Chapter 10

Biocompatibility and Biological Performance of the Improved Polyurethane Membranes for Medical Applications

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1. Introduction

Polyurethanes (PUs) are one of the most “pluripotent” synthetic polymer classes used in medical applications. Due to their structural versatility, they have been widely discussed as materials appropriate for biomedical applications (Abd El-Rehim & El-Amaouty, 2004; Guelcher et. al., 2007; Guelcher, 2008; Kavlock et. al, 2007; J.S. Lee et. al., 2001; Lelah & Cooper, 1987; Siepe et. al., 2007). Up to now, new PUs have been synthesized that possess good mechanical properties. Most of them are considered biocompatible on account of *in vitro* cytotoxicity evaluation.

However, it is well known that structural and mechanical adaptability of PUs is not always accompanied by cell and tissue biocompatibility. Therefore, numerous data in the literature are focused on biocompatibilization or functionalization of PUs (Yao, 2008; Sartori, 2008, Huang & Xu, 2010). Some promising methods for the improvement of biological response of PUs are conjugation, blending or coating with natural polymers. Thus, polysaccharides as chitosan, cellulose and their derivatives (Raschip, 2009; Zia, 2009; Zuo, 2009), proteins and glycoproteins as collagen, fibrin, fibronectin (R. Chen et. al., 2010; Sartori et. al., 2008), proteoglycans and glycosaminoglycans (Gong et. al., 2010) and other molecules (Hwang & Meyerhoff, 2008; Hsu et. al., 2004; Makala et. al., 2006; Song et. al., 2005; Verma & Marsden, 2005) are employed successfully for PUs modification. Owing its specific properties, hydroxypropylcellulose (HPC) is already used as binder, thickener, lubricating material (artificial tears) and emulsion stabilizer in pharmaceutical and food industry. Moreover, HPC may provide interactions through its hydroxyl radicals, being an excellent compound for copolymerization in scaffolds for tissue engineering and in drug delivery systems (Berthier et. al., 2011; D. Chen & Sun, 2000; Gutowska et. al., 2001; Raschip et. al., 2009;

Valenta & Auner, 2004). In previous studies we found that when added to PU structure, HPC improves hydrophilicity and mechanical properties of PUs by increasing the elasticity of the resulted materials (Macocinschi et. al., 2009).

Considering the reviewed concept of biocompatibility as "the ability to exist in contact with tissues of the human body without causing an unacceptable degree of harm to the body" (Williams, 2008), our interdisciplinary work was focused on the synthesis of PU-based materials with improved ability to long-time functional integration. PU/HPC membranes were prepared by blending method. HPC was chosen due to its physical-chemical properties, its demonstrated biocompatibility and accessibility. The aim of the chapter is to highlight the most important criteria, able to predict the behaviour of material-tissue interfaces and the long-term material-tissue integration, in order to select most suitable compositions and morphologies for specific medical application. Thus, surface zeta (ζ) potential, wettability (as contact angle measurement and water uptake), pH modification after long time hydration and autoclaving, protein adsorption at protein physiological concentration and some relevant elements of bulk and surface morphology are treated as screening criteria for suitable membrane choice in the first part of the chapter. Biological performance evaluation, such as oxidative stress action, thrombogenicity and *in vivo* behaviour of PU/HPC membranes are further discussed.

2. Materials and methods

2.1. Preparation of polymer samples

Preparation of PU/HPC samples was performed according to Fig. 1 as previously reported (Macocinschi et. al. 2009; Vlad et. al, 2010).

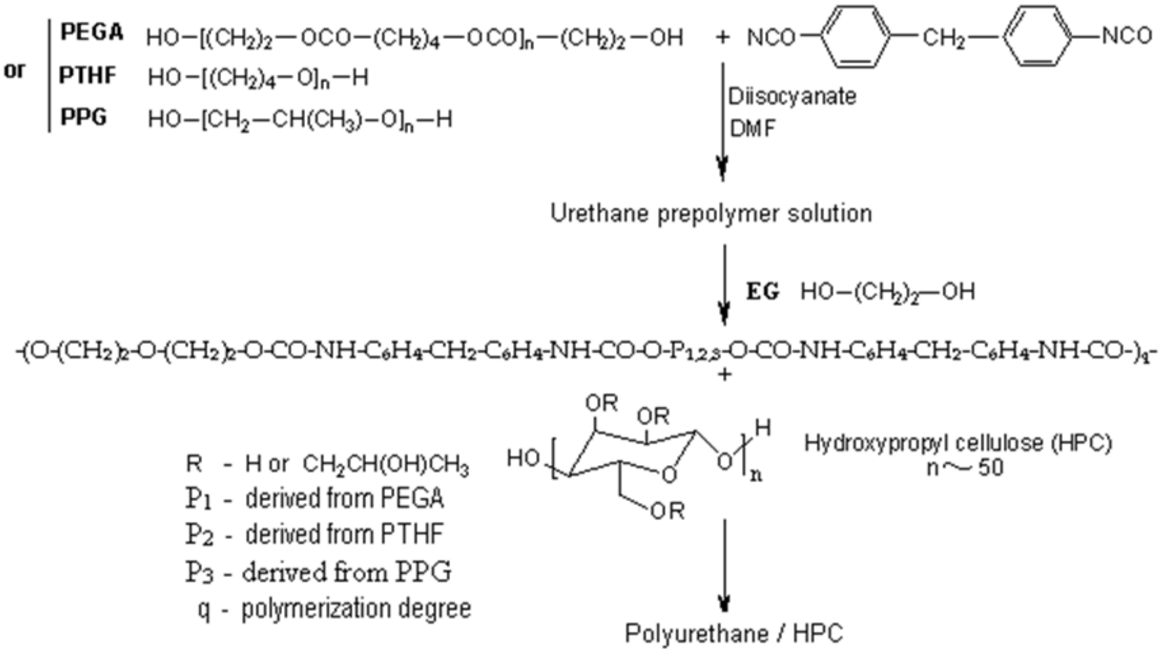

Figure 1. Scheme of chemical structure and synthesis way of PUs/HPC

Briefly, isocyanate terminated urethane prepolymers were first synthesized by the polyaddition reactions between 4',4'-diphenylmethane diisocyanate (MDI) and macrodiols in N,N-dimethylformamide (DMF) as solvent. Poly(ethylene adipate)diol (PEGA, Mn = 2000 g/mol), polytetrahydrofuran (PTHF, Mn = 2000 g/mol) or poly(propylene)glycol (PPG, Mn = 2000 g/mol) were used as macrodiols. The urethane prepolymers were treated in a subsequent step with ethylene glycol (EG) as chain extender. Finally, HPC (average weight molecular weight Mw = 95 000 g/mol) was added to PU solutions to obtain the following compositions for all PU/HPC samples: macrodiol/MDI/EG/HPC = 52.24 /36.57/7.27/3.92 (weight ratios). As the molar ratio between isocyanate groups in MDI and the sum of hydroxylic groups in macrodiol and EG was 1.02, the excess of isocyanate groups linked to PU prepolymers were available to bind a part of HPC chains. Membranes with about 1 mm thickness were prepared by pouring PU/HPC DMF solutions in distilled water, at 40 °C. The formed films were then dried under vacuum for several days and kept in distilled water for solvent removing.

To half of PUs with PEGA macrodiol in the soft segment no HPC was added to obtain PU-PEGA reference sample. HPC containing samples based on PEGA, PTHF and PPG macrodiols were codified as PU-PEGA/HPC; PU-PTHF/HPC and PU-PPG/HPC, respectively.

2.2. ζ potential determination

ζ potential of the PU membranes was measured by streaming potential method using a commercial electrokinetic analyzer SurPASS, (Anton Paar GmbH, Graz, Austria). For each sample, ζ potential has been measured in 0.1 M NaCl solution at physiological 7.4 pH value, a 300 mbar electrolyte pressure and a 80 ml/min flow rate. For statistical reasons, four streaming potentials were measured. The mean value of these data was used for potential calculation by Fairbrother–Mastin equation, considering also the effect of surface conductivity (Luxbacher, 2006)

2.3. Wettability

Wettability of the PU membranes was determined by measuring the surface contact angle and water uptake. For surface contact angle, uniform drops of the tested liquid (double-distilled water) with a volume of 2 μl were deposited on the film surface and the contact angles were measured after 30 s, using a video-based optical contact angle measuring device equipped with a Hamilton syringe in a temperature-controlled environmental chamber. All measurements were performed at room temperature of 25 °C. Repeated measurements of a given contact angle were all within the range of ± 3 degrees. *Water uptake* was calculated as the ratio between fully hydrated and dried sample weights.

2.4. Material extraction in a simulated biological microenvironment

Material extraction in a simulated biological microenvironment was done for long period of time (over 2 months) in Hank's Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+}, with glucose, and phenol red as pH indicator. For extraction experiments, 0.2 g of each membrane, cut in very small pieces (see Fig. 2), were incubated in 2 ml of HBSS solution at 37°C. pH variation was monitored daily, based on phenol red indicator colour and measured after 1, 2, 3, 30 and 60 days of incubation using Mettler Toledo SevenGo SG2ELK pH-meter.

2.5. Scanning Electron Microscopy (SEM)

SEM analysis of PU/HPC membrane cross-sections was performed using a VEGA TESCAN microscope, in high vacuum mode, at an acceleration voltage of 30 kV.

2.6. Protein adsorption

Amount of protein adsorption on membrane surfaces was measured in three different conditions: (a) on individual protein solutions of fibrinogen (FB) at 3 mg/ml (95% clotable from Sigma-Aldrich) and serum albumin (SA) at 45 mg/ml (bovine SA (BSA) from Sigma-Aldrich); (b) FB and BSA mixed solutions of physiological concentrations (3 mg/ml for BSA and 45 mg/ml for FB); (c) complex protein conditions (platelet poor blood plasma (PPP)). Prior adsorption experiment, the PU/HPC films were brought to equilibrium with phosphate buffer saline (PBS) up to reaching maximum hydration, for about 72 h. Briefly, PU/HPC hydrated membranes with 0.5 cm x 0.5 cm surface area were covered with 0.25 ml of one of the protein solutions or with blood plasma and kept at 37 °C for 30 min. FB and BSA concentration in incubated medium was determined before and after incubation. A turbidimetric method based on the formation of an insoluble complex with Na_2SO_4 was used for FB determination. The method based on antigen–antibody reaction was performed for SA measuring, using a Dialab kit, Austria. FB and SA reaction products were assessed on a Piccos 05 UV–VIS spectrophotometer at λ = 530 nm for FB and λ = 340 nm for SA. The adsorbed amount of proteins was calculated with the following relation:

$$\text{Adsorbed proetin}(\text{mg/cm}^2) = \frac{(Co - Ce)\cdot V}{S} \tag{1}$$

where *Co* and *Ce* are the initial and post-incubation concentrations of protein solution (mg/ml), *V* is the incubated volume of the protein solution (ml) and *S* is the surface of the incubated PU/HPC sample

2.7. Total Antioxidant Status (TAS)

TAS was measured in blood plasma obtained by human blood centrifugation at 1000 G for 20 min. PU samples were incubated in blood plasma for 1, 2 and 3 days at 37 °C and mild orbital shacking. The TAS measurement was made by standard protocol provided by Randox TAS kit. Thus, 2,2′-azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS)® was incubated with a peroxidase (metmyoglobine) and H_2O_2 to produce the $ABTS^{®+}$ radical cations having a stable blue-green colour that was measured at 600 nm on a spectrophotometer mentioned in the previous section. By adding blood plasma containing antioxidants a suppression of this colour to a degree which is proportional to their concentration is observed. Control serum ("standard" provided by the determination kit) was used for data validation. TAS values were calculated based on the measured absorbance in the standard, blood plasma sample and blank (buffer provided by the kit) before and after H_2O_2 adding. The absorbance differences (ΔA) between measurement before and after H_2O_2 adding for standard, sample or blank solutions were used for calculation of TAS concentration according to relations 2 and 3:

$$\text{Factor} = \frac{\text{concentration of standard}}{\Delta\,\text{A blank} - \Delta\text{A standard}} \tag{2}$$

$$\text{TAS mMol/L} = \text{Factor} \cdot \Delta\,\text{A blank} - \Delta\text{A sample} \tag{3}$$

2.8. Haemocompatibility testing

Haemocompatibility of membrane surface was evaluated by haemolysis and coagulation tests. All tests were performed on well swollen PU samples in PBS. *Haemolysis* was determined using 0.25 ml of blood (human blood from healthy voluntary donors, collected on 3.8 % sodium citrate solution as anticoagulant in 9:1 v/v ratio) that was incubated with 1 cm^2 surface area PU samples for 30 min at 37 ^oC. Haemoglobin released from lysed erythrocytes was measured by spectrophotometric method at $\lambda = 545$. *Prothrombin time* was measured after 1 hour incubation of polymer sample in blood plasma. Standard laboratory method was applied using PT kit (Biodevice, Italy) and ACL 100 coagulometer. Blood plasma was obtained by blood centrifugation at 1000 G for 10 min.

Platelet adhesion on material surface was determined based on number of platelet counted in 0.1 ml platelet rich blood plasma (PRP), before and after membrane (0.5 cm x 0.5 cm) incubation for 1 hour at 37 ^oC. PRP was obtained by blood centrifugation at 400 G for 20 min. Improved Neubauer haemocytometer was used for platelet counting. *Clot weight test* was performed by adding 0.2 ml of human blood upon well swollen samples with 1cm^2 surface area. The thrombus formation was started by adding 0.05 ml $CaCl_2$ solution (0.025 mol/l). Each formed thrombus was weighed and compared with control. Collagen film was used as positive pro-coagulant control and normal blood plasma without polymer sample as negative control.

2.9. *In vivo* biocompatibility

Subcutaneous implantation experiment was performed on Wistar 200 g weight male rats. Testing protocol was designed according to ISO 10993-2 (Animal Welfare Requirements) and the guidelines of Council for International Organizations of Medical Sciences (CIOMS). The pieces of autoclaved purified or unpurified membranes (0.5 x 0.5 cm size) were implanted under both sites (right and left) of dorso-lateral skin. Material purification was performed by immersion in sterile distilled water for 1 week and equilibration in physiological salted sterile solution for 24 hours before subcutaneous implantation. All surgical procedures were done under thiopental anaesthesia, using a dosage of 35 mg/kg body weight. Lots of six animals for each material were taken in each experiment. The period of 10 or 30 days was chosen for material examination. Explanted samples together with surrounding tissue were fixed in 10% formaldehyde solution embedded in paraffin wax, sliced in 15 µm pieces and stained using Hematoxylin – Eosin (HE) method for cell examination and Masson's trichrome for collagen fibres.

3. Results and discussions

3.1. Primary screening criteria for the appropriate selection of PU/HPC membranes for medical usage

Biocompatibility of PUs, seen in terms of specific application, is a result of a "bio-appropriate" expression of surface and bulk properties achieved by synthesis and scaffold fabrication methods. Thus, surface ζ potential and surface wettability are important characteristics responsible for specific tissue-material interaction mechanisms, starting with protein adsorption that can be influenced in turn by specific physiological/pathological tissue environment.

3.1.1. Surface ζ potential and wettability

Surface charge plays an important and active role in tissue-material interaction and must be considered in accordance with the targeted application. The importance of surface charge on cell adhesion, biofilm formation or thrombogenesis was demonstrated (Cai et. al., 2006; Colman & Schmaier, 1997; Kang et. al., 2006; Khorasani et. al., 2006). These phenomena are a consequence of adsorptive behavior of proteins on charged surface rather than the effect of electrostatic interactions with cells (Keselowsky et. al., 2003; Wilson et. al., 2005). Many data refer to the effect of surface charge on biological phenomena (Jelinek et. al., 2010; Kang et. al., 2006). However, there are not many data reporting surface charge and its clear relevance for biocompatibility of PU-based membranes. Moreover, it is difficult to estimate the electro-kinetic properties of such surfaces, mainly due to the complexity of the chemical composition but also due to membrane variable porosity and swelling behavior that can influence surface charge values (Yaroshchuk & Luxbacher, 2010).). Surface ζ potential of material is a property that reflects surface charge. Some reported data have shown that poly(ether-urethane)s exhibit a very negative (– 25 mV) ζ potential, while poly(ester-urethane)s are less negative (- 12 mV). Contradictory data were published on the beneficial effect of positively (Khorasani et. al., 2006) or negatively charged surfaces (Sanders et. al., 2005) on cells attachment and proliferation.

Thus, this section is aimed to predict the influence of surface potential and wettability on the biocompatibility and biological performances of PU-based samples. Table 1 shows hydrophilic/hydrophobic properties and ζ potential of examined PU-based samples.

Material samples	Contact angle						WU (%)	ζ (mV)
	First immersion			*Second immersion*				
	θ_{adv}(°)	θ_{rec}(°)	H(%)	θ_{adv}(°)	θ_{rec}(°)	H(%)		
PU-PEGA	85.3±1.1	54.3±0.6	36.3	51.0±0.5	54.1±0.6	5.6	141±10	- 4.31
PU-PEGA/HPC	84.8±1.1	44.2±0.5	47.9	52.6±0.5	43.7±0.5	16.9	140±4	+ 3.14
PU-PTHF/HPC	77.4±1.1	42.9±0.5	44.5	31.6±0.4	42.3±0.4	25.2	167±3	+ 0.78
PU-PPG/HPC	85.6±1.1	44.8±0.5	47.7	60.3±0.6	44.1±0.5	27.0	92±6	+ 4.85

Table 1. Dynamic contact angle values (θ) in contact with water, hysteresis (H) resulted from advanced (*adv*) and receded (*rec*) contact angles, water uptake (WU) (Macoconschi et. al., 2009) and ζ potential of the PU samples

As one can see from Table 1, PU-PEGA has a slightly negative ζ potential, probably due to the presence of carboxylic groups resulted by the hydrolysis of residual isocyanate groups during membrane precipitation in water. After blending with HPC, the residual isocyanated groups linked to PU prepolymer are reacted with the hydroxyl groups of HPC and all PU/HPC membranes showed a slightly positive surface. The most hydrophilic sample (PU-PTHF/HPC) exhibited the most neutral ζ potential. This observation is in accordance to other data that report dependence of surface charge on water swelling capacity (Aranberri-Askargorta et. al., 2003).

3.1.2. Extraction microenvironment

The material biocompatibility can be appreciated through its effects on the physico-chemical properties of the physiological environment, especially on the pH. Thus, pH modification of HBSS buffer solutions after unsterilized and sterilized membranes incubation was measured. The results are shown in Figs 2 and 3 (1, PU-PEGA; 2- PU-PEGA/HPC; 3, PU-PTHF/HPC; 4, PU-PPG/HPC).

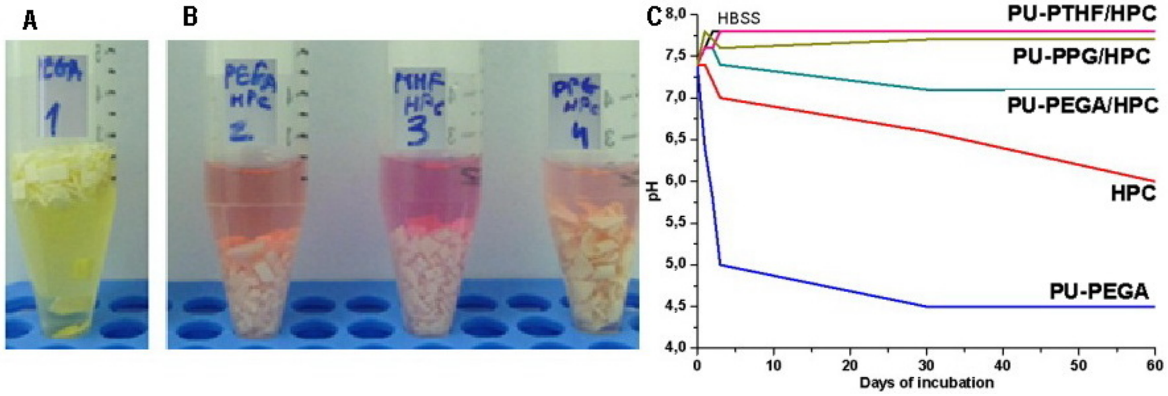

Figure 2. pH variation of HBSS buffer in which unsterilized membranes were incubated: A, PU-PEGA; B, PUs/HPC; C, pH variation curves

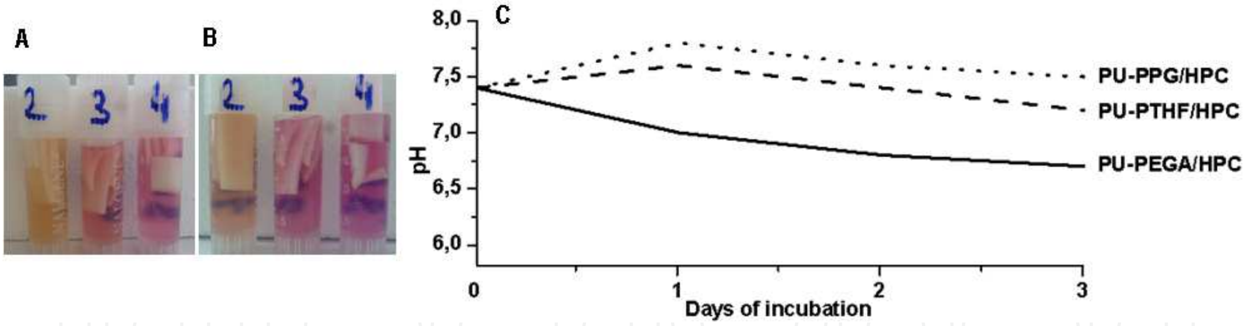

Figure 3. pH variation of HBSS buffer in which autoclaved PU/HPC membranes were incubated: A, 24 h of incubation; B, 72 h of incubation; C, pH variation curves

As one can see from Figs. 2 and 3, a long-period of incubation of unsterilized and sterilized (by autoclaving at 121 °C and 1 atm) PU/HPC membranes in simulated biological fluid did not meaningfully modify the physiological range pH value of the incubation environment, while a pronounced decrease of the environment pH was observed for pure PU-PEGA sample (Fig. 2 A). Thus, one can say that HPC gives an important contribution to hydrolytic stability of urethane and ester bonds of PU chains.

For autoclaved samples, the variation of pH values of the environment for poly(ether-urethane)s remains in the range of the physiological value, while PU-PEGA/HPC membrane induced a higher decrease of pH (Fig. 3), a normal result owing the higher thermal degradability of poly(ester-urethane)s (Guelcher, 2008).

Another property that was changed by modifying PU membranes with HPC was the floatability (see Fig. 2B). As the surface wettability and water uptake for PU-PEGA sample is similar to HPC modified one (see Table 1), the reason of these different behavior could reside in different morphologies, as seen from SEM images of membrane cross-sections (Fig. 4).

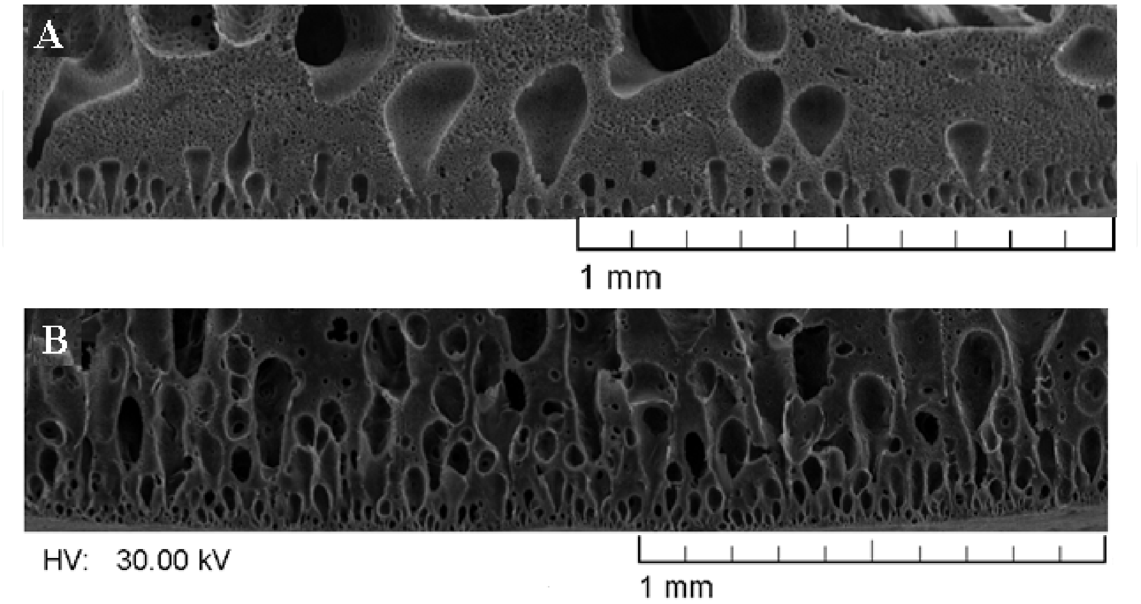

Figure 4. SEM image of PU-PEGA (A) and PU-PEGA/HPC (B) membrane cross-sections

PU-PEGA sample showed important bulk microporosity, with isolated pores, while PU-PEGA/HPC presented smaller but interconnected pores allowing water diffusion and the decrease of the floatability.

Thus one can conclude that PU/HPC membranes are slightly positively charged and they possess interconnected porous morphology influencing the wettability and floatability. They also showed a less pronounced influence on the biological media as compared to the pure PU membrane.

3.2. Protein adsorption

There are many data concerning mechanisms of protein adsorption on different surfaces (Gray, 2004; Scott & Elbert, 2007; Van Tassel, 2006; Wilson et. al., 2005). It was clearly demonstrated that proteins have amphoteric properties, being able to adsorb on both negatively and positively charged surfaces (Michelsen et. al., 2000; Van Tassel, 2006). The amount of adsorbed proteins is depending on their isoelectric points as well as on surface chemistry and hydrophilicity (Keselowsky et. al., 2003; Wertz & Santore, 2001). Hydrophobic surfaces manly interact with hydrophobic protein core that leads to the modification of the protein physiological conformation and its functionality. Opposite to hydrophobicity, superficial water maintains native protein conformation and specific functionality (Keselowsky et. al., 2003; Noinville & Revault, 2006).

Many authors have reported protein adsorption behaviour on different surfaces using simulated solutions and highly sensible methods in which very low protein concentrations are detected. Thus, the adsorption of albumin solutions of different concentrations on pure silica or on silica modified with NH_2 and CH_3 terminated self-assembled monolayers (SAMs) (Noinville & Revault, 2006) and on silica-titanium surfaces (Kurrat et. al. 1997) was studied. Other authors reported the competitive adsorption of fibrinogen on mica (Gettens et. al., 2005; Tsapikouni & Missirlis, 2007). Surface adsorption of SA, FB, fibronectin (FN), immunoglobulins (IGs) and lysozyme were investigated to evaluate the surface biocompatibility (Bernsmann et. al., 2010; Pompe et. al., 2006; Rezwan et. al., 2005), each class of these proteins providing specific surface properties for targeted application. Thus, FN adsorption is relevant for the prediction of cell adhesion, lysozyme – for enzymatic degradability predisposition, IGs - for immune-specific interactions, while SA and FB adsorption have haemocompatibility predictive value.

In order to estimate protein adsorption (retention) capacity of materials at blood or tissues contact, simulated physiological environment, close to normal blood conditions is required. For example, Bajpai, 2005 followed SA adsorption capacity of biomaterials at SA bulk concentration from 1 to 6 mg/ml, while Alves et. al., 2010 used mix protein conditions, considering physiological value for each protein. The mix protein adsorption conditions are considered to better reflect the complex interactions that occur between different proteins (Latour, 2008).

PU/HPC membranes were previously demonstrated to possess good mechanical properties (elongation at break for dried/hydrated PU-PEGA/HPC = 71/84; for PU-PTHF/HPC = 72/159 and for PU-PPG/HPC = 53/55), appropriate for cardio-vascular applications (Macocinschi et. al., 2009). The physisorption of SA and FB is further highlighted as screening criteria for biocompatibility and, more specifically, haemocompatibility. Very short characteristics of SA and FB, important for protein-material interaction are given below.

SA is a protein belonging to the so called "soft" class of proteins, with a molar mass of about 65 kD for BSA and 67 kD for human SA (HSA). This protein represents about 60% of the blood proteins. Normal blood concentration of HSA is 35 – 50 mg/ml. This protein is involved in many physiological phenomena as carrier protein for fatty acids, metals, cholesterol, bile pigment, hormones and drugs. SA is also characterised by antioxidant properties (Bourdon et. al. 1999; Kouoh et. al., 1999; H. Lee et. al., 2000) that is higher in alkaline pH, up to 8 (H. Lee et. al., 2000). SA is preponderantly negatively charged, its isoelectric point being close to 4.8 (Carter & Ho 1994; Noinville & Revault, 2006). Approximately 67% of the secondary SA structure is represented by the α-helix. It was demonstrated that the stability of SA secondary structure strictly depends on pH (Freeman, 2006) that influence the protein conformation. Thus, at pH = 5, SA takes almost spherical, native, unfolded shape that forms a thick layer on the adsorptive surfaces. At pH = 7 (close to physiological pH), due to molecular spreading, SA forms an extended contact area with adsorptive surfaces. This behavior can be influenced by surface charge, surface functionality and functionality distribution, surface morphology or wettability conditions (Wilson et. al., 2005). The role of adsorbed SA on biomaterial biocompatibility is still ambiguously described in the literature. While some authors have demonstrated biocompatibility improvement of material with increased adsorption of SA (Eberhart et.al. 1987; Marconi et. al., 1996; Randrasana et. al., 1994), others demonstrated a better biocompatibility of SA-resistant surfaces (Ostuni et. al., 2001; Wan et. al., 2006).

FB is a high molecular weight (340 kD) complex glycoprotein that has 2 molecular domains, each of them consisting of three polypeptide chains called Aα, Bβ and γ. Molecular updated analysis of FB can be found in recent reports (Cardinali et.al, 2010). FB is an important factor of haemostasis. Through fibrin network formation as first cell scaffold, FB is involved in wound healing and tissue regeneration. Its normal blood concentration varies from 2 to 4 mg/ml. In inflammations or in other pathological statuses - as cardiovascular diseases - FB can reach up to 7 mg/ml, therefore adsorption properties of biomaterials for this protein should be carefully analysed, especially for those targeted for blood contact applications.

The results obtained in adsorption experiments of SA and FB from both individual and mixed solutions on PU/HPC membranes are presented in Fig. 5.

No significant differences between adsorption behavior of both proteins in their pure and mixed solutions were registered, except a small tendency to decrease adsorbed BSA from mixed solution as compared to individual solution, especially on PU-PEGA and PU-PEGA/HPC membranes, where FB, with a higher molecular weight, showed a higher affinity.

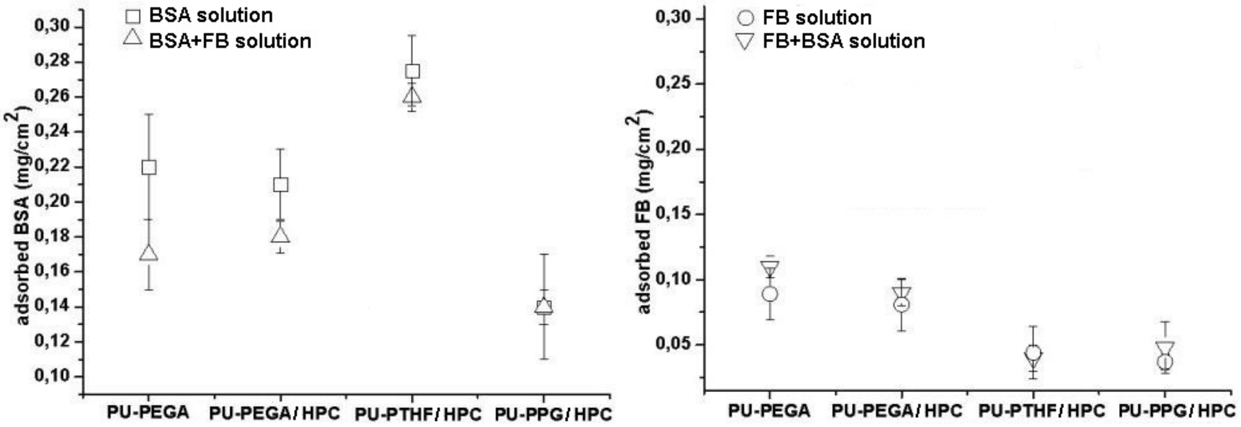

Figure 5. Amount of adsorbed BSA (left) and FB (right) from individual protein solutions and in co-adsorptive environment (mixed protein solution) of physiological concentrations, i.e., 3 mg/ml for FB and 45 mg/ml for BSA

Figure 6 shows the ratios of adsorbed BSA and FB from mixed protein solutions and from blood plasma. In both studied conditions and for all membranes, the amount of adsorbed SA is higher than that of adsorbed FB, a normal result considering the lower concentration of FB in solutions. The total amount of the adsorbed SA and FB proteins from blood plasma is lower as compared to that adsorbed from mixed solutions due to the competitive adsorption of some other blood plasma proteins. Moreover, the ratio between adsorbed FB and SA is lower in blood plasma than in mixed solutions.

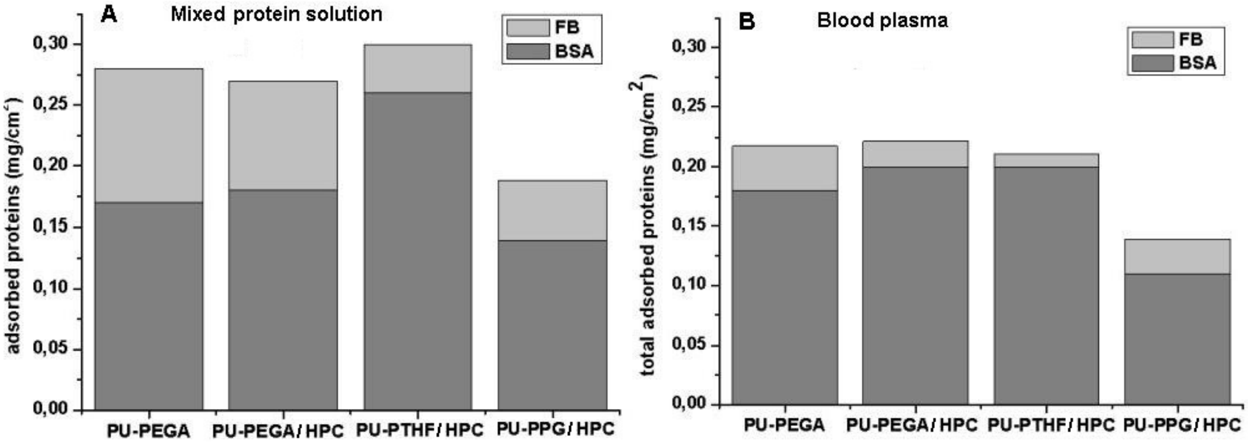

Figure 6. Total amount of adsorbed FB and BSA from: A – mixed protein solution at 3 mg/ml FB and 45 mg/ml BS physiological concentration; B - human blood plasma with 2.98 mg/ml initial FB concentration and 45.3 mg/ml initial SA concentration

As conclusion, comparing PU-PEGA and PU-PEGA/HPC membranes one can observe that small amount of polysaccharide rich in functional substituents can bio-stabilize PU structures and improve their resistance for autoclaving procedures as important step in ready to use biomaterials preparation. From all the data presented in this section, one can say that the more hydrophilic PU-PTHF/HPC membrane could be the most appropriate for biomedical applications.

3.3. *In vitro* and *in vivo* performances of PU/HPC membranes

The biocompatibility of PUs are widely discussed and questioned, mostly in the past. In the last two decades new generation of PUs that combine mechanical advantages with the biological performances emerged (Gisselfa et. al., 2002; Jordan & Chaikof et. al., 2007; Jun et. al., 2005; Kavlock et. al., 2007; Parveen et. al., 2008). For many years it has been considered that PUs biocompatibility is spotless due to their products of degradation, e.g., aromatic polyamines. As it is well known for the most part of biocompatible materials, the life time of their *in vitro* functionality is quite short. This is a consequence of their intrinsic physico-chemical properties, on one hand, and of the tissue action on the material, on the other hand (Anderson, 2001; Guelcher, 2008; Shen & Horbett, 2001).

3.3.1. Oxidative in vitro behavior

Oxidative degradation of PUs caused by hydrolytic or enzymatic mechanism was intensively discussed (Christenson et. al., 2004; Guelcher, 2008; Gary & Howard, 2002; Sutherland et. al., 1993). First of all, PUs designed for tissue-contact devices undergo hydrolytic degradation as a result of watering with physiological solutions. This process has an impact especially on poly(ester-urethane)s that can generate hydroxy-acids, being susceptible to induce reactive oxygen species (ROS) production following the material-tissues interaction. By means of this mechanism, PUs can be implied in the sustained oxidative degradation and a wide range of pathological states.

As it is well known, ROS can trigger subtle mechanisms responsible for diseases generation through the peroxidation of cell membrane lipids and DNA damage (Marnett, 2002; Tribble et. al., 1987; Yagi, 1987). The most susceptible organs to oxidative aggression are the heart, vessels, lung, gut, liver, brain and nerves (Ames et. al., 1993; Förstermann, 2008; Paradis et. al., 1997; Rahman et. al., 2002; Sayre et. al., 1997).

In a normal body state, ROS appear constantly as a result of some biological errors or as a consequence of some short living reactive intermediate products generated by the cell aerobic metabolism. Endogenous enzymatic and nonenzymatic pathways are responsible for the formation of free radicals. These pathways are balanced by two endogenous antioxidant pathways, which form the TAS (see fig. 7).

While some harmful material characteristics can be marked as cytotoxic or proinflammatory by standard testing, others, such as oxidative stress (that causes long-time material failure), are undetectable by using short period testing. Thus, well known biocompatible materials were found to display surface alteration or cracking after long-time implantation. Adding antioxidant compounds to materials can improve their resistance against tissue degradation (Oral et. al., 2006; Stachelek et. al., 2006; Wattamwar et. al., 2010).

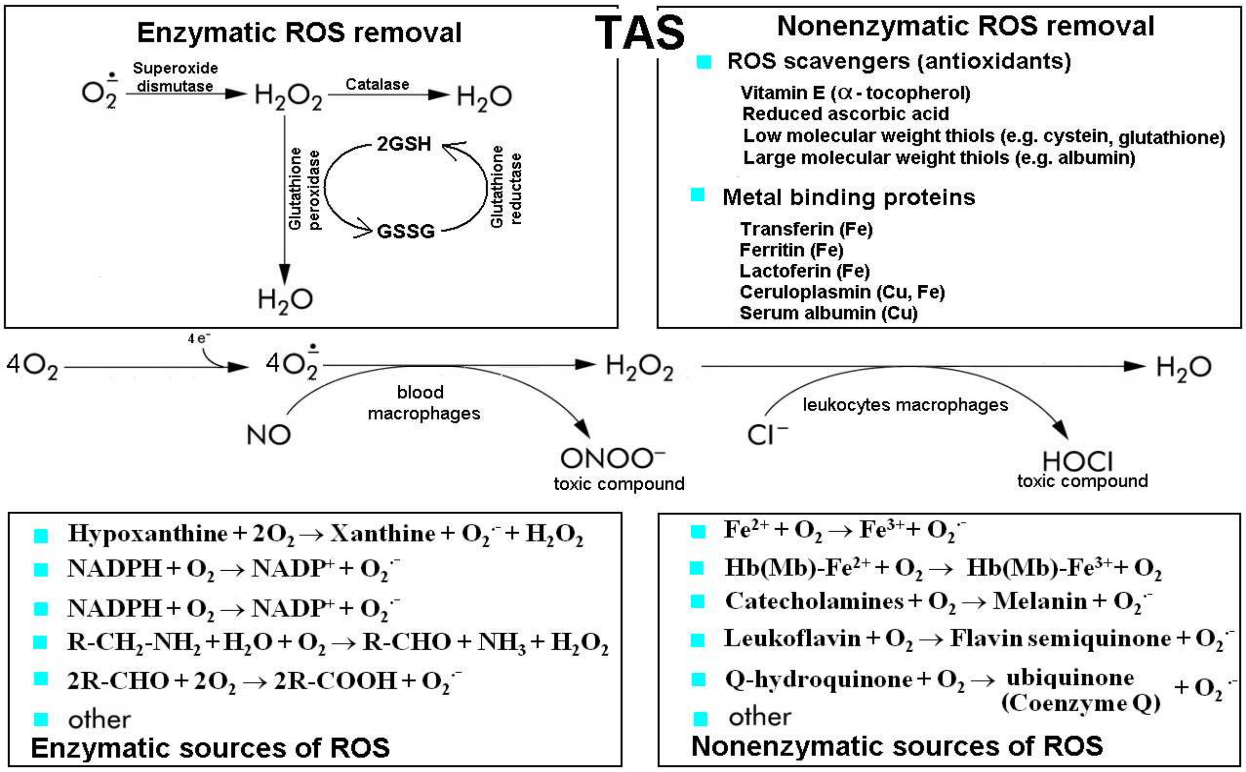

Figure 7. Schematic representation of the oxidative/antioxidative balance with enzymatic and nonenzymatic tissue pathways

Antioxidant defensive systems are present in both cells and extracellular environment. SA molecules are the most important antioxidants in blood. Due to their high concentration and polyvalent possibilities to fit with oxygen free radicals, SA molecules are considered to be the main plasmatic components of defence that assure neutralisation of more than 70% of ROS (Bourdon & Blache, 2001).

Assigning to SA molecules the main role in protective effect, we analysed the interaction of PU/HPC membranes with blood plasma, following the plasma antioxidant status. To define the importance of SA adsorption on material surface, the membranes were incubated at 37 °C in blood plasma and TAS was measured periodically. The results are shown in Fig. 8.

Two PU samples (PU-PEGA and the more hydrophobic PU-PPG/HPC) had significant tendency to quickly decrease TAS activity in the first 48 hours. Due to the complexity of TAS, it is difficult to speculate on the mechanism by which the decreasing phenomenon arises and certainly more examinations are needed. However, one can suppose that PU-PEGA alter the TAS activity as a result of plasma pH modification that leads to sustained free radical generation in the presence of the material. The mechanisms by which TAS activity is lowered after PU-PPG/HPC incubation could not be related directly to SA antioxidant activity, but to some other oxidant pathways that need further investigations.

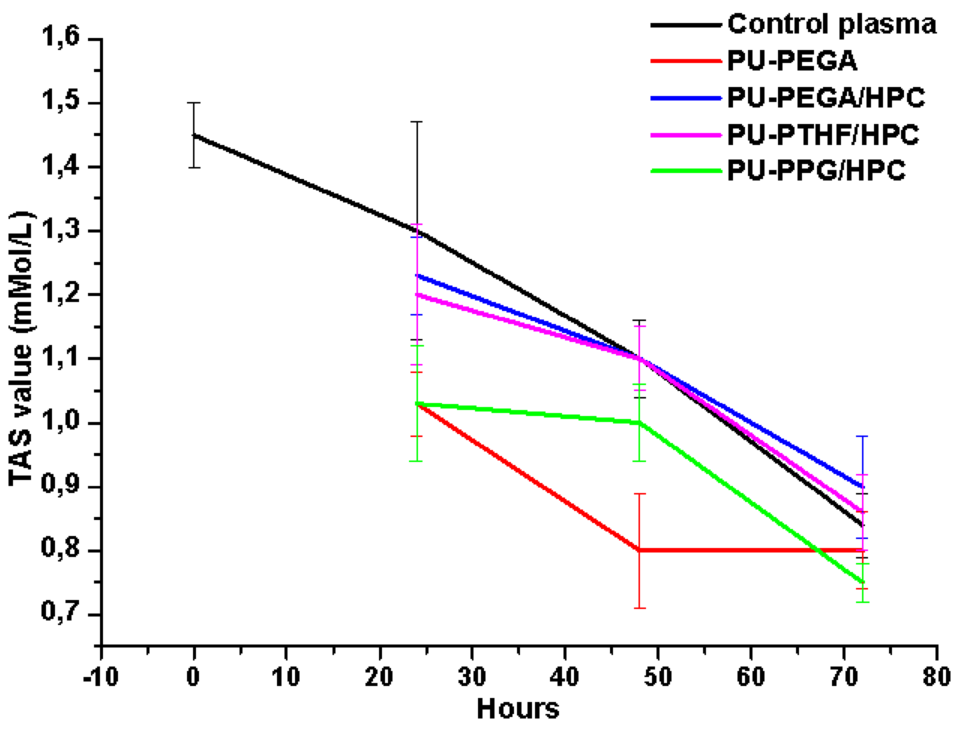

Figure 8. TAS evolution after PU/HPC incubation in blood plasma at 37 °C

3.3.2. In vitro haemocompatibility

Haemocompatibility involves compatibility with blood cells and blood plasma in other words, nonhaemolytic and nonthrombogenic behavior. Haemolysis is a mechanism by which erythrocytes (red blood cells) are destroyed through cell membrane lyses. Erythrocyte membrane lyses may occur as a result of environment pH modification or by cytotoxic action on erythrocyte membrane. Thus, both lipid (by lipid peroxidation) and protein (by protein modification) compounds can be affected.

Thrombogenesis is a complex phenomenon by which thrombus is formed by blood clotting. As a physiological event, haemostasis implies the activation of the enzymatic cascades in which three main factors are involved – vascular, cellular and plasmatic (Edmunds, 1998).

A synthetic material can induce haemostasis activation by surface charge, hydrophobicity and/or released products of degradation. It is widely recognised that both positively and negatively charged as well as hydrophobic surfaces can induce thrombus formation. This can be explained by involvement of several mechanisms as presented in Fig. 9.

A positive charge can be favourable for FB adsorption, followed by its conformational modification and adhesion of platelets and leukocytes (monocytes). Adherent cells are activated and they release numerous molecules that lead finally to FB cleavage with fibrin network formation (clot). Among platelet secreted factors are platelet thromboplastin, fibrin stabilizing factor, serotonin, anti-heparin factor, and others. Adherent (activated) monocyte releases thrombogen tissue factor (TF). Mechanism triggered by positive and hydrophobic surfaces is mainly related to extrinsic coagulation pathway (B. Furie & B. C. Furie, 2008).

A negative charge acts as an activator of plasmatic factor XII (Hageman factor) that involves contact system and intrinsic coagulation pathway (Zhuo et.al. 2006). This mechanism also involves high molecular weight and positively charged kininogen (HMW) and plasma thromboplastin (factor XI). Contact mechanism is tightly related to inflammatory events because some intrinsic pathways factors are direct activators of neutrophils. Whole mechanism of contact blood coagulation is still unclear. Some authors hypothesized that it can also be induced by adsorbed FB (Colman & Schmaier, 1997) and hydrophobic surfaces (Zhuo et. al., 2006). As for intrinsic coagulation mechanism of thrombus formation, this can also be activated by negatively charged low density lipoproteins (LDL), the molecules that adhere to the vessel walls in some pathologic conditions associated with cardiovascular risks (Krieter et. al., 2005). This possible mechanism should be taken into account because almost all pathological situations in which blood-assisted devices are used are accompanied by high level of cardiovascular risk factors (high level of LDL, cholesterol, triglycerides and modified blood pressure).

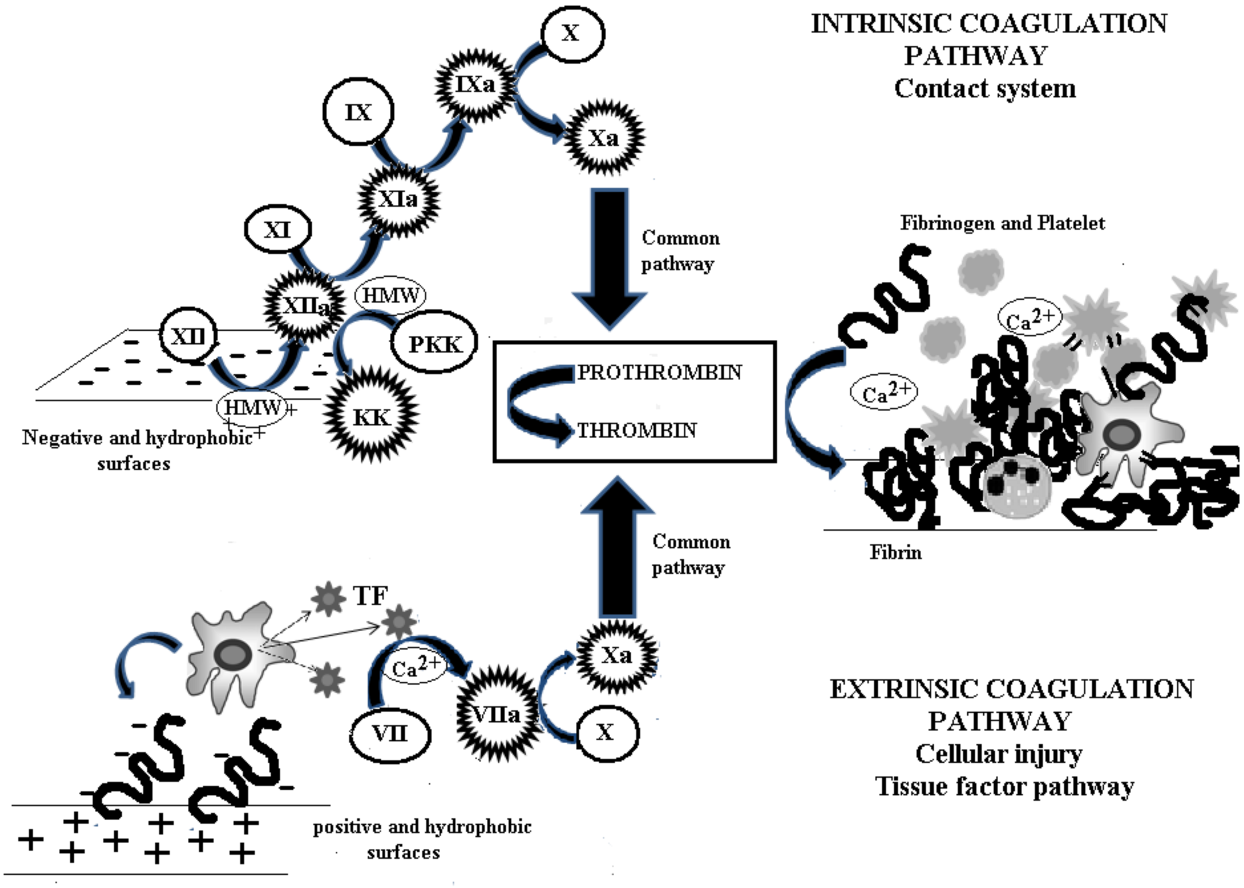

Figure 9. Contact coagulation and extrinsic (tissue factor) coagulation blood pathways

PUs are promising materials for implantable and non-implantable blood-interacting devices. They combine an increased elasticity with good mechanical resistance. For haemocompatibility evaluation of PU/HPC materials discussed above, the haemolytic and thrombotic potentials were determined by standard and adapted methods (see section 2). The obtained results are summarised in Table 2.

All studied membranes showed a low haemolytic activity, lower for PU/HPC than for pure PU-PEGA sample.

Material samples	Haemolytic potential	Thrombotic potential			
	Released Hb (%)[1]	FB (mg/ml)[2]	PT (s)[3]	Adhered platelet (cells x 10^5/mm^2)	% blood clot amount[4]
PU-PEGA	6,7±0,2	2,79±0,04	11,06±0,4	1,40±0,08	40%
PU-PEGA/HPC	5,2±0,1	2,87±0,04	10,9±0,09	0,82±0,05	29%
PU-PTHF/HPC	4,2±0,2	2,90±0,01	10,9±0,09	0,86±0,05	15%
PU-PPG/HPC	5,5±0,1	2,77±0,07	10,9±0,07	1,25±0,09	89%

Table 2. Haemolytic and thrombotic potential of the PU/HPC samples: Hb-haemoglobin; FB-fibrinogen; PT- prothrombin time

As for thrombotic action, a correlation between adsorbed FB, platelet adhesion and amount of formed clot was registered, while no significant variation was recorded for PT. This latter parameter was kept within the normal limits (see footnote 3).

The judgement strictly based on the haemocompatibility results permits to state that all examined materials have an acceptable thrombotic potential (referring to physiological requirements). Considering clot amount and all the other characteristics discussed above, it is obvious that PU-PEGA and PU-PPG/HPC are not suitable for long-time functional integration.

3.3.3. *In vivo biocompatibility and performance*

The technological progress achieved in the last decades in apparently unrelated areas (biomaterials, biotechnology, cell and molecular biology, tissue engineering, and polymer science) has generated a boost in the development and use of devices for medical and/or other type of applications (e.g. artificial organs, biosensors, catheters, heart valves) (Shastri, 2003). In spite of real improvement of this sort of devices there are still some important problems to face since implanted medical devices usually reveal different degree of loss of functionality over time after insertion (Göpferich, 1996). Tissue or blood-device interface interactions or a lack of biocompatibility resulting from the normal homeostatic response of the body to the implantation injury, determining an inadequate *in vivo* functionality and longevity, remains a serious concern (Callahan & Natale, 2008; Fujimoto et. al., 2007; Morais et. al., 2010).

In order to protect the body from the foreign object, under normal physiological conditions, the body reacts by several nonspecific mechanisms (immune and inflammatory cells recruitment), usually termed foreign body reaction (FBR) (Anderson, 2001). There is an imperative call for knowing the degree to which the pathophysiological conditions are

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[1] Percentage of released Hb over negative control

[2] FB concentration remained in blood plasma after incubation. FB control was 2,98 ± 0,04 mg/ml

[3] Physiological normal value according to related laboratory are between 8,3 s and 11,3 s

[4] Percentage of blood clotting over negative control (blood without incubated material)

created, the homeostatic mechanisms are disturbed, and the resolution of the inflammatory response (simple put, the measure of the host reaction). All of these will finally establish the effective compatibility of a specific device. In the same time, understanding these reactions (the implant versus the host and the host versus the implanted device) will reduce health problems to the beneficiary of the device and device malfunction. Usually, for practical reasons, the homeostatic mechanisms are separately assessed even if it is well known that they are profoundly interrelated (Sieminski & Gooch, 2000).

The first event after a device/material insertion is that the body generates quickly a sort of "interface" *via* nonspecific adsorption of plasma/tissue soluble proteins on the implant surface (Shen & Horbett, 2001). There are some well identified elements that determine the FBR strength: device material composition, surface chemistry, size and shape, porosity, degradation, velocity as well as the place of device insertion (Ratner & Bryant, 2004)

As presented shortly below, tissue injury associated with device implantation, initiates a complex set of events (nonspecific inflammatory reaction and wound healing responses) that will bring about a FBR (Wahl et. al., 1989). The stages of inflammatory responses are well studied and can be separate in acute and chronic inflammatory periods.

The initial phase, acute stage, starts quickly in matter of hours, lasts for several days (up to 14 days) and is underlined by rapid device interface generation and typical for this phase, different degree of neutrophil leucocytes responses (Jiang et. al., 2007). The main result of this stage is the building of temporary interface material-tissue, the cleaning-up of the injury place and the vasodilation that bring more blood in the affected area.

The acute inflammatory reaction typically decline in maximum 14 days with a "biocompatible" material. Some local conditions (extent of surgical injury, body reactivity) or properties of the implanted device can trigger a chronic inflammatory evolution (Kirkpatrick et al., 1998).

Numerous blood and tissue proteins such as cytokines (e.g. tumor necrosis factor (TNF), interleukins (IL-6, IL-8), matrix metalloproteases (MMP-1, MMP-3), granulocyte-macrophage growth factors (GM-CSF)) are released, and leukocytes adhere to the endothelium of the blood vessels and infiltrate the injury site. These proteins are strong calling factors for monocytes, cells which will migrate to the site of inflammation where they will differentiate into macrophage. If inflammatory stimuli persist, the conditions that can lead to chronic inflammation are created. Cell population of this stage of inflammatory reaction is usually characterized by the presence of monocytes, macrophages, and lymphocytes (Bhardwaj et al., 2010). Also, in this step it can be noticed that the proliferation of blood vessels (angiogenesis), and connective tissue occurs that participate in remodelling of the affected area. The formation of blood vessels is crucial for wound healing, supplying necessary factors for tissues reconstruction. In the end, the granulomatous tissue is replaced by an extracellular matrix (ECM) that acts not only as a physical scaffold but also as an essential modulator of the biological processes, including differentiation, development, regeneration, repair, as well as tumour progression. The end phase of the FBR draws in wrapping the implant by a collagenic fibrous capsule that limits the implant and therefore prevents it from interacting with the surrounding tissue. The main tissue events of the material-tissue interaction and wound healing are schematically presented in Fig. 10.

Morphologic aspects (light microscopy) of the acute tissue reaction to subcutaneous implanted polyurethane (PU-PTHF/HPC) at 10 days of implantation and chronic inflammation at 30 days of implantation are shown in Fig. 11. The study was conducted on Wistar male rats using the protocol described in section 2.

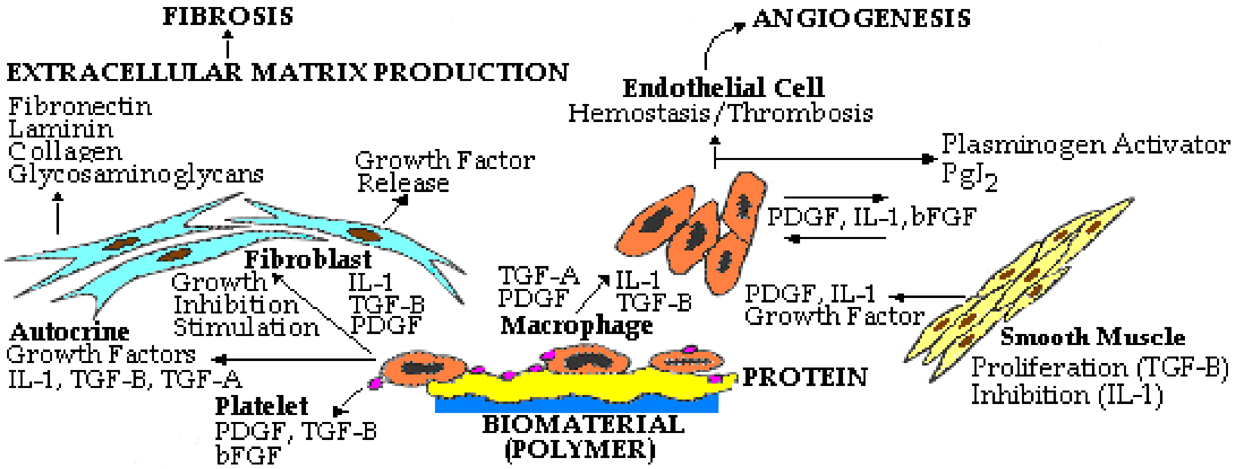

Figure 10. Fibrosis and fibrous encapsulation. End stage healing response to biomaterials. GF – growth factor (PD – platelet derived, T – transforming, bF – basic fibroblastic); IL – interleukin; PGL – prostaglandin.

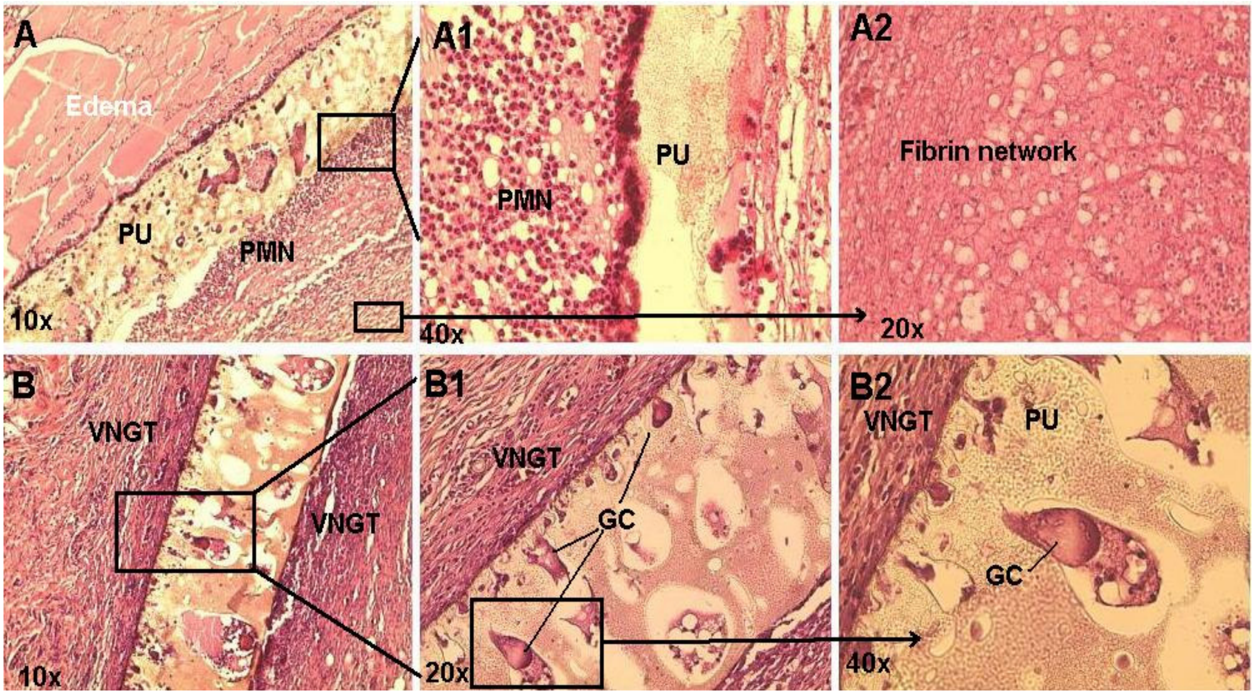

Figure 11. Light microscopy images of tissue response to implantation of subcutaneous non-washed PU-PTHF/HPC (PU): A - A2, 10 days of implantation; B -B2, 30 days of implantation. All images – HE staining. Objective magnifications are indicated in the left bottom corner

Unwashed (unpurified) material was implanted first, to highlight the importance of the properly prepared biomaterial for medical usage. From Fig. 11, A-A2 images, it can be easily seen as an intense acute inflammation reaction with numerous neutrophil polymorphonucleate leucocytes (PMN), edema and early fibrin network formation away from implantation site. These results suggest that an inappropriately prepared material at some stage in manufacture and/or manipulation can delay wound healing. As we expected, at 30 days of implantation (Fig.11, B-B2 images), inflammatory chronic reaction was really strong for related material, with the characters of neovascularised granulomatous tissue (VNGT) and giant cells (GC).

In the end of this chapter, comparative study concerning long-time potential functionality based on evolution of chronic inflammation of PUs/HPC discussed above was done. The histological images of 30 days implanted, properly purified PUs/HPC are shown in Fig. 12.

There were found chronic inflammations with VNGT and FBR with GC for PU-PEGA/HPC (A-A2 images) and PU-PPG/HPC samples (C-C2 images). Moreover, granuloma formation (G) as result of macrophage material degradation was present at material-tissue interface of PU-PPG/HPC (C and C1 images in Fig. 12).

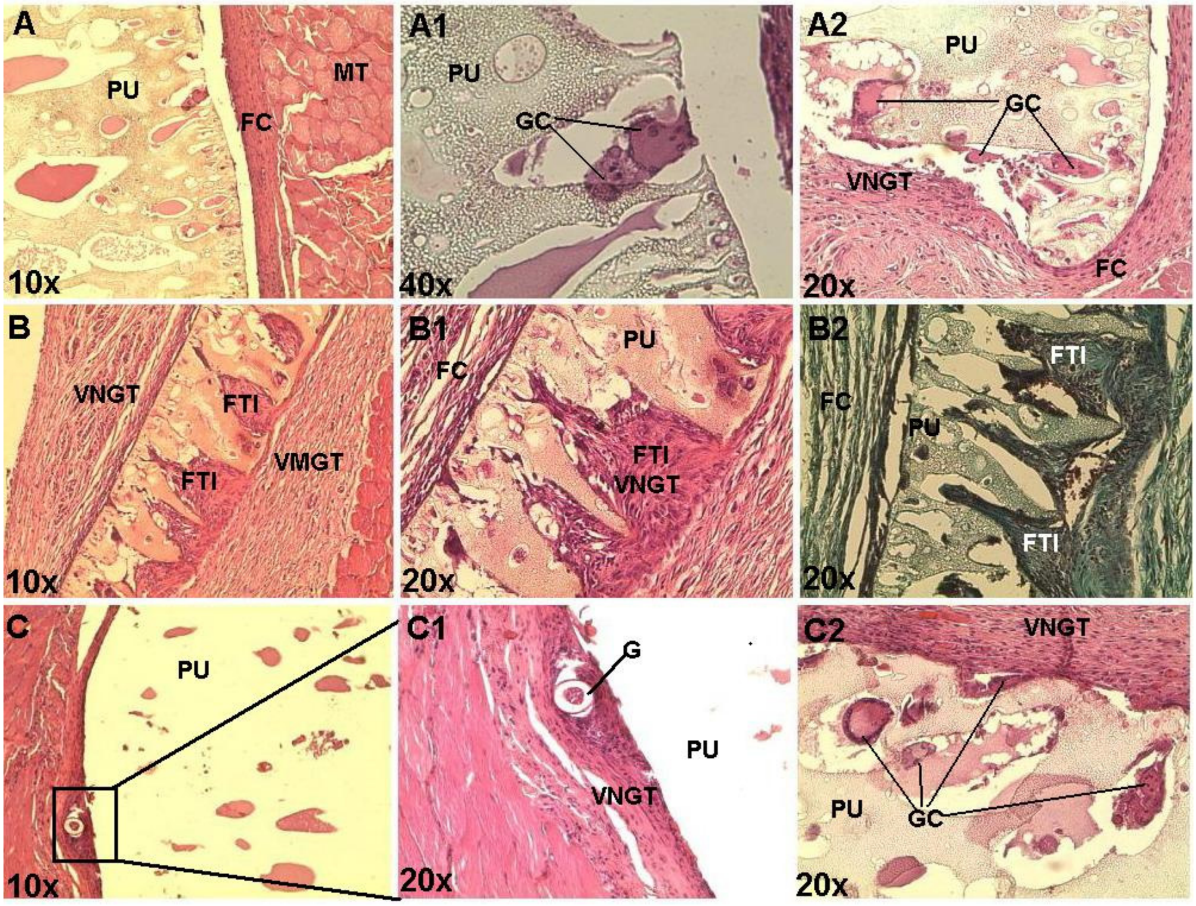

Figure 12. Light microscopy images of tissue response following 30 days subcutaneous implantation of washed PUs/HPC. A–A2, PU-PEGA/HPC; B-B2, PU-PTHF/HPC and C-C2, PU-PPG/HPC. B2, Masson's trchrome staining; all other images - HE staining. Objective magnifications are indicated in left bottom corner

The absence of GC and rich granulomatous tissue ingrowth through large material pores was observed for PU-PTHF/HPC sample (Fig.12, B-B2 images). Morphological aspect for PU-PTHF/HPC implant suggests a material-tissue integration and regenerative remodelling. Moreover, the fibroblast-rich tissue ingrowth only from one side of the membrane highlights the bifacial behavior of the implanted sample, with potentially tubular or cavity-like device performances. Thus, considering PU-PTHF/HPC increased haemocompatibility, oxidative and other biocompatibility advantages discussed above, we presume a cardiovascular-device performance for this PU sample.

4. Conclusions and further perspectives

Polyester and polyether urethane structures with improved bulk and surface characteristics by blending with small amount of biocompatible cellulose derivative, HPC, are screened for long-time functional integration. The stability of the pH value of biological media and the ratio of adsorbed albumin and fibrinogen from blood plasma were found to be the most valuable screening criteria to evaluate the blood-interface functionality, but not only. These criteria could provide information on material capacity to keep stability of the main body balances (oxidant/antioxidant, haemostasis/haemolysis) that are responsible for material acceptance in the early phase, followed by structural and functional integration in the later stages. These characteristics together with other important material properties as surface neutral charge and desired porous structure are keys points for good results expectance as was demonstrated. Another PU characteristic highlighted in our study was washability for potentially proinflammatory compounds removal. Due to interconnected mechanisms of thrombosis and inflammation, even haemocompatible PU, but with chronic prolonged inflammatory capacity (through itself or some released compound) will certainly get to fail its haemocompatibility *in vivo*. From this point of view we demonstrate an acceptable stability of some PU membrane by autoclaving and long-time watering in biological buffers. Further studies are necessary on extended classes of polyurethanes in the aim to prepare and keep ready to use pre-equilibrated and safe PUs for medical applications.

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