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PDMS content affects in vitro hemocompatibility of synthetic vascular grafts

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Abstract An unsolved problem when employing small-diameter vascular grafts for aorto-coronary by-pass and peripheral reconstruction is the early thrombotic occlusion. The PEtU-PDMS is a new elastomeric material, composed of poly (ether) urethane and polydimethylsiloxane, synthesized to realize grafts with improved hemocompatibility characteristics. In order to investigate the effect of PDMS content on hemocompatibility, three different percentages of PDMS containing grafts (10, 25 and 40) were evaluated. Grafts realized with Estane 5714-F1® and silicone medical grade tubes were used as references. The hemocompatibility was investigated by an in vitro circuit in which human anticoagulated blood was circulated into grafts by a peristaltic pump modified to obtain a passive flow. For each experiment, 40 cm length graft was closed into a circular loop and put in rotation for 2 h at 37°C. At the end of the experiments different parameters regarding platelet adhesion and activation were evaluated: circulating platelets count, β-thromboglobulin release, platelet CD62P expression and amount of monocyte-platelet conjugates. PEtU-PDMS grafts with 25 and 40% of PDMS induced the lowest platelet adhesion, plasma level of β-TG and amount of monocyte-platelet conjugates. No significative variations were observed in CD62P expression. In conclusion, PDMS content significatively affects blood-graft surface interaction, in fact higher PDMS percentage containing grafts showed the best in vitro hemocompatibility.

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1 Introduction

Surgical treatment of patients affected by peripheral atherosclerotic or cardiac disease, such as coronary ischemia, needs vascular graft to re-establish the vascular flow. At the moment, autologous saphenous vein represents the conduit of choice for arterial replacement because of its availability in sufficient quantity for multiple grafts and its ease of surgical preparation [1]. However, despite improvements in methods of harvesting, preservation, and early anticoagulant treatment, 15–30% of venous grafts becomes occluded in the first year, one-half of which within the first month. The subsequent annual occlusion rate is about 5–10%, therefore more than 15% of patients who has coronary artery by-pass with saphenous vein requires re-operation within 10 years [2–5]. From the above, artificial vascular grafts are interesting to replace damaged blood vessels. Up to now vascular grafts with high diameter (≥6 mm), realized in polyethylene terephtalate and expanded polytetrafluoroethylene, allow a good patency for replacement of the abdominal artery, while no artificial conduit has shown satisfactory results in small diameters (<6 mm).

In this study a standard aromatic poly(ether)urethane (PEtU) was modified with the addition of

increasing quantities of a reactive polydimethylsiloxane (PDMS) to realize a new PEtU-PDMS material which combines in one step the good properties of both PEtUs and silicones. In fact the attributes that make PEtUs attractive as materials for biomedical applications are their good biocompatibility and excellent physical-mechanical properties [6], however, they show the phenomenon of biodegradation that occurs in long-term implantation [7, 8]. On the other end silicones have shown good hemocompatibility, low toxicity and a long-term biostability [9]. Besides polyurethane chemically bound to silicone, has shown to improve the elasticity and the hemocompatibility when compared with commercial polyurethanes, giving the necessary properties to be used for biomedical devices realization [10].

This new elastomeric formulation (PEtU-PDMS) could find an application as biocompatible material in many different areas of medical devices, in particular in the field of cardiovascular graft where hemocompatibility and elasticity are primary features. With regard to this observation vascular grafts with different PDMS content were realized using a spray-technology. This technology, which utilizes the principle of phase-inversion, produce a sudden precipitation of the polymer solution onto a rotating mandrel and allows realising materials (graft or film) with an even distribution of PDMS and PEtU. In previous studies we have realized by spraying, and analysed by IR, film surfaces of pure PEtU, pure PDMS, and of PEtU-PDMS containing growing concentration of PDMS (10, 20, 30, 40, 50, 60, 80, and 100%). The IR analysis of the internal surface showed that exists a critical concentration of PDMS after that we have two different situation in the materials: from 10 to 30% we have the preferential exposition of characteristic PU groups on the material surface, while from 40 to 100%, we preferentially have the characteristic PDMS groups exposed. In other words, between 30 and 40% of PDMS content, the material surface begin to behave as a ''silicon-like'' surface. In particular, but related to the work described above, this paper focus on the hemocompatibility evaluation of PEtU-PDMS grafts material in relation to three different PDMS contents.

Grafts hemocompatibility was investigated by a circuit in which human anticoagulated blood was circulated into different PDMS containing grafts (10, 25, 40%). The circuit was constructed according to the model described by Tepe in 2002, briefly, a peristaltic pump was modified in order to obtain a passive blood flow into the grafts and to avoid hemolysis during the experiment. At the end of each experiment different parameters regarding platelet adhesion and activation were evaluated: circulating platelets count with an electronic globule-counter; release of β-thromboglobulin from activated platelets by ELISA, platelet P-selectin (CD62P) surface expression as marker of platelet degranulation and amount of monocyte-platelet conjugates using cytofluorimetric analysis. These parameters were chosen according to ISO 10993 Part 4 [11].

2 Materials and methods

2.1 PEtU-PDMS and reference materials

The PEtU-PDMS synthesis and processing to realize vascular grafts were carried out as previously described by Soldani [12]. In particular, PEtU-PDMS materials containing 10, 25 and 40% of PDMS (w/w) were prepared.

A medical grade silicone and the Estane 5714-F1®, commercial materials known for their biocompatibility and hemocompatibility, were chosen as references according to ISO 10993 Part 12 [13, 14]. The medical grade silicone was supplied in tubing form (code 96410-25, Masterflex, Vernon Hills, IL, USA), while the Estane 5714-F1® was supplied in grain form (BFGoodrich Chemical-Italia, Milano, Italy) and dissolved in tetrahydrofurane-dioxane (THF-DX) 1:1 (v/v) to obtain a 3% (w/v) final concentration solution.

The working solutions of PEtU-PDMS and Estane 5714-F1® materials were brought near to their precipitation point by adding 17% (v/v) of distilled water (non-solvent). Then, using an instrument named spray-machine, the unstable solutions were processed by a spray-technology associated with phase-inversion of the material, to realize vascular grafts of 10 cm length, 5.0 mm I.D. and 0.5 mm wall thickness [15, 16]. Briefly, pre-phase inversion solution and distilled water were simultaneously sprayed to intersect onto a rotating Teflon® mandrel, through modified spray-guns, which were mounted onto a sliding carriage. The combining polymeric solution and non-solvent induces a sudden phase-inversion of the polymer from the solution and results in the deposition of an even distribution of porous, or microporous, material onto the mandrel.

This technique allows to obtain grafts with different internal microgeometry in relation to fabrication parameters such as pressure and flow rate of both polymer and non-solvent solutions, amount of non-solvent added and concentration of the polymer solution.

Previous experimental observations showed the importance of the internal surface microgeometry of

grafts in inducing thrombus formation, in particular it was demonstrated that a highly porous surface is less thrombogenic than a non-porous one, because it provides a minimal surface area of interaction with blood [17]. In this study PEtU-PDMS grafts with the same porous surface microgeometry were produced using a 0.2% (w/v) polymer concentration and varying the PDMS content as above mentioned.

2.2 The in vitro circuit

Grafts hemocompatibility was investigated by a circuit in which human blood was circulated into PEtU-PDMS grafts containing different percentages of PDMS.

The circuit was constructed according to the model described by Tepe with slightly modifications [18]. Briefly, for each experiment, 40 cm length graft was closed into a circular loop, partially filled with blood, placed on a rotating cross-support and put in rotation by a rotating pump (code 7523–37, Masterflex) in order to obtain an indicative 24 cm/s flow speed for 2 h. In this way a passive blood flow, opposite to the direction of graft rotation, was obtained avoiding hemolysis during the experiment. The temperature was stabilized at 37°C by a thermostatic chamber. A schematic representation of the circuit is shown in Fig. 1.

The same closed circuit, made of 40 cm of length, 5.0 mm I.D. medical grade silicone tubing (code 96410–24, Masterflex), has been used to evaluate the hemolysis in preliminary experiments.

Before assembling the circuit, PEtU-PDMS grafts were sterilized in HCl 0.4 N under sonication for 30 min., then they were thoroughly rinsed in sterile distilled water. Finally, grafts were externally coated with a highly concentrated PEtU-PDMS material solution (about 13% w/v) to make them impermeable to the circulating blood.

2.3 Blood collection

Human blood was drawn from healthy, non-smoking, 20–35 years old donors who had not taken anticoagulant and antiaggregant medication for at least 2 weeks prior to the study. For each experiment 15 ml of blood was collected in sodium-citrate 3.8% (1:10 v/v), from the antecubital vein using a 19 G butterfly needle to minimize ex vivo activation of platelets [19]. Before each experiment donors hematocrit was measured and resulted within normal range.

Fig. 1 Schematic diagram of modified Tepe loop system: (1) modified roller pump to obtain a passive blood flow inside the grafts; (2) cross bearing to lodge the grafts closed to a loop; (3) PEtU-PDMS grafts or reference materials partially filled with anticoagulated blood; (a) anticlockwise = direction of graft rotation; (c) clockwise = direction of blood flow into the grafts

2.4 Hemolysis evaluation

Blood samples drawn after 2 h of circulation were centrifuged at 2500 g for 30 min. The resulting platelet poor plasma (PPP) was collected and examined spectrophotometrically (UVIKON 943, Kontron Instruments, Milano, Italy) at 542 nm to quantify free hemoglobin released into plasma. Samples diluted 1:200 with Lyse S (Coulter, Milano, Italy) to lyse all erythrocytes served as positive control. The hemolysis was calculated in percentage in relation to the total hemoglobin [20]. The percentage of hemolysis after 2 h of circulation was about 2%, a low value considered acceptable [21].

2.5 Platelet adhesion

The platelet adhesion on the grafts internal surface was indirectly determined by circulating platelets count. Immediately after the venipuncture (basal) and at the end of circulation time (2 h), the number of platelets was automatically counted in whole blood by an electronic counter (Sysmex SF3000, Kobe, Japan).

2.6 β-thromboglobulin release assay

At the beginning and at the end of circulation period blood samples were collected into pre-chilled centri-

fuge tubes containing a mixture of theophylline (1 mM final concentration; Sigma Co., St. Louis, MO) and prostaglandin E_1 (0.033 μg/ml final concentration; Sigma). This mixture, called Edinburgh anticoagulant, was used to arrest β-thromboglobulin (β-TG) release during the PPP preparation, in fact it blocks the degranulation reaction and prevents further platelet activation [22, 23]. After allowing the tubes to cool in an ice bath for 30 min., they were centrifuged at 2500 *g* and 4°C for 30 min. One third of the volume of the resulting PPP supernatant was collected in the middle portion of the plasma and then stored at –80°C prior to β-TG level determination by a commercial ELISA (Asserachrom β-TG, Boehring-Mannheim, Milano, Italy) and a multiplate reader (SPECTRAFluor PLUS, Tecan, Milano, Italy).

2.7 Sample preparation for flow cytometric analysis

In order to quantify platelet activation and monocyte-platelet conjugates in the circulating blood, tests were carried out by flow cytometry using monoclonal mouse antibodies supplied by Valter Occhiena (Torino, Italy). Flow cytometric analysis was performed by FACScan instrument and CellQuest software (Becton Dickinson, San Jose, CA, USA).

2.7.1 CD62P expression

As previously described [24], 35 μl of diluted blood samples (1:10 in PBS-EDTA 9 mM, pH 7.2) were incubated for 1 h at 4°C with 5 μl of anti-CD41 conjugated with phycoerythrin (PE) antibody and 10 μl of anti-CD62P conjugated with fluorescein-isothiocyanate (FITC). IgG1 (FITC) was used as isotype control.

At the end of the incubation period, samples were diluted with PBS-EDTA and analyzed by FACScan. By evaluating the cells size (forward scatter) and granularity (side scatter, SSC) the gate of platelets was identified and within this area 20,000 events were collected. The percentage of activated platelets (CD62P positive) was determined within the gate of the platelets population (CD41 positive) in comparison with the baseline isotype control.

2.7.2 Monocyte-platelet conjugates

Appropriate volumes of the following antibodies were added to 10 μl of diluted blood (1:5 with PBS-HEPES 10 mM): anti-CD45 (PE), anti-CD14 (FITC) and anti-CD41 and a mouse isotype control both of them conjugated with peridinin chlorophyl (PerCP). Samples were incubated for 20 min. at r. t. in the dark. Finally, samples were diluted 1:10 with paraformaldehyde (0.2% in PBS) and analyzed by FACScan. The acquisition process was stopped at 10,000 events using a dot-blot of SSC versus fluorescence 2 (FL2). The collecting data were analyzed quantifying the percentage of CD14/41 double positive events, representing monocyte forming conjugates with platelets and the mean fluorescence intensity (MFI) expressed as linear fluorescence channels after subtracting the median value of the isotype-matched control histogram from that corresponding to the specific marker, representing the number of monocyte conjugated with platelets.

2.8 Statistical analysis

For each type of grafts five independent experiments of in vitro circulation were carried out and the results expressed as mean ± S.D. Data from all experiments were statistically evaluated using StatView™ 5.0 software (SAS Institute, Cary, NC, USA) by Student *t* test ($p < 0.05$ was considered significative).

3 Results

3.1 Platelet adhesion

The number of platelets was automatically counted in whole blood at the beginning (basal) and at the end of circulation time (2 h), this value is indirectly correlated to platelet adhesion on the grafts internal surface. Platelet count in the basal samples was within the normal range. The platelet number at 2 h was expressed in percentage in relation to the platelet count in the basal sample, that was considered as 100%.

The effect of circulation on the count is shown for all the types of grafts in Fig. 2. The percentage of circulating platelets after 2 h decreases for all the tested grafts, but it results that grafts with the two higher PDMS content (25 and 40%) capture a minor amount of platelets related to grafts with the lower PDMS content (10%) and Estane 5714-F1® grafts. Moreover, the Estane 5714-F1® grafts capture a number of platelet significatively lower than the silicone medical grade tubing.

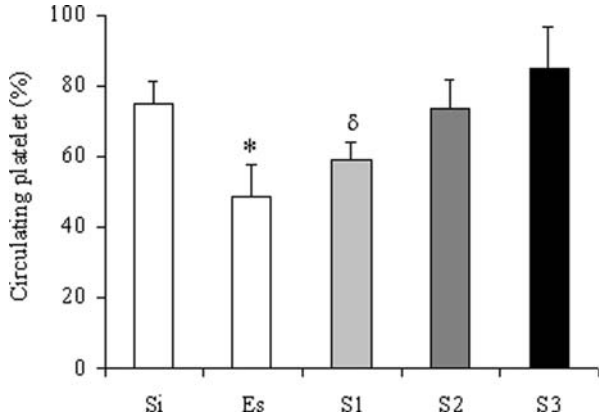

Fig. 2 Platelet count on circulating blood sample at the end of circulation time for PEtU-PDMS grafts and reference materials. Si = Silicone medical grade tubing, Es = Estane 5714-F1®, S1 = PEtU-PDMS (10%), S2 = PEtU-PDMS (25%), S3 = PEtU-PDMS (40%). Statistical analysis by Student *t* test ($p < 0.05$) * versus Si, S2 and S3; $^{\delta}$ versus S3

3.2 β-TG release assay

The β-TG level in plasma samples taken immediately after the blood venipuncture was about 60 ng/ml, an acceptable baseline of β-TG level considering that several minutes elapsed before the sodium-citrate anticoagulated blood was added to the Edinburgh anticoagulant to arrest further platelet activation. The β-TG release from platelets into plasma at the beginning and at the end of circulation time is shown in Fig. 3. The statistical analysis showed that grafts with the two higher PDMS content (25 and 40%) induce a significative lower β-TG release in comparison with PEtU-PDMS grafts containing 10% of PDMS and both reference materials.

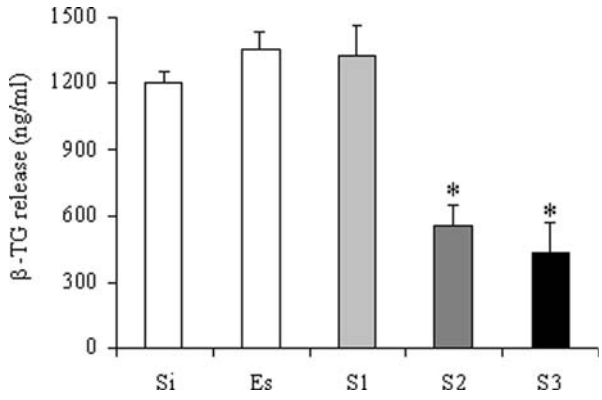

Fig. 3 β–TG release on circulating blood sample at the end of circulation time for PEtU-PDMS grafts and reference materials. Si = Silicone medical grade tubing, Es = Estane 5714-F1®, S1 = PEtU-PDMS (10%), S2 = PEtU-PDMS (25%), S3 = PEtU-PDMS (40%). Statistical analysis by Student *t* test ($p < 0.05$) * versus Si, Es and S1

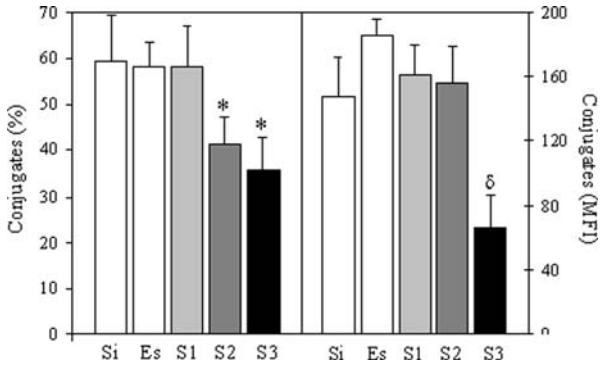

Fig. 4 Monocyte-platelet conjugates expressed as percentage (on the left) and MFI (on the right) on circulating blood sample at the end of circulation time for PEtU-PDMS grafts and reference materials. Si = Silicone medical grade tubing, Es = Estane 5714-F1®, S1 = PEtU-PDMS (10%), S2 = PEtU-PDMS (25%), S3 = PEtU-PDMS (40%). Statistical analysis by Student *t* test ($p < 0.05$) * versus Si, Es and S1; $^{\delta}$ versus Si, Es, S1 and S2

3.3 Flow cytometric analysis

3.3.1 CD62P expression

P-selectin expression on the platelet surface was evaluated by cytofluorimetric analysis. The average percentage of CD62P positive platelets was the same from the beginning to the end of each experiment for all the PEtU-PDMS grafts and reference materials tested (data not shown).

3.3.2 Monocyte-platelet conjugates

The statistical analysis showed that PEtU-PDMS grafts containing 25 e 40% of PDMS induce a significatively lower percentage of monocyte-platelets conjugates and MFI in comparison to PEtU-PDMS grafts containing 10% of PDMS and reference materials (Fig. 4).

4 Discussion

It is widely believed that most of the complications occurring with vascular grafts after the surgical implant are due to thrombus formation as result of blood–material surface contact. Because the interaction between blood and materials occurs at their interface, the hemocompatibility of materials is primarily determined by their chemical characteristics. Since the important biological interactions occur on the polymeric surface, representative approaches to improve hemocompatibility of blood contacting device have focused on immobilization of heparin, poly(ethylene glycol), phospholipids polymers and amphiphilic

copolymers on hydrophobic polymers [10]. Moreover hydrophilicity and surface chemical composition have been proposed to relate hemocompatibility of polymers, in particular polyurethanes [6, 25].

The PDMS is a polymeric material often utilized for the manufacture of biomedical devices because of its superior biocompatibility, low toxicity, oxidative and thermal stability as well as wide service temperature. Besides, although PDMS adsorbs and denatures fibrinogen, PDMS surfaces are known to discourage cell adhesion, due to the high chain flexibility [26, 27]. These advantages also allow its use in combination with polyurethanes (PUs) for medical device application; these PDMS-based PUs polymers exhibit better blood contact properties reducing significantly platelet adhesion when compared with reference commercial PUs [7, 10].

The driving hypothesis of this study is that the PDMS exposed on the luminal surface of small-diameter grafts affects initial thrombus formation and subsequent graft performance in terms of patency rate. In the past, most methods to assess hemocompatibility focused on platelet adhesion and adherent thrombus as the major undesirable events [28]. More recently, experimental animal models employing baboon and dog [29, 30] were used to test chronic arteriovenous shunt, but this type of experiments are expensive and the question of equivalent reactivity between humans and other animal species remains [31]. The goal of this work was to develop an inexpensive and simple in vitro hemocompatibility test to predict the performance of synthetic material for small-diameter grafts in vivo. In fact measuring biomaterial induced platelet activation in vitro presents a challenge. One major problem is the ever present background level of platelet activation in vitro from the venipuncture, blood manipulation, temperature changes and contact with non-test surfaces in the experimental system [19]. Therefore the in vitro evaluation of biomaterial-induced platelet activation needs to reduce the noise of background activation. A way to optimize this signal to noise ratio is to expose the blood to a large surface area of test material [28].

In a recent study a standardized model was set up to evaluate the thrombogenicity of different peripheral vascular stent types in vitro in order to minimize the above mentioned background effects [18]. Briefly, different stents were implanted into closed polyvinyl chloride (PVC) tubing loops and the loops were carefully filled with freshly drawn donor blood and placed on a rotating cylinder thanks to a modified roller pump. In this way a passive blood flow was obtained into the tubing avoiding hemolysis due to an actively circulated volume pumped by a roller. However, a major flaw in this model of in vitro circuit is the presence of an air bubble that, although a basic state to obtain the passive blood circulation, may induce protein aggregation and denaturation at the blood-air interface affecting platelet-surface interactions. It has been demonstrated, in experimental systems that even the presence of microscopic gas nuclei on surfaces enhance platelet adhesion and complement activation more than an order of magnitude over that observed on denucleated surfaces [32, 33]. Therefore, keeping in mind the explained drawback we developed a circulation system, according to the model described by Tepe, to evaluate in vitro the influence of PDMS content in PEtU-PDMS grafts on the initial reactions of blood with material. The circuit was modified replacing the PVC tubing with 40 cm length PEtU-PDMS grafts to amplify the biomaterial effect on blood. In order to minimize the effect of air-induced platelet activation we took care that the air bubble kept steady during the whole period of circulation and for all the independent experiments.

The spray-machine was used to realize grafts with increasing PDMS content. Luminal surface of all grafts in this study has an high porous microstructure because in previous in vitro and in vivo studies it was demonstrated that a small-diameter vascular graft having a luminal surface with high porosity has a better hemocompatibility than a low porosity one in terms of platelet adhesion and β-TG release [16, 17].

The circuit was used for the screening of hemocompatibility of grafts with different PDMS content (10, 25 e 40%) and silicone medical grade tubing and Estane 5714-F1® grafts were chosen as reference materials. In vitro platelet adhesion and activation tests were used to evaluate the PEtU-PDMS grafts hemocompatibility because platelets play a key role in thrombus formation during the contact of blood with foreign surfaces [34].

Platelet adhesion, indirectly quantified by an automatic counter, was found to correlate to the PDMS content, in fact grafts with higher PDMS content capture a small quantity of platelets compared to grafts with lower content and Estane 5714-F1® graft. Platelet activation, quantified by β-TG release into plasma following the degranulation reaction, revealed that grafts with higher PDMS content induce lesser β-TG release than grafts with lower PDMS content and reference materials. The flow cytometer was used to evaluate the platelet activation in whole blood samples avoiding the artifact due to PPP preparation. Using anti CD62P antibody, P-selectin expression in circulating platelets was analyzed because this antigen

appears to be associated with an increase in thrombotic risk [35, 36]. However, any variation in the percentage of activated platelets was registered with each type of PEtU-PDMS grafts and the PEtU-PDMS graft induced activation was similar to reference materials. Moreover, through cytofluorimetric analysis, the formation of monocyte-platelet conjugates was monitorized, in fact this parameter was found to promote the integrin surface expression increase on leucocyte (CD18-CD11b complex) and consequently it has a key role in acute phase inflammatory reaction, thrombotic process and cardiac ischemia [37, 38]. The data expressed as percentage of positive cells and MFI showed that PEtU-PDMS grafts with 25 and 40% of PDMS induced a lower conjugates number in relation to PEtU-PDMS grafts with 10% of PDMS and reference materials. These results related to cytofluorimetric analysis are in accordance to the in vivo study of Michelson et al. in 2002, in which the monocyte-platelet conjugates quantification revealed to be a more sensitive marker to evaluate platelet activation than CD62P surface expression [39]. Regarding the apparent contradictory response of the silicone tubing, which shows higher platelet activation respect to the higher PDMS containing graft (40%), we have to consider that this kind of commercial silicone was chosen because it is well known for its biocompatibility and hemocompatibility, but its chemical structure is different from the PDMS we used in the synthesis of the PEtU-PDMS material due to the presence of methylic group. Moreover it was supplied in tubing form having a smooth closed (non porous) internal surface, while the PEtU-PDMS grafts material have a microporous internal surface (average pore size about 120 μm), in fact this kind of structure showed a better hemocompatibility in relation to a non-porous one [17]. In addition, the more likely explanation for the apparent discrepancy between P-selectin expression and β-TG values is probably due to the contact of blood with material surface that activates platelets; therefore the activated platelets, that release β-TG into plasma, adhere onto the luminal surface and are not present in the circulating blood.

In conclusion, the in vitro circulation system, developed in this study, appears to be a useful tool for the hemocompatibility evaluation of synthetic vascular grafts in terms of platelet adhesion and activation. In particular the short-term in vitro experiments demonstrated that PDMS content in PEtU grafts positively affects their hemocompatibility, in fact higher PDMS content vascular grafts (25 and 40%) induced the lowest platelet adhesion and activation, monitorized by platelet count, β-TG release and monocyte-platelet conjugates. However, in the long-term it will be important to determine through in vivo experiments how the small-diameter vascular grafts having higher percentages of PDMS content will perform in terms of hemocompatibility, intimal hyperplasia development and inflammatory reaction.

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