IN VITRO EVALUATION OF PLATELET ADHESION ON POLYURETHANE FILMS AND MEMBRANES

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Abstract. The *in vitro* response of human platelets was studied upon adherence to films/membranes of polyurethanes with different soft segments: polyurethane with segment lactate comparatively with poly(esterurethane) precursor, in order to promote poly(lactateurethane) as candidate for limit blood-contacting materials. For optimizing the material, the surface energy was increased by dielectric barrier discharge in He and the results were compared with those of pristine films/membranes. *In vitro* platelet adhesion and the degree of hemolysis were determined by preliminary blood compatibility assessment, using the direct contact method, and the morphology of adhering platelets was studied by scanning electron microscopy. The most hydrophilic surfaces appear to cause least activation and aggregation of platelets.

Key words: poly(lactateurethane), poly(esterurethane), helium plasma treatment, blood compatibility.

INTRODUCTION

Considering their unique chemical structure, polyurethanes are among the most versatile materials that can be used in medical applications. Strong elastomers or rigid plastics, polyurethanes are resistant, biocompatible and hemocompatible.

For improving the biocompatibility of polyurethanes researchers have concentrated on bulk and surface modifications [12]. In the case of materials that enter in contact with blood important are their surface characteristics, which influence proteins adsorption responsible for platelets activation and aggregation. A way to improve surface characteristics of a biomaterial is plasma treatment.

In our previous *in vivo* study [8, 10] we referred to surface films improvements, by dielectric barrier discharge in He (He-DBD) and Ar^+ ion beam treatments, of new polyurethane with lactate segment, poly(lactateurethane) (PL), comparatively with poly(esterurethane) (PU) precursor, in order to promote PL as candidate for limit blood-contacting materials.

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The aim of this study is to determine in vitro platelet adhesion on PL films and membranes treated by He-DBD comparatively with PL and PU as prepared and on post treated films/membranes, by preliminary blood compatibility assessment, using the direct contact method.

MATERIALS AND METHODS

The synthesis of PL and precursor PU was described in reference [9]. From these polymers two types of asymmetric membranes/films were processed by phase inversion precipitation methods. The asymmetric membranes were prepared by the wet phase inversion method and the dense films were obtained by the dry phase inversion method. The nonsolvents used were either distilled water or DMF-water solutions of various concentrations. After preparation, the films and membranes have been post treated in similar thermal conditions as in reference [11]. The phase inversion and post thermal conditions of films and membranes are presented in Table 1.

The obtained films/membranes were cut in two series of samples: voucher of 10×10 mm^2 and 20×26 mm^2 eprouvette for He-DBD treatment. The resulted samples were cleaned with isopropyl alcohol under sonication (5 min, 150 W), dried under a nitrogen flow and sterilized by exposure at UV for 30 min (15 min of exposure for each face).

The 20×26 mm^2 standard eprouvette samples were treated by DBD in He at atmospheric pressure.

The He-DBD used for surface treatment of the polymer sample is evenly distributed in any direction parallel to the electrodes. The experimental set-up of DBD [1, 2] consists of two copper plane-parallel electrodes with a surface of 10.4 cm^2 each. Two glass plates of 1.1 mm thick and 30 cm^2 surface area each were used as dielectric barrier between the two copper electrodes. The distance between the two glass plates was 3 mm. One electrode was connected on high voltage and the other one was grounded. The high voltage power supply (1 to 20 kV) generates mono or bipolar sine pulses of 10 to 45 ms width and frequency in the range of 10 Hz to 10 kHz. The total pressure of the gas was atmospheric pressure. The polymer sample was placed on a crutch between the two glass plates using a frame of 4.2×3 cm^2 made of a rigid polymer. This crutch was manipulated with the help of a shaft attached to the frame. The frame was placed between two electrodes at approximately the same distance of both electrodes.

This geometry plasma discharge is different from the DBD-filamentary regime used in the previous studies [10] and has the advantage that the discharge is uniformly distributed on the surface, thus allowing the treatment of porous polymer membranes.

During the plasma treatment of both polymer surfaces, the electrical parameters of He-DBD, which we have maintained constant, are: discharge voltage of 2 kV, discharge current of 110 mA, pulse voltage width of 20 ms and frequency of 2 kHz. The exposure time to plasma treatment of each face was 90 s and the mass flow rate of helium was 100 cm^3/min. Each He-DBD 20×26 mm^2 standard eprouvette was cut in 10×10 mm^2 pieces eliminating the weak treated border.

The surface energies of the voucher and of the He-DBD treated samples were determined by contact angle measurements, using the sessile drops method. A Bauch & Lomb microscope with a Philips Pro II PCVC840 camera image-analyzing system was used for the image processing of the 1 μl volume drops.

The surface energy was determined by using a software program, FTA32 [14]. Two methods were used for determining the surface energy: the Owens-Wendt Method – geometric mean combining rule (Owens), and the Wu Method – harmonic mean combining rule (Wu).

For the 10×10 mm^2 voucher series and He-DBD treated samples a hemolysis test was done by the direct contact method. Blood was obtained from healthy volunteers by venipuncture and collected in EDTA KE/2.6 mL SARSTED Monovette. Polymer samples were placed in the 2.6 mL SARSTED Monovette. The contents of all tubes were gently mixed and incubated at 37 ^oC for 30 min. All tubes were centrifuged at 2500 rpm for 10 min after removing the polymers. Centrifugation causes separation of lysed and unlysed cells. Lysed cells remain in solution and unlysed cells form a pellet at the bottom of the tubes.

The amount of cell lysis or released hemoglobin is then determined spectrophotometrically with a LKB ULTROSPEC 4052 TDS spectrophotometer. From each incubated and centrifuged tube, 1 mL of blood was diluted with 9 mL of PBS (pH 7.4) in order to perform the absorbance analysis ($A_{\text{test sol}}$). To determine negative and positive absorbance control two additional probes were incubated and centrifuged in the same conditions as above: 1 mL of blood was diluted with 9 mL of PBS for minimum hemolysis (A_{PBS} – 0% hemolysis) and 1 mL of blood was diluted with 9 mL of water representing maximum hemolysis ($A_{100\%}$ – 100% hemolysis).

Absorbance was assessed at 545 nm and the percentage of hemolysis after material incubation was calculated using the following equation:

$$\% \text{ Hemolysis} = \frac{A_{\text{test sol}} - A_{\text{PBS}}}{A_{100\%} - A_{\text{PBS}}} \times 100 \tag{1}$$

One standard procedure for biological specimen preparation, in order to obtain high quality micrographs with electron microscopy, involves double fixation using glutaraldehyde (GA) as the primary fixative followed by osmium tetraoxide, OsO_4. In the first step, as primary fixation, the samples were held in 2.5% GA for 2.5 hours. GA stabilizes tissues by cross-linking proteins, rendering them insoluble and immobile. Afterwards the samples were washed in phosphate buffer saline

(PBS) three times for 15 minutes until the entire GA was removed. In the second step the samples were held in OsO_4 for 1 hour and 45 minutes at room temperature and away from light. Osmium tetraoxide reacts with lipids and certain proteins but also provides electron density to the tissue [13]. After washing with water, three times for 15 minutes, dehydration was achieved by transferring the material through an ascending alcohol series (25, 50, 75, 99%), for 15 minutes each time.

The morphology of adhering platelets was studied by scanning electron microscopy (SEM) on a TESLA-BS-300 microscope.

RESULTS

In Table 1 are synthesized the results regarding pristine and He-DBD post-treated PU and PL membranes and films: the degree of hemolysis and energy of bioadhesion depending on the various conditions during the preparation process.

Some general remarks need to be highlighted. Our comparative study with a specific thrombin inhibitor and with untreated blood for the two polyurethanes shows that citrate does not significantly reduce early platelet deposition. Of course 15 min experiments were not possible in untreated blood due to clotting.

The He-DBD plasma treatment increases the energy of bioadhesion, excepting PU1 films for which probably the dry phase inversion at 110 °C for 2 hours creates an energetic barrier at the surface that cannot be reached by the He-DBD plasma treatment. The increase of energy of bioadhesion leads to the increase of the degree of hemolysis, excepting PU1 treated and untreated films.

Table 1

The characteristics of PU and PL specimens, surface energy and the percentage of hemolysis, depending on the preparation method and post treatment conditions (temperature of post thermal process and time of exposure)

Sample			PU1	PL1	PU3	PL3	PU5	PL5	PU7	PL7
Preparation process	Wet phase inversion	*T*(°C)/*t*(h)	–		–		45/0.3		45/0.3	
	Dry phase inversion	*T*(°C)/*t*(h)	110/2		–		–		110/2	
	Evaporation	*T*(°C)/*t*(h)	25/24		25/24		25/24		25/24	
%Hemolysis		Pristine	1.86	1.37	6.70	2.23	2.80	4.65	6.51	5.51
		Post-treated	1.86	2.23	7.07	3.53	3.53	6.70	7.07	6.33
Surface energy (N/m)	Wu	Pristine	45.40	49.02	45.56	25.31	42.04	45.53	59.13	37.26
		Post-treated	41.48	68.40	54.23	37.55	68.6	52.01	67.18	69.63
	Owens	Pristine	44.84	51.92	47.34	21.08	40.38	46.52	58.53	35.50
		Post-treated	43.5	65.88	53.11	34.01	80.64	50.33	79.46	76.68

Surface morphologies of pristine and He-DBD plasma treated PU and PL films and membranes are presented in Figures 1 and 2 respectively.

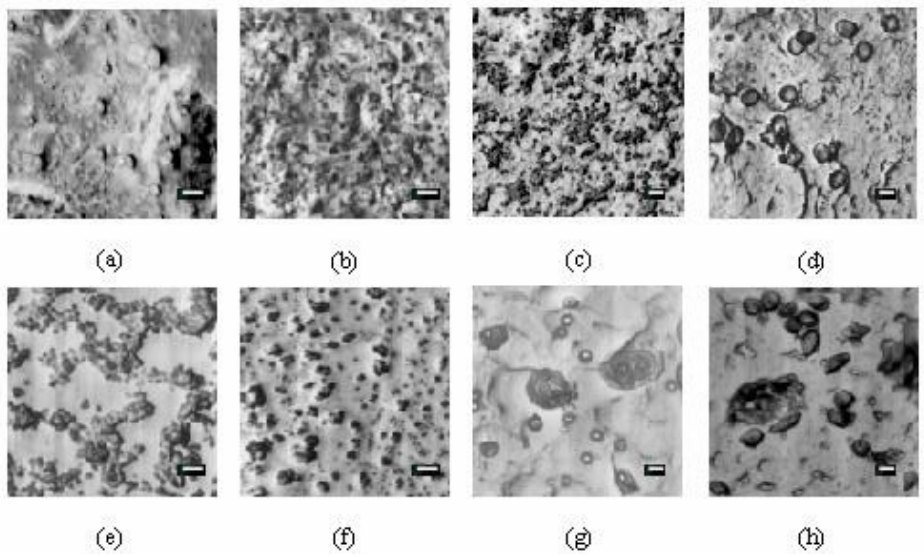

Fig. 1. The surface morphology (SEM micrographs) of pristine PU (top) and PL (bottom) films (a, b, e, f) and membranes (c, d, g, h); white stick mark is 4 μm.

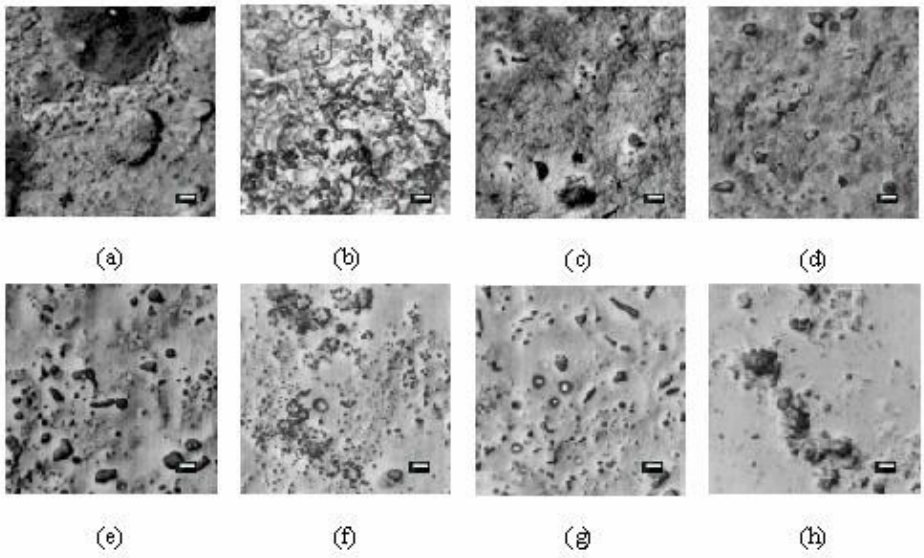

Fig. 2. The surface morphology (SEM micrographs) of pristine PU (top) and PL (bottom) films (a, b, e, f) and membranes (c, d, g, h); white stick mark is 4 μm.

The representative SEMs of the internal morphologies of the films and membranes are shown in Fig. 3. It is noticeable that while the films are very compact (Fig. 3a), in membranes pores are interconnected in the matrix and form a network-like structure (see detail from Fig. 3c).

The behavior of these morphologies makes the films act like a barrier for fluid blood, while the membranes can be penetrated by it. The figurate elements of the blood penetrate both pristine and He-DBD plasma treated membranes, preponderant to the asymmetric face of the membranes (Fig. 3b). The penetration speed of blood for the pristine and He-DBD plasma treated membranes seems to be the same because of the short contact time of blood with the membranes.

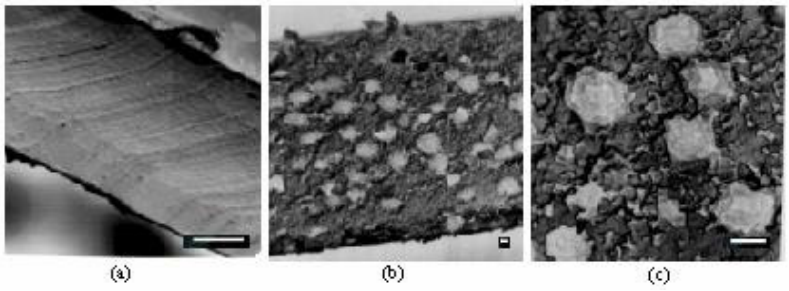

Fig. 3. The surface morphology (SEM micrographs) of pristine PU (top) and PL (bottom) films (a, b, e, f) and membranes (c, d, g, h); white stick mark is 4 μm.

DISCUSSIONS

Platelets are anuclear discoid blood cells with a diameter ranging between 1.5–3 μm. In response to an activating stimulus like adsorbed adhesion proteins, platelets become activated and release several chemical factors that cause other platelets in the proximity to adhere to one another. This phenomenon is called "platelet aggregation".

Some authors have correlated the blood-contacting behavior of surfaces with their interfacial free energy and surface chemistry [5, 6]. They demonstrated a relationship between surface hydrophilicity and the *in vitro* activation of coagulation, fibrinolysis and platelet deposition. The most hydrophobic surfaces appeared to cause least activation of coagulation but most activation of platelets.

Depending on the atmosphere of the discharge and on the energy received by the gas from the energetic electrons, determined by the discharge regime, certain chemically reactive species would be present in the discharge phase. Although, in theory, there are no chemically reactive species in pure He, processes involving cross-linking and double bonding can be observed. In a He discharge, which implies a high enough level of energy, all the other types of molecules, present in the gap, become excited and ionized.

The plasma has active constituents which can be chemically reactive species like O_2, O^- or N^- involved in the formation of new chemical functions and adding of new atoms groups to the surface or species involved in bonds breaking such as photons, electrons, nonreactive ions (He^+) and nonreactive excited atoms (He*) or molecules (N_2*). This nonreactive species can excite the polymer and break bonds like C–C or C–H that have as result the appearance of polymer radicals, if they have proper energy.

When oxygen is present in the plasma atmosphere, on the polymer surface groups like hydroxyl, carbonyl, carboxylate and carboxylic acid are formed and the number of processes involving double bonding is increasing with the duration of plasma treatment. The presence of N_2 from air can lead to the formation of functional groups containing nitrogen atoms like amine, imide, nitrile and also amide [7].

In the previous works it had been shown that in the DBD processing of polymer surfaces the breaking of polymer C–H bonds is favored in comparison with the breakage of C–C bonds from the polymer backbone [3].

In our case, the separation of the hard phase segment and the existence of a higher order degree in PL crystalline phase as compared with PU were shown by DSC, WAXRD and ATR-FTIR experiments [9, 10]. While PU has a homogeneous medium crystallinity, PL is formed of a soft amorphous phase with hard crystalline nuclei. Because of the lactate segment inside PL, the phase separation was favored and the percentage of crystalline phase, which coexists within the amorphous one, favored an increased degree order of complexity. These features lead to a different surface energy behavior of the PU and PL films/membranes to the various conditions of the dry/wet inversion preparation process and post-thermal treatments.

As a result of the differences from hard-to-soft segment ratio of PU and PL, in the case of post-thermal treatment at 110 °C, the surface hydrophilicity of the films and membranes post-treated by He-DBD plasma, is higher for PL than for PU, while in the case of the air dried films/membranes the separation of hard-soft phase is higher for PU, thus resulting an increased hydrophilicity. These can be argued by the reason that the observed reduction in contact angle comes in greater part from the initial creation of C–O single bonds, C=O and O–C=O more oxidized forms, responsible for increasing the surface hydrophilicity by increasing both the London dispersive component and the specific polar component of surface free energy [4].

These results are also reflected by the SEM images of the pristine and He-DBD plasma treated films and membranes in figures 1 and 2, respectively. For the pristine PU and PL films and membranes the thermal treatment did not reveal major differences regarding the platelet adhesion, excepting air dried PL3 films (Fig. 1f) for which any oxidation surface reaction leads to a low surface energy and an increased number of adhered platelets.

Regarding the He-DBD plasma treatment, there is an obvious correlation between the increase of the surface energy and the reduced adherence of the blood platelets to the treated PU and PL films and membranes comparatively with the pristine films and membranes. The most hydrophilic surfaces appear to cause the least activation and aggregation of platelets (Figs. 1 and 2 comparatively).

In the case of pristine (Figs. 1c, d, g and h) and He-DBD plasma treated (Figs. 2c, d, g and h) membranes, besides platelets the SEM images also show erythrocytes, mostly on the pristine ones; pores occupy large surface areas of the polyurethane membranes (Figs. 3b and c) thus increasing the available area for the cellular contact and cell attachment.

CONCLUSIONS

For the pristine PU and PL films and membranes the thermal treatment did not reveal major differences regarding the platelet adhesion, excepting air-dried PL films.

The surface hydrophilicity of the films and membranes post-treated by He-DBD plasma, in the case of post-thermal treatment at 110 °C, is higher for PL than PU, while in the case of the air dried the separation of hard-soft phase is higher for PU, thus resulting an increased hydrophilicity.

The increase of energy of bioadhesion leads to the increase of the degree of hemolysis, excepting PU films post-thermal treated at 110 °C.

The ratio of soft/hard segments and He-DBD plasma treatment (which determine the hydrophilicity) of the PL and PU polyurethanes showed a direct relationship with the degree of platelets adhesion and aggregation. The most hydrophilic surfaces appear to cause the least activation and aggregation of platelets.

SEMs of the internal morphologies show that while the films are very compact, in membranes pores are interconnected in the matrix and form a network-like structure; films act like a barrier for the blood fluid, while the membranes can be penetrated by it.

The figurate elements of the blood penetrate both pristine and He-DBD plasma treated membranes, preponderant to the asymmetric face of the membranes.

Corroborated with PL films/membranes biodegradability [1], this structural morphology and surface could be suitable for cell ingrowths and new tissue formation.

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