

In vitro Biocompatibility of HPC - modified Polyurethane Membranes

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Polyurethanes (PU) are synthetic materials with large structural possibilities and mechanical adaptability that have promising perspectives in medical applications. This paper is focused on the hydrolytic stability and biocompatibility of poly(ester-urethane) and poly(ether-urethane) membranes blended with hydroxypropylcellulose (HPC). FTIR analysis and extended biocompatibility tests were performed. Based on our in vitro results this PU sample is expected to promote a better biological tolerance and lower pro-inflammatory reaction in vivo.

Keywords: HPC-modified polyurethanes, hydrolytic stability, cytotoxicity, pro-inflammatory potential

Polyurethanes (PUs) are well known synthetic polymers used for many medical applications [1-4]. Their block-polymer structure could provide a large spectrum of physico-chemical properties and degradability, reasons for which the biological performances of PUs have been widely discussed in the last decade [5-8]. While the structural and mechanical adaptability of the PUs is indisputable, their bio-functionalization for a better biocompatibility is still a challenge. Most efforts are now focused on molecular conjugation, blending or coating as methods for functional improvement of the PU [9-12]. From chemical point of view, the approach is directed to the molecules that provide a high level of molecular interactions, for example through hydroxyl radicals of polysaccharides including those of cellulose derivatives [13,14]. Previously we demonstrated that small amounts of HPC in some PU structures can enhance surface hydrophilicity, neutralize surface charge, decrease surface roughness and improve bulk porosity of the resulted membranes. All these property modifications were accompanied with change in protein adsorption, improvement of the haemocompatibility and oxidative stress resistance at the material-blood interface [15-16]. In this paper the hydrolytic modifications of the material structures, *in vitro* biocompatibility as well as polymorphonuclear (PMN) leucocytes activation as a result of material extracts are discussed.

Experimental part

Materials and reagents: Poly(ethylene adipate)diol (PEGA, Mn = 2,000 g/mol) from Fibrex SA Savinesti, Romania; polytetrahydrofuran (PTHF, Mn = 2,000 g/mol) and poly(propylene)glycol (PPG, Mn = 2,000 g/mol) from BASF; ethylene glycol (EG), N,N-dimethylformamide (DMF) and 4,4-diphenylmethane diisocyanate (MDI, distilled under reduced pressure prior to utilization) from Merck; Hydroxypropylcellulose LF (HPC with average weight molecular weight Mw = 95 000 g/mol) from Klucel. All other materials and reagents were purchased from Sigma-Aldrich unless otherwise mentioned (Thiazolyl Blue Tetrazolium Bromide (MTT), suitable for cell culture;

Dulbecco's Phosphate Buffered Saline (DPBS), modified, without calcium chloride and magnesium chloride, liquid, sterile-filtered, suitable for cell culture; Hank's Balanced Salt Solution (HBSS) based on HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), modified, without calcium chloride and magnesium chloride, liquid, sterile-filtered, suitable for cell culture; Dulbecco's Modified Eagle Medium (DMEM), high glucose with L-glutamate and Pyruvate; Penicillin-Streptomycin-Neomycin (PSM) solution (5,000 units penicillin, 5 mg streptomycin and 10 mg neomycin/mL) stabilized, sterile-filtered, suitable for cell culture; Fetal Bovine Serum (FBS) heat inactivated, non-USA origin, sterile-filtered, suitable for cell culture; dextran T500; bovine serum albumin (BSA); 0.4% Tripan Blue solution).

Preparation of PU/HPC samples was previously reported [17]. Briefly, isocyanate terminated urethane prepolymers were first synthesized by the polyaddition reactions between MDI and macrodiols in DMF as solvent. PEGA, PTHF or PPG were used as macrodiols. The urethane prepolymers were treated in a subsequent step with EG as chain extender. Finally, HPC 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 dried under vacuum for several days and kept in distilled water for solvent removing. HPC functionalized samples based on PEGA, PTHF and PPG macrodiols were named as PU/HPC-PEGA; PU/HPC-PTHF and PU/HPC-PPG. For the aimed experiments the PU/HPC membranes were cut in 6 mm discs and decontaminated by their immersion in 70% sterile ethylic alcohol for 20 min. In order to perform *in vitro* cytotoxicity tests the sterilized membranes were washed 3 times in sterile DPBS and then incubated overnight in DMEM culture media supplemented with 1% PSN at 37°C, 5% CO₂ and 95% humidity. For FTIR

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analysis and the polymorphonuclear (PMN) leucocytes migration experiments, the sterilized samples were cut in smaller pieces (about 2 mm² membrane surfaces) and then incubated in HBSS for 30 days at 37°C and normal atmospheric condition. 0.1g of each polymer pieces were used for 1 ml of HBSS. The pH of the HBSS media was measured each 2 days between 0 and 30 days of incubation, using Hanna Instrument pH meter. The extended pH measurement of the experimental HBSS media was performed in day 60. After incubation, the HBSS was removed and further used for PMN migration (PU extract) while PU pieces were analysed by FTIR spectroscopy. Before FTIR analysis the PUs were washed 3 times in a large amount of ultrapure water and dried at room temperature for several days. The non-incubated PU samples, prepared in the same way, were used as non-degraded controls.

FTIR analysis was performed using Bruker Vertex 70 FT-IR instrument, equipped with a Golden Gate single reflection ATR accessory, spectrum range 600-4000 cm⁻¹, at ambient temperature.

In vitro cytotoxicity was performed on primary rat fibroblasts isolated from rat skin, using the explant method [18]. Briefly, 1cm² of skin was decontaminated using 3-step washing procedure in DMEM with decreasing PSN concentration (4%, 2% and 0%). After the washing procedure, the hypodermic part of the skin was removed and dermis was cut in small (1-2mm) pieces and plated on the bottom of a petri dish, covered with a thin layer of FBS. The skin tissue pieces were covered with 1.5 ml of DMEM supplied with 15% FBS and 1% PSN and incubated at 37°C, 5% CO₂ and 95% humidity. Culture media was changed every 3 days, for about 2-3 weeks, until a confluent cell radiated corona was formed around the plated tissue pieces. The migrated cells were passed on 75cm² culture area flask, according to standard subculture technique [18], until a confluence monolayer was reached. Cells from 2 and 3 passages were used for biocompatibility studies. Each pre-equilibrated polyurethane disc, prepared as mentioned above, was placed over pre-plated fibroblasts at 2.5x10⁴/well cell density, in 24-well plate. The cytotoxicity assay was performed using standard MTT technique [18]. The obtained results were normalized to negative control (cultures kept in the same conditions, but not incubated with materials). The cytotoxicity experiment was performed in parallel with the morphological assessment of the cells. Briefly, the cell cultures were washed with DPBS, fixed for 20 min in 4% glutaraldehyde solution then stained using hematoxylin - eosin (HE) staining protocol [19]. The stained cells were analysed using Leica DMIL inverted microscope at 10x magnification objective.

The polymorphonuclear (PMN) leucocytes migration experiment was performed on PMNs isolated from human blood of healthy volunteer donor, using gradient separation method described in literature [20]. The PMN cells were

counted using improved Neubauer hemacytometer. Cell viability was determined by widely used 0.2% Trypan Blue solution in DPBS [18]. Only the cell suspensions with over 90% of cell viability were considered appropriate for the experiments. PMN migration experiment was performed using Falcon HTS Transwell-24 plates with 8µm pore size membrane and 1 x 10⁵/cm² pore density. The lower compartments of the plates were filled with 0,1 mL of PU extract, while the upper compartments were filled with 0.5 mL of PMN suspension in HBSS, using 1 x 10⁶ cells/well. The PMNs were activated by adding to the cell suspension 10 µL of 1 mM calcium chloride. The plates were kept for 30 min at 37°C and 95% humidified atmosphere followed by 10 min incubation at +4°C. After incubation the upper insert compartment was removed and the PMN cells migrated in the lower compartment were counted using inverted phase-contrast optical microscope. The migration experiment was performed in triplicate for each PU extract. As negative control HBSS was used.

Results and discussions

FTIR analysis. It is generally accepted that hard block-polymer part of PUs is responsible for their physical resistance and hardness while soft part assures functionality and degradability. It is known as well that poly (ester) urethanes and poly (ether) urethanes are susceptible to hydrolytic and oxidative degradation that could lead to loss of their biocompatibility in time.

The comparative FTIR spectra of 30-days incubated PU samples compared to the initial materials are presented in figures 1 and 2. All spectra present typical urethane absorption bands centred between 1703 and 1710 cm⁻¹ and C-O bands in the region 1220-1000 cm⁻¹.

According to the spectra, the long-term incubation of PU/HPC-samples does not induce pronounced structure modifications. However, comparing the spectra of PU-PEGA (fig.1a) and PU/HPC-PEGA (fig. 1b) samples one may observe an increase of the C-O-C band at 1060 cm⁻¹. The band increase could be attributed to the superposition of the C-O-C bands of PEGA moiety in polyurethane and HPC. Following the degradation (fig. 1c) this band is decreasing again, denoting the degradation of the C-O-C linkages, most probably in HPC. Moreover, comparing the intensities ratio of C=O ester band of PEGA and of urethane band at 1728 and 1710 cm⁻¹, respectively, in initial PU/HPC-PEGA (fig. 2b) with this ratio in incubated sample (figure 2c), one may observe a small decrease of ester band following the incubation. Thus, the hydrolysis of the PEGA ester bonds has to be also considered.

Similar behaviour was observed for the non-degraded and degraded PU/HPC-PTHF (figs. 2a and b) and PU/HPC-PPG (figures 2c and d), except the C=O ester absorption

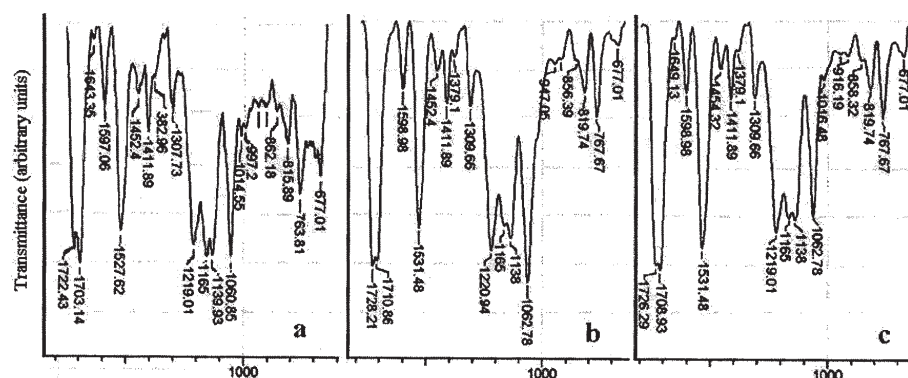


Fig. 1. FTIR spectra of the PU-PEGA samples: (a), PU-PEGA reference sample; (b), non-degraded PU/HPC-PEGA sample; (c), degraded PU/HPC-PEGA sample

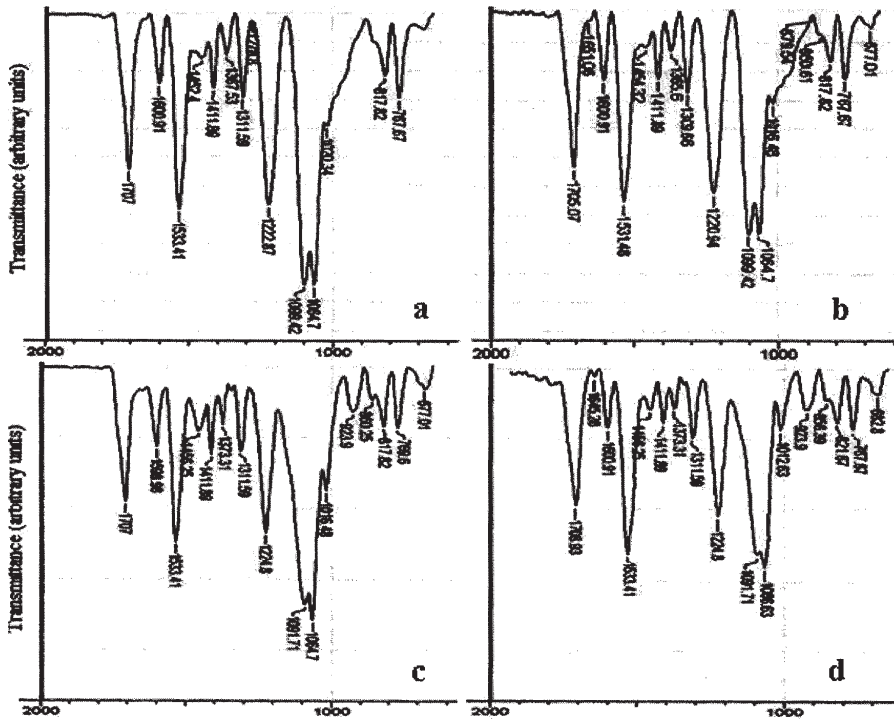


Fig. 2. FTIR spectra of the PU/HPC-PTHF and PU/HPC-PPG samples (*a* – non-degraded and *b* – degraded PU/HPC-PTHF sample; *c* – non-degraded and *d* – degraded PU/HPC-PPG sample)

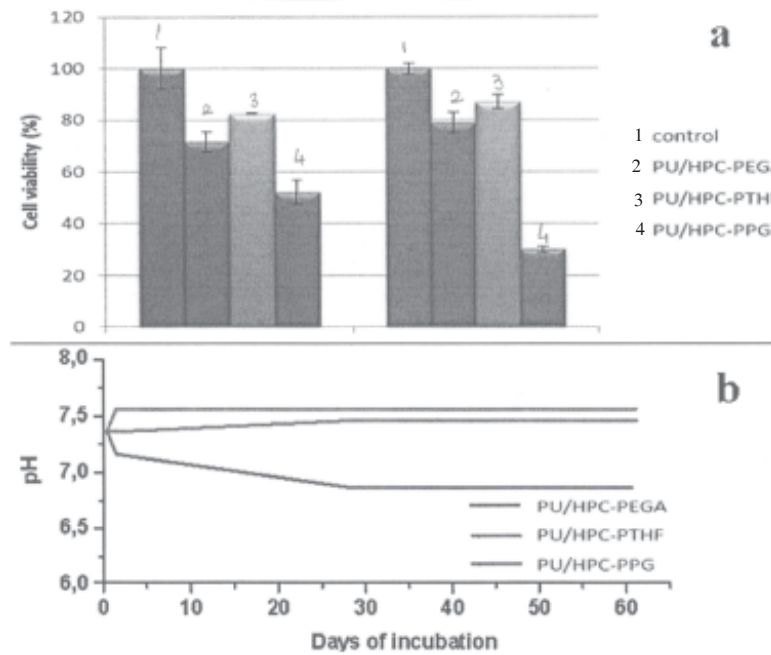


Fig. 3. (a), Cell viability of the primary fibroblasts after 24 and 48 h of incubation with PU/HPC samples; (b), pH dynamics in culture media in which PU samples are incubated at 37°C

that is absent in the spectra of poly(ether-urethane) samples.

In vitro biocompatibility of the PU/HPC polyurethanes. The results of cytotoxicity test on PU/HPC samples are presented in figure 3a. According to this figure, the least cytotoxic and most biocompatible PU sample was PU/HPC-PTHF that assures about 87% of cell viability after 48 hours of cells-material incubation.

The most cytotoxic PU sample was PU/HPC-PPG. After 48 hours this material decreased cell viability to 30% compared to the control. The PU/HPC-PEGA sample expresses a low cytotoxic effect in the first 24 h of culture. After 48 h the cell viability in the presence of this sample has grown and became more comparable with those expressed by PU/HPC-PTHF. The slight cytotoxicity expressed by PU/HPC-PEGA could be mostly explained by the pH dropping observed in the cultures (fig. 3b). The pH instability induced by this sample is probably due to hydrolytically instable polyester bonds of this sample, also observed in IR spectrum of PUHPC-PEGA sample (figure 2c). As one may see from figure 3b, the pH of the culture

is more stable for incubated PU/HPC-PTHF and PU/PG poly(ether-urethane) samples.

The cytotoxicity study was accompanied by the morphological cell assessment. Figure 4 presents the relevant cell culture images for 24 and 48 hours of cells-material incubation.

According to figure 4, no morphological changes have been observed for PU/HPC-PEGA (images b and f) and PU/HPC-PTHF (images c and g), compared to the control cultures (images a and e). For PU/HPC-PPG sample, abnormal cell morphology and very low cell density have been observed even in the first 24 h of culture (images d and h).

The PMN leucocytes migration experiment. PMN leucocytes are white blood cells that are involved in non-specific body defence. These are the cells that will first react to a microbial invasion and tissue injuries including those associated with implantable devices. The mechanism by which PMN are attracted to the affected area is chemotaxis, the phenomenon by which the cells move toward the highest concentration of some molecules named chemoattractants. Reaching the destination, the

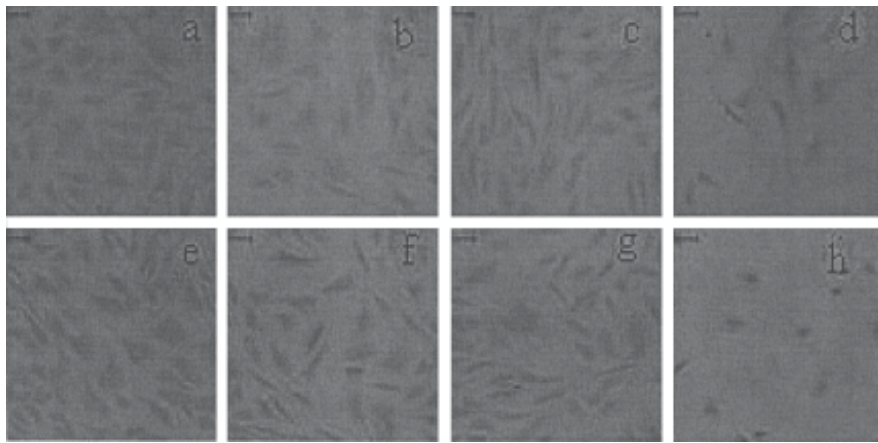


Fig. 4. The cell morphology of the cells cultured with PU samples: (a-d), cultures at 24 h; (e-h), cultures at 48 of h; (a) and (e), control samples; (b) and (f), PU/HPC-PEGA; (c) and (g), PU/HPC-PTHF; (d) and (h), PU/HPC-PPG). The scales of the images are equal to 50 µm

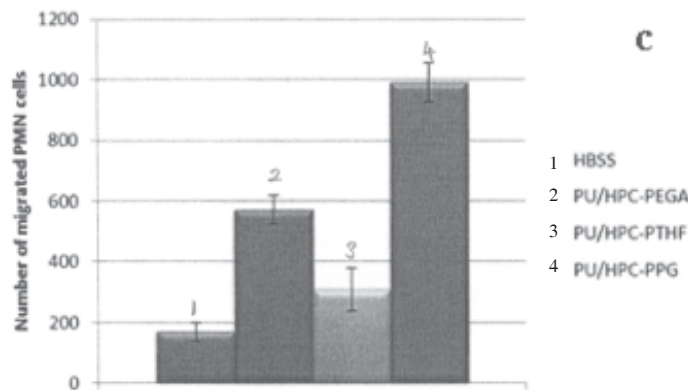
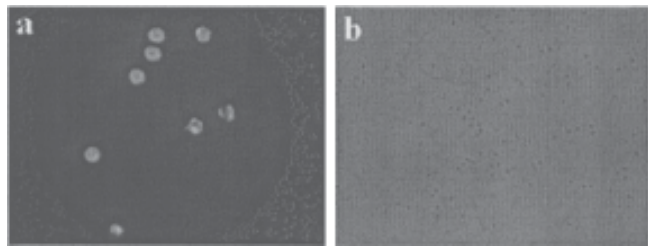


Fig. 5. PMN migration: (a), the porous membrane that separates the upper cell culture compartment from the lower, at 40x objective magnification; (b), image of the migrated leucocytes in the lower culture compartment, at 10x objective magnification; (c), number of the migrated leucocytes in the lower culture compartment.

leucocytes induce a complex acute inflammatory reaction that is continued with a chronic phase and finally with rejection or isolation of the affected area in a thick fibrous capsule [21]. The solvents, additives, synthesis process contaminants, residues and degradation products are the most frequent PMN calling signals issued by the materials. Thus, an *in vitro* method for PMN calling power could have a predictive value for *in vivo* behaviour.

Figure 5 shows the porous membrane that separates two culture compartments used for PMN migration (a), PMN cells migrated in the lower compartment (b) and the number of the cells migrated through the membrane (c). In the first image, some leucocytes trapped in the pore's hole of the membrane are seen. From figure 5c, one can resume that all materials have some PMN calling power, significantly higher than blank control. The highest chemotactic property is expressed by PU/HPC-PPG while the lowest one by PU/HPC-PTHF. These results are well correlated with the cytotoxicity data.

The found PMN calling effect and cytotoxicity of the PU/HPC-PPG sample might be due to the internal porous structure, consisting in small, less interconnected pores as well as due to higher hydrophobicity of this sample compared to the other two, data reported in our previous paper [22]. The mentioned PU/HPC-PPG characteristics most probably disrupts the release of the residual compounds from the material structure. The results

obtained in this section provide a prediction for a proinflammatory behaviour of the PU samples *in vivo*.

Conclusions

In this paper we argue the hydrolytic behavior of HPC-modified PU samples and their extended biocompatibility with a potential predictive value for the *in vivo* situation. We found that a long period of incubation in physiological condition could affect mostly the poly(ester-urethane) sample (PU/HPC-PEGA). The ester bonds degradation could be associated with the pH modification of the biological environment and less biocompatibility. Regarding the poly(ether-urethane) samples, their biocompatibility is soft-compound dependent. All biocompatibility assessments performed have demonstrated better biological performances of the PU/HPC-PTHF sample.

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