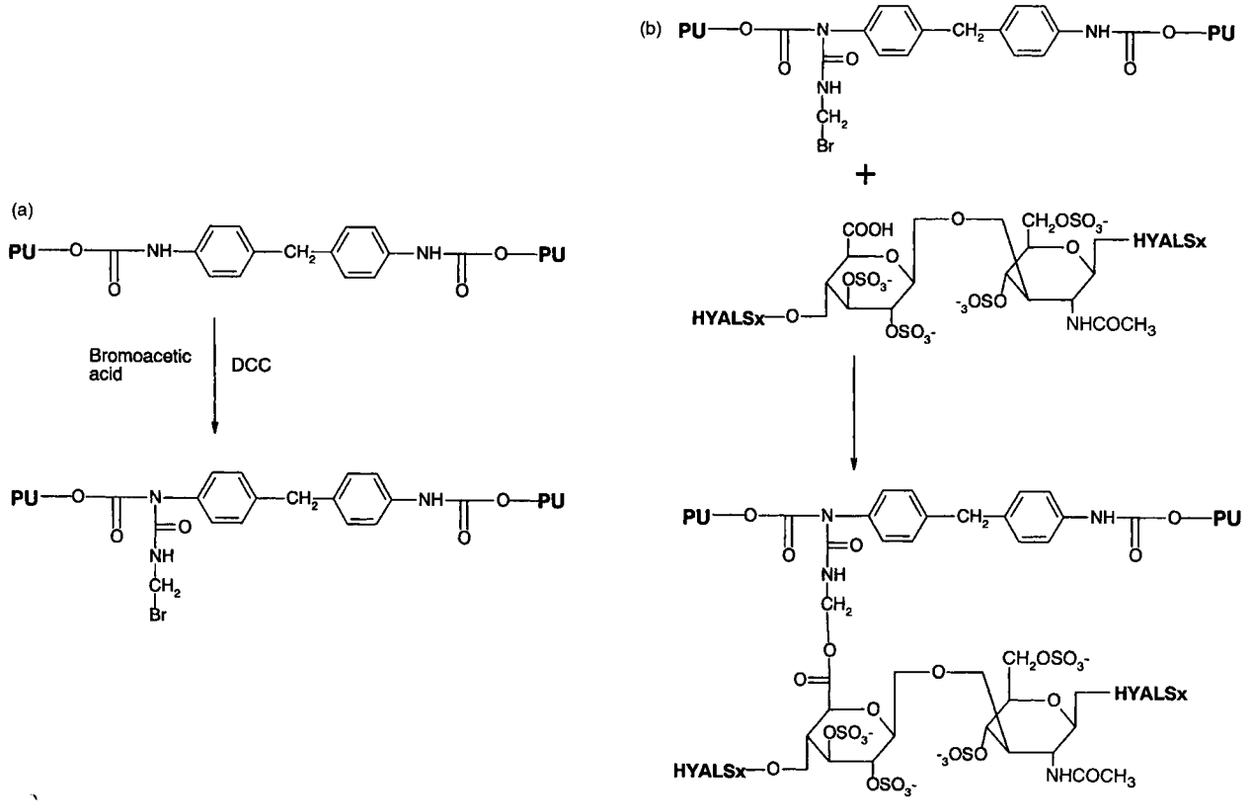

Fig. 1 Scheme of Pubrac synthesis: a) PU functionalization, b) S_N2 substitution.

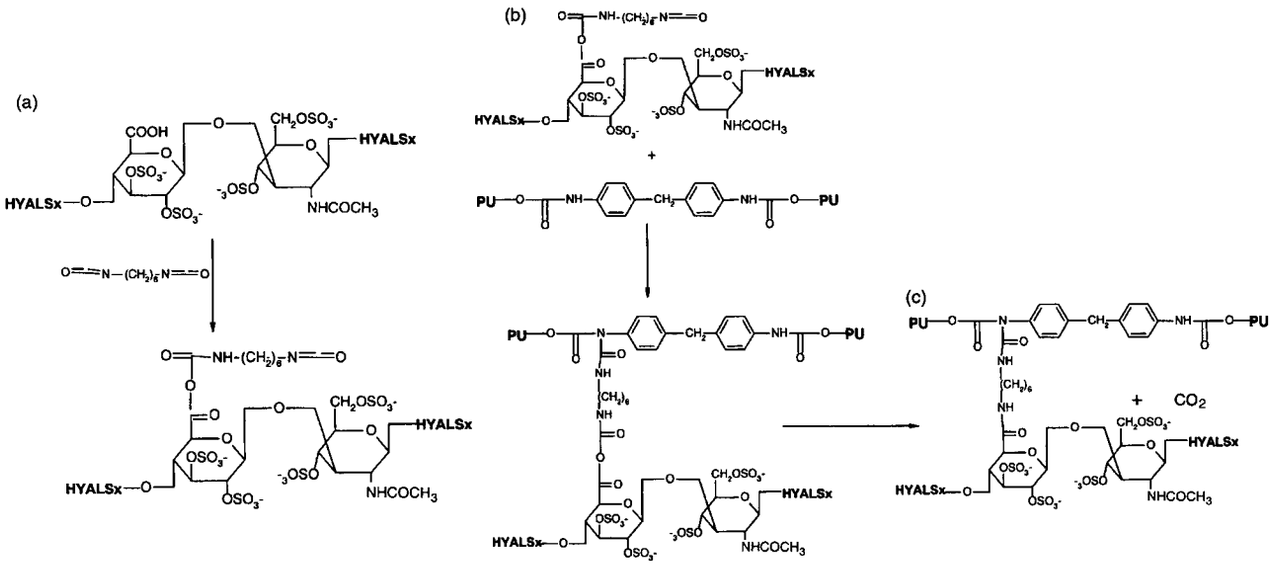

Fig. 2 Scheme of Puhmdi synthesis: a) $HyalS_{3.5}$ functionalization, b) PU cross-linking, c) CO_2 elimination.

trisodium citrate as anticoagulant at a volume ratio of 9 parts blood to 1 part citrate. Platelet-rich plasma (PRP) was generated by spinning whole blood at 150 g for 15 min at room temperature.

4.2 Whole blood coagulation time (WBCT)

3 ml of whole blood (without anticoagulant treatment) was added to a test tube whose internal surface was covered with the material. The same procedure was performed on a non-covered test tube as a control. The test tubes were gently shaken. The formation of the thrombus was verified by direct observation and the time was measured with a chronometer. The measurement was repeated three times for each sample.

4.3 Thrombin time (TT)

2 ml of human plasma was added to a small circular portion of material (= 0.8 cm) and incubated at 37 °C for 10 min. 0.2 ml of human thrombin was added to the plasma in contact with the sample. The formation of the clot was revealed manually and the TT was measured with a chronometer. The measurement was repeated five times for each sample.

The TT was also determined for plasma alone, after it had been in contact with the material for 10 min at 37 °C, in order to check for the release of $HyalS_{3.5}$ from the material.

The reptilase time (RT) was also measured to exclude the possibility of aspecific adsorption of fibrinogen on the materials.

4.4 Platelet adhesion

1 ml of PRP was added to a small piece of material (1 × 1 cm) and incubated for 3 h at room temperature. Each sample was washed in phosphate buffer to remove the non-adhering platelets and then treated with a glutaraldehyde solution 2.5% (w/w) and sodium cacodylate solution 100 mM at pH 7.4 for 30 min. The samples were than washed in 100 mM cacodylate buffer for 30 s, rinsed with distilled water, and left standing in primary dehydration solution [70% (v/v) ethanol in H_2O] for 15 min. They were then transferred to a secondary dehydration solution [90% (v/v) ethanol in H_2O] for 15 min and subsequently absolute ethanol for 15 min for a total platelet dehydration. Finally the samples were desiccated overnight under a vacuum, gold sputtered, and analyzed by SEM.

5 Results and discussion

5.1 Synthesis

The Pubrac and Puhmdi materials were both synthesized *via* a two-step reaction, as shown in Fig. 1 and 2.

In the first step of the Pubrac synthesis (Fig. 1a) the polyurethane functionalization with bromomethyl groups was obtained through the reaction between the NH-urethane group and bromoacetic acid by *N*,*N*′-dicyclohexylcarbodiimide (DCC). The reaction proceeded with the formation of an amidic bond between the carboxy group of bromoacetic acid and the NH-urethane group of PU.[21,22]

In the second step (Fig. 1b) S_N2 reaction took place[23,24] between the COO^- group of $HyalS_{3.5}$ and the modified-PU through the formation of an esteric bond.

The reaction was performed with a bromoacetic acid/DCC molar ratio of 1.5. The excess of bromoacetic acid is necessary to eliminate all the DCC from the reaction moisture. The success of the second-step reaction (Fig. 1b) was demonstrated by the presence of bromine ion in solution, detected by the addition of silver nitrate solution.

In the synthesis of Puhmdi the first-step reaction (Fig. 2a) consisted of the functionalization of the $HyalS_{3.5}$-TBA salt by hexamethylene diisocyanate. The reaction proceeded with the formation of an anhydride bond between the aliphatic diisocyanate moiety and the aliphatic carboxy group. Once formed, the anhydride compound decomposes (Fig. 2b) at room temperature into amide and CO_2.[25]

In the second step the functionalized $HyalS_{3.5}$ reacts with the NH-urethane group through the formation of an allophanate bond.

This reaction was carried out in an excess of HMDI, which may also induce a cross-linking between the polyurethane chains themselves.

The presence of immobilized $HyalS_{3.5}$ on both materials was revealed by the toluidine test: in both cases the surface of the material assumed a violet color diagnostic of the presence of sulfate groups.[26]

5.2 FTIR-ATR analysis

Representative IR spectra of the two materials are shown in Fig. 3 and 4. The assignments of the main wave numbers for Pubrac and Puhmdi are reported in Tables 1 and 2 respectively.

In Fig. 3 and 4 the spectra of Pubrac and Puhmdi, respectively, are compared with the spectrum of the native

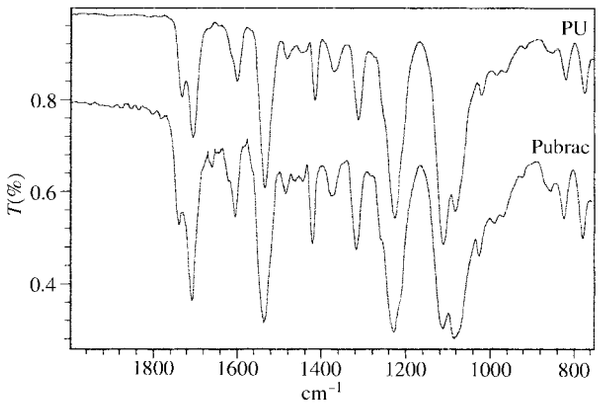

Fig. 3 ATR-FTIR spectrum of Pubrac.

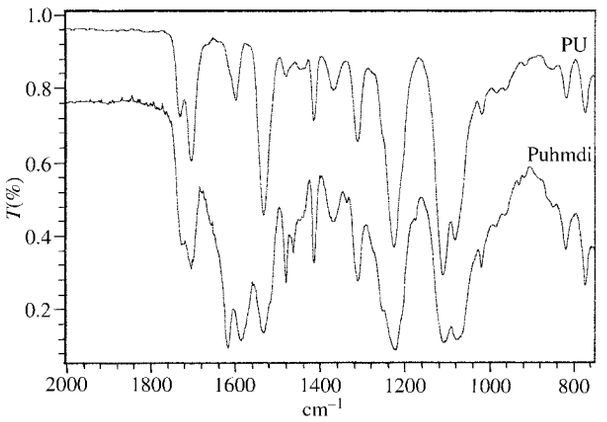

Fig. 4 ATR-FTIR spectrum of Puhmdi.

PU.[27,28] The largest differences between the two spectra are observed in the 1800–1500 cm^{-1} and 1300–900 cm^{-1} regions.

In the spectrum of Pubrac (Fig. 3) the absorption at 1654 cm^{-1}, assigned to the amide C=O stretching, can be related to the presence of $HyalS_{3.5}$.

In the spectrum of Puhmdi (Fig. 4) the bands at 1615 cm^{-1} and 1585 cm^{-1}, assigned to the amide I and amide II vibrations respectively, can be related to the presence of ureic carbonyl groups formed in the cross-linking reaction. The shoulder at 1650 cm^{-1} and the peak at 1631 cm^{-1}, assigned to the amide C=O stretching, can be related to the presence of $HyalS_{3.5}$.

Both materials show a shoulder at 1250 cm^{-1} and an increase of the intensity at 1020 cm^{-1} and 1000 cm^{-1} due to the S=O and SO_3^- stretching frequencies, respectively, of the sulfate groups present in the $HyalS_{3.5}$ molecules.

Table 1 Main wavenumbers observed in the IR spectra of Pubrac

Wavenumber/cm^{-1}[a]	Assignment
1732 s	Urethane C=O stretching (free)
1703 s	Urethane C=O stretching
1654 sh	C=O amide stretching
1615 m	Urea C=O + benzene C=C stretching
1597 m	Benzene C=C stretching
1530 s	CN + NH stretching (amide II)
1450 m	CH_2 bending
1413 m	Benzene C=C stretching
1250 m	S=O stretching
1220 m	CN + NH stretching (amide I)
1107 vs	C–O–C stretching
1076 s	O=C–O stretching
1064 m	C–O–C stretching
1020 m	SO_3^- stretching
1000 m	SO_3^- stretching

[a] s = strong; m = medium; w = weak; vs = very strong; sh = shoulder.

Table 2 Main wavenumbers observed in the IR spectra of Puhmdi

Wavenumber/cm^{-1}[a]	Assignment
1732 s	Urethane C=O stretching (free)
1703 s	Urethane C=O stretching
1650 sh	Amide C=O stretching $HyalS_{3.5}$
1631 sh	Amide C=O stretching
1615 s	Urea C=O + benzene C=C stretching
1597 s	Benzene C=C stretching
1585 s	CN + NH stretching
1530 s	CN + NH stretching (amide II)
1450 m	CH_2 bending
1413 m	Benzene C=C stretching
1107 vs	C–O–C stretching
1250 m	S=O stretching
1076 s	O=C–O stretching
1060 m	C–O–C stretching
1020 m	SO_3^- stretching
1000 m	SO_3^- stretching

[a] s = strong; m = medium; w = weak; vs = very strong; sh = shoulder.

Fig. 5 and 6 show the spectra of the hydrated Pubrac and Puhmdi compared with those of the corresponding non-hydrated materials. At the bottom of each figure the most significant regions of the spectra are overlaid and magnified. The hydrated samples were obtained simply by exposure of the material to an environment with controlled humidity.

As expected the spectra of the hydrated samples show an increased intensity of the bands due to their hydrophilic component ($HyalS_{3.5}$); migration of the hydrophilic compound from the bulk to the surface occurs in the hydrophilic environment. The surface reorganization of these materials is reversible when the samples are dehydrated and their IR spectra revert to those of the original cast samples. The same mechanism of migration of some hydrophilic components from the bulk to the surface of the hydrophobic matrix of styrene, when the environment is changed from dry to aqueous, has already been reported in the literature.[10]

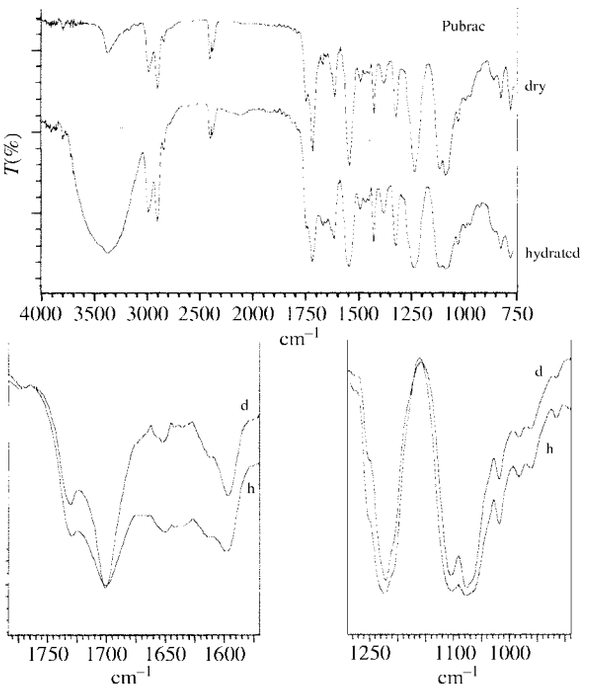

Fig. 5 ATR-FTIR spectrum of hydrated Pubrac; magnification of the 1750–1500 cm^{-1} and 1250–950 cm^{-1} ranges.

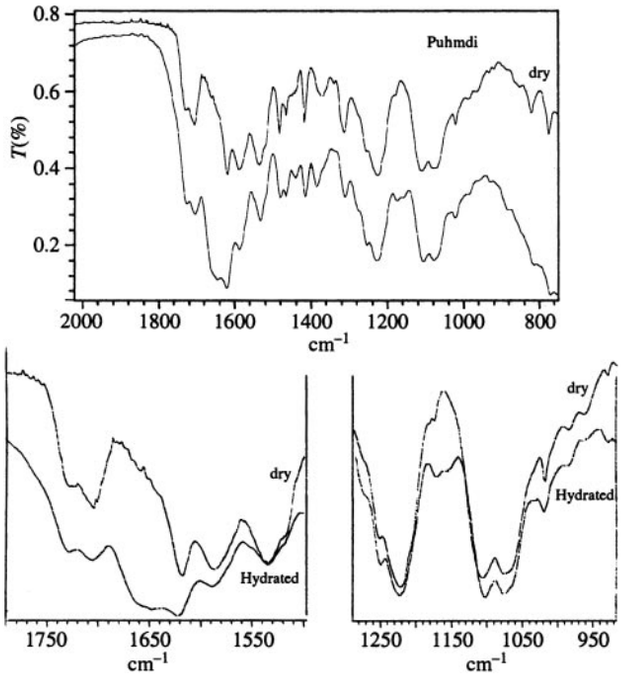

Fig. 6 ATR-FTIR spectrum of hydrated Puhmdi; magnification of the 1750–1500 cm^{-1} and 1250–950 cm^{-1} ranges.

5.3 Biological properties

The blood compatibility of the materials was evaluated in terms of anticoagulant, non-thrombogenic activity and inhibition of platelet adhesion.

Table 3 reports the values of the whole blood coagulation time (WBCT) and thrombin time (TT) determined for the different samples. The Puhmdi material shows the same WBCT as the control material and the native PU. The Pubrac material instead exhibits a strong increase in WBCT. Thus, we can infer that this material has an anticoagulant activity.

The TT test was performed on each material to investigate the terminal part of the plasmatic phase of coagulation, in particular the transformation of fibrinogen to fibrin catalyzed by thrombin. This test also revealed that Pubrac is able to inhibit the coagulation process, as does $HyalS_{3.5}$ in solution,[20,19] while Puhmdi does not show any biological activity. The TT performed on the plasma alone, after being in contact with the material, was the same as that of the control sample, which excludes any possible interference of $HyalS_{3.5}$ released from the sample or of other factors.

Fig. 7a, b and c show the SEM pictures of the materials in contact with Platelet Rich Plasma (PRP). The native PU (Fig. 7a) was the control material.

On Puhmdi (Fig. 7b), as on the control material, the platelets were connected and formed an aggregation, which prevented analysis of their distribution. In contrast, on the surface of Pubrac (Fig. 7c) there were very few platelets, which confirms the extreme biological activity of the immobilized $HyalS_{3.5}$.

Table 3 WBCT determined on a test tube whose internal surface was covered with the material and TT determined on a small circular portion of material (∅ = 0.8 cm)

Material	WBCT/min	TT/s[a]
Polystyrene	25 ± 2	12.1 ± 0.9
PU	26 ± 2	12.5 ± 0.4
Pubrac	> 120	> 120
Puhmdi	26 ± 2	16.3 ± 0.2

[a]For all samples, the reptilase time (RT) was the same as that of the control.

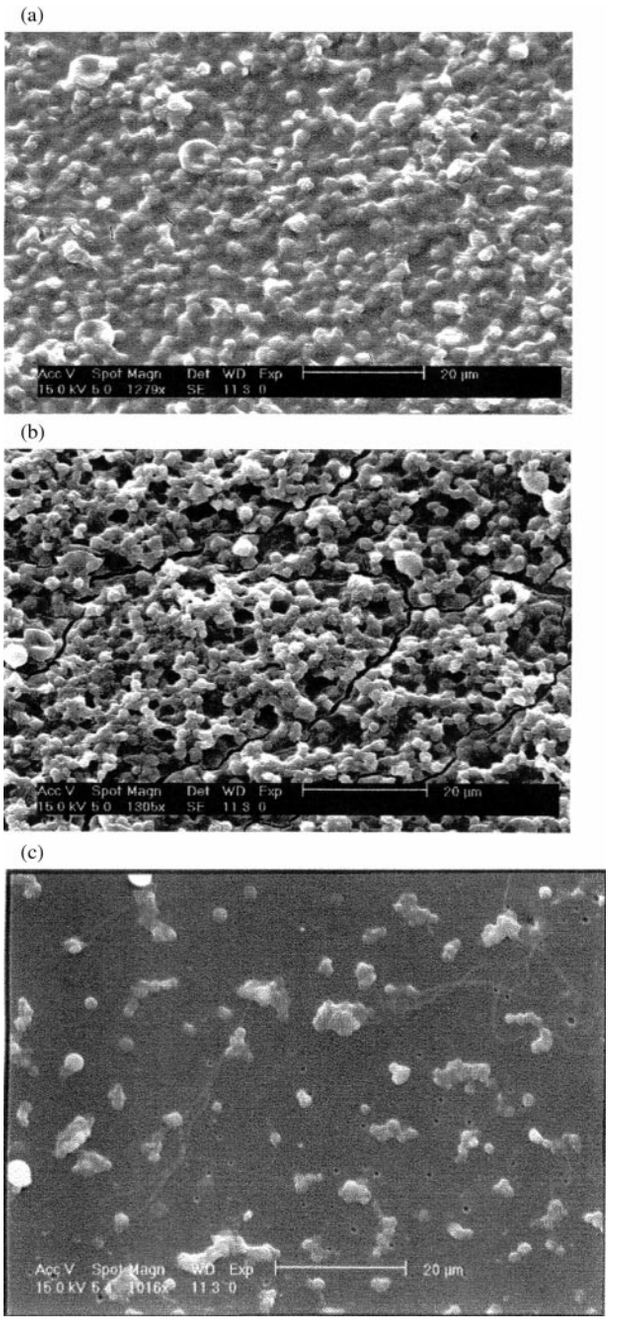

Fig. 7 SEM pictures of the materials in contact with PRP: a) Pellethane, b) Puhmdi, c) Pubrac.

6 Conclusions

Both methods of synthesis are able to link $HyalS_{3.5}$ to the PU chains. Both materials consist of a hydrophilic component ($HyalS_{3.5}$) and a hydrophobic component (PU). The chemical composition of the surfaces of the original cast samples was essentially polyurethane-like. When the environment is changed from dry to hydrated, the hydrophilic component ($HyalS_{3.5}$) migrates from the bulk to the surface in both materials.

The surface of Pubrac possesses anticoagulant and non-thrombogenic activity and inhibits the adhesion, activation and aggregation of platelets. In contrast, Puhmdi does not show any biological activity.

Since Pubrac is soluble in organic solvent, it is possible to use a more volatile solvent to improve its surface morphology, as demonstrated by the SEM photos.

The most significant chemical differences between the two

materials are related to the fact that in Puhmdi most of the $HyalS_{3.5}$ molecules are cross-linked through the carboxy groups to the PU chains, whereas in Pubrac only a few carboxy groups of $HyalS_{3.5}$ are involved in the linking reaction to PU because of their lower reactivity under the conditions of this chemical route. The smoothness of the surface of Pubrac could play an important role in the biological activity of the material, as demonstrated by the different growth of human umbilical vein endothelial cells on smooth or rough surfaces.[29] However, we believe that in Puhmdi most of the COO^- groups are involved in the reaction with HMDI, whereas in Pubrac they are not. The carboxy group thus plays an important role in the biological response of immobilized $HyalS_{3.5}$. In fact, a study of some partially esterified $HyalS_{3.5}$ demonstrated[30] that the partial hindering of the carboxy group induces a reduction of the TT with respect to unmodified $HyalS_{3.5}$. Moreover, total esterification does not produce any lengthening of TT as in the commercial Hyaff.

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