

In vitro evaluation of the PEtU-PDMS material immunocompatibility: the influence of surface topography and PDMS content

D. Spiller · C. Mirtelli · P. Losi · E. Briganti ·
S. Sbrana · C. Counoupas · S. Kull ·
S. Tonlorenzi · G. Soldani

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Abstract The aim of the present work is to evaluate the in vitro immunocompatibility of an elastomeric material with feasible applications in the cardiovascular field. In particular, since it is well known that surface chemistry and topography play a key role in the foreign body response, their influence on human monocytes was evaluated. The material, constituted by a poly(ether)urethane (PEtU) and a polydimethylsiloxane (PDMS), was synthesized to manufacture films and small-diameter vascular grafts with three different surface topographical features, smooth, rough and porous, and siloxane rates, 10, 30 and 40. Human THP-1 monocytes have been cultured for 72 h on the films and human blood has been circulating for 2 h into the grafts to assess leukocyte adhesion and cytokine releases. Materials extracts were utilized to evaluate monocyte apoptosis. Smooth films showed lower cell adhesion degrees than rough and porous ones. All the PEtU-PDMS

(poly(ether)urethane-polydimethylsiloxane) films and vascular grafts induced a narrow inflammatory response, as demonstrated by slight cytokine secretion levels, in particular samples with the highest PDMS contents (30 and 40%) induced the lowest IL-1 β secretion. Moreover, an absence of monocyte apoptosis advises that the negligible release values have not to be ascribed to material toxicity. In the end, surface topography showed to affect only monocyte adhesion while siloxane content the cytokine release. Therefore, the possibility to modify the above tested parameters during material synthesis and manufacture could allow to bound the inflammatory potency of the PEtU-PDMS devices and render them excellent candidates for cardiovascular reconstruction.

1 Introduction

The implantation of biomedical devices into the human body sets off an inflammatory response, described as the foreign body response, that is a result of the inflicting surgical trauma and of the foreign materials presence [1].

Immediately after implant, the material surface is covered with plasma proteins that further direct cellular adhesion and activation [2], the inflammatory response ensues and monocytes adhere to the surface through interactions with adsorbed proteins. Adherent monocytes differentiate into macrophages which produce a wide variety of pro-inflammatory cytokines comprising interleukin-1 beta (IL-1 β), tumor necrosis factor-alpha (TNF- α), interleukin-6 (IL-6) and anti-inflammatory cytokines such as interleukin-10 (IL-10) [3]. These soluble factors collectively mediate cellular participation in the foreign body response and the ultimate wound healing, both up-regulating and down-regulating inflammation. Furthermore,

D. Spiller (✉) · C. Mirtelli · P. Losi · E. Briganti ·
C. Counoupas · S. Kull · S. Tonlorenzi · G. Soldani
Istituto di Fisiologia Clinica del Consiglio Nazionale delle
Ricerche (IFC-CNR), Laboratorio di Biomateriali, Ospedale G.
Pasquinucci, 54100 Massa, Italy
e-mail: spiller@ifc.cnr.it

D. Spiller
Scuola Sant'Anna di Studi Universitari e di Perfezionamento,
Pisa, Italy

S. Sbrana
Istituto di Fisiologia Clinica del Consiglio Nazionale delle
Ricerche (IFC-CNR), Laboratorio di Ematologia e Citometria a
Flusso, Ospedale G. Pasquinucci, 54100 Massa, Italy

C. Counoupas
Dipartimento di Patologia Sperimentale, Biotecnologie Mediche,
Infettivologia ed Epidemiologia, University of Pisa, 56127 Pisa,
Italy

adherent macrophages may fuse to form foreign body giant cells: the role of these cells is not still clearly understood but it seems that they may concentrate degradative and phagocytic properties leading to structural and chemical damage of the implant [4].

An active area of research has turned its attention towards the development of new biocompatible devices which can minimize the foreign body response, some of the most promising candidates are those based on silicone-modified polyurethanes. The poly(ether)urethane-polydimethylsiloxane (PEtU-PDMS) elastomeric materials combine in one step the good biocompatibility and mechanical resistance of the PEtU with those of good blood compatibility and long-term biostability of the PDMS. These materials can be synthesized with different concentration of the two polymeric components and manufactured by distinct methods to obtain biomedical devices with desirable structural and surface micro-geometries.

Besides the well-documented effects of material chemistry on the function of cells [5], it is currently accepted that surface topography influences the cell response, e.g., modulating macrophage production of cytokines and chemokines [6]. Therefore, the knowledge of topographic effects especially on inflammatory cells is mandatory.

The aim of this study was to investigate the in vitro PEtU-PDMS material immunocompatibility and, in particular, the influence of PDMS content and surface topography. PEtU-PDMS films with different topographical features (smooth, rough and porous) and siloxane concentrations (10, 30 and 40%) were manufactured by solvent casting and spray phase inversion methods and monocyte adhesion, cytokine release and apoptosis induction were measured in static condition.

In previous in vitro studies, PEtU-PDMS material in form of small-diameter vascular grafts, fabricated by spray phase inversion, showed good blood compatibility and low toxicity, suggesting their potential application for vascular reconstructions [7, 8]. Up to now, the immune compatibility of the above mentioned grafts has not been studied, consequently, the cytokine release and the leukocyte adhesion were measured in an in vitro perfusion system in which human whole blood has been circulating for 2 h into PEtU-PDMS vascular grafts.

2 Materials and methods

2.1 PEtU-PDMS and reference material

The PEtU, supplied in grain form (Estane[®] 5714, Lubrizol Advanced Materials, Inc., Cleveland, OH, USA), was purified using a Soxhlet apparatus in a 1:1 (v/v) methanol:acetone mixture before being dissolved in a 1:1 (v/v)

tetrahydrofuran (THF): 1,4-dioxane (DX) mixture to a concentration of 3% (w/v).

The silicon used in the present work is a diacetoxy silyl terminated (tetra-acetoxy functional) polydimethylsiloxane (PDMS) (United Chemical Technologies Inc., Bristol, PA, USA) that was supplied in fluid form at 55% (w/v) in 1:1 (v/v) THF-DX and diluted with the same mixture to obtain a 3% (w/v) final concentration solution. The solvents, purchased from Carlo Erba Reagenti (Milano, Italy), were purified by distillation before use.

The chemical reaction between PEtU and PDMS was performed as previously described by Briganti et al. [7] resulting in a semi-interpenetrating polymer network. The reaction allowed to obtain PEtU-PDMS materials containing different percentages of PDMS (10, 30 and 40) with respect to the PEtU. Afterwards, the PEtU-PDMS was stored as a solution of 3% (w/v) in THF-DX 1/1 (v/v) and protected from light.

A low density polyethylene (LPDE) was chosen as reference material according to the ISO 10993-Part 12 “Biological evaluation of medical devices: Samples preparation and reference material”. The LDPE was supplied in form of film (USP_{TM}, Rockville, MD, USA).

Pure PEtU and pure PDMS were used as comparison materials.

2.2 Samples manufacture

In this study PEtU-PDMS films, fabricated using two manufacturing methods, solvent casting and spray phase inversion, were used for the static culture experiments. PEtU-PDMS vascular grafts, fabricated by spray phase inversion, were used for the perfusion experiments.

2.2.1 Solvent casting processing

This technology is a versatile tool for thin film manufacture based on the solidification of a polymeric solution into a mould. In brief, 15 ml of 3% (w/v) PEtU-PDMS solutions were cast in glass molds, 60 mm Petri dishes, and were let solidifying in air at room temperature in order to obtain smooth films. After 48 h, the films were gently detached from the mould, sterilized in an ultrasonic bath by 30' incubation with 0.4 N HCl solution, thoroughly rinsed and stored in sterile distilled water.

2.2.2 Spray phase inversion processing

The bespoke “spray-machine”, described in detail by Okoshi [9], that makes use of the spray technology combined with the phase inversion principle of polymeric solutions, was used to obtain microfibrillar films and vascular grafts with different micro-geometries along the surface and the wall thickness.

PEtU-PDMS working solutions at 2% (w/v) and 0.2% (w/v), to which it was added 17% (v/v) of distilled water, were used to fabricate films featuring rough and porous surfaces respectively.

Working solutions at 0.2% (w/v), diluted with 17% (v/v) of distilled water, were used to fabricate vascular grafts featuring a luminal porous surface, as explained elsewhere [10, 11].

All the films and vascular grafts were maintained for a minimum of 16 h in distilled water at room temperature to allow the solvent removal and, afterwards, were sterilized and stored as above described.

2.2.3 Reference and comparison materials processing

In the static culture experiments, LDPE reference films were used as supplied, pure PEtU comparison films were manufactured by both the above described methods while pure PDMS comparison films only by solvent casting due to the siloxane high viscosity and poor phase separation. In the perfusion experiments, pure PEtU comparison vascular grafts were fabricated as above described. All the reference and comparison samples were sterilized and stored as the PEtU-PDMS ones.

2.3 Morphological analysis

The scanning electron microscopic (SEM) and stereo (SM) observation allowed to perform the morphological analysis of sample surface and to measure surface pore diameter and wall thickness respectively.

Surface morphology of samples were observed by SEM (Jeol 5600, Jeol Italia, Milano, Italy) after gold–palladium metallization as described by Losi et al. [12]. Micro-photographs were collected at 14 KV accelerating voltage and at 800× magnification.

The wall thickness and surface porosity of samples were measured upon staining with a Sudan Black B solution (0.3% w/v in absolute ethanol, Carlo Erba, Milan, Italy) and subsequent SM observation with the Olympus SZH10 microscope (Olympus optical Co., Tokyo, Japan) [13]. A video camera (KY-F32, JVC, Milan, Italy) connected to the microscope allowed to acquire representative images of sample surface at 60× original magnification. The quantitative measurements were determined using a software for image acquisition and elaboration (KS300, Carl Zeiss, Jena, Germany).

2.4 Static culture experiments

2.4.1 Monocyte culture

THP-1 human monocytes (code ICLC HTL97014, National Institute of Cancer Research, Genova, Italy),

derived from peripheral blood of individuals affected by chronic myeloid leukemia, were used for cell adhesion and cytokine release evaluation. Cell density was maintained between $2\text{--}9 \times 10^5$ cells/ml in RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin. Each experiment was performed on cells that demonstrated viability rates >95% after staining with Trypan blue dye for dead cell exclusion. Culture media, culture reagents and Trypan blue dye were supplied by Sigma Chemical Co., St. Louis, MO, USA; FBS was supplied from Gibco® (Invitrogen s.r.l., Milano, Italy).

2.4.2 Monocyte incubation

The films were placed at the bottom of a 24 wells plate and 1 ml of THP-1 suspension in complete culture medium (2.5×10^5 cells/well) was added to each well. The monocyte incubation was performed at 37°C for 72 h. As negative control, monocytes were cultured in complete medium without samples. As positive control, cells have been incubated with *E. coli* lipopolysaccharide (LPS, 10 µg/ml) for 24 h according to Wataha et al. [14]. At the end of the incubation period, the samples were washed with PBS and fixed with 70% methanol solution for the assessment of monocyte adhesion. Cell-free culture supernatants were harvested by centrifugation and stored at -80°C for the cytokine release assessment.

2.5 Perfusion experiments

An in vitro perfusion system, described by Losi and colleagues [10], allowed the direct contact between whole blood and vascular grafts. Human blood was drawn from the antecubital vein of healthy, non-smoking donors, who had not taken anti-inflammatory drugs for at least 2 weeks prior to the study, and collected in heparin (50 U/ml).

The human anticoagulated blood has been circulating for 2 h into PEtU-PDMS or PEtU grafts (50 cm of length, 5 mm I.D.), connected with a medical grade silicone tubing (30 cm of length, 5 mm ID) (Masterflex, Cole-Parmer Instrument Company Ltd., London, UK) on which a peristaltic pump (Masterflex) operated by compression. The entire system has been maintained at 37°C in a thermostated bath for the whole duration of the experiment.

In each experiment, at the beginning and at the end of the circulation, blood samples were collected for the automatic leukocyte count and other samples were centrifuged at $2500 \times g$ for 30' at 4°C to obtain platelet poor plasma (PPP) that was collected and stored at -80°C to measure the cytokine release.

2.6 Assessment of leukocyte adhesion

In the static culture experiments, adherent THP-1 monocytes were stained by a Giemsa solution (0.1%, Sigma Chemical Co.), washed with PBS and observed by the Olympus Stereo Microscope. Representative images of the adherent cells were acquired by the KY-F32 JVC video camera at 60× original magnification. The cell number was determined through computerized count using the KS300 software. Four areas of about 1 mm² were randomly counted for each sample.

In the perfusion experiments at basal and 2 h time points, the monocyte, lymphocyte and polymorphonuclear cell (PMN) adhesion on the grafts lumen surface was determined in whole blood by circulating cell count through an electronic counter (Sysmex SF3000, Kobe, Japan).

The results are expressed as percentage of adherent cells. The percentage was indirectly obtained by the number of cells counted in blood samples at the end of the circulation reported to that counted in the basal time point (considered as 100% of circulating cells).

2.7 Assessment of apoptosis induction

2.7.1 Extract preparation

The samples were immersed in medium RPMI 1640 containing 10% FBS that was chosen as extraction vehicle. The ratio of sample surface area/extracting medium volume was 3 cm²/ml as specified in the ISO 10993-12 for the regularly shaped device with 0.5–1 mm of wall thickness. The surface area was calculated on the basis of the overall sample dimensions, not taking into account surface topography. The extraction has been performed in chemically inert containers at 37°C for 72 h under low stirring condition (60 rpm). As negative control, the same extracting medium was incubated without any sample in the above mentioned conditions. At the end of the extraction period, samples were aseptically removed and the extracts were stored at 4°C and used within 24 h.

2.7.2 Annexin-V-Propidium Iodide (PI) staining

The apoptosis induction was estimated on THP-1 monocytes using Annexin-V-Propidium Iodide (PI) staining kit (Bender MedSystems, Vienna, Austria). Fluorescein isothiocyanate (FITC)-conjugated Annexin V allows to detect the redistribution of phosphatidylserine to the outer leaflet of the plasma membrane and PI allows to appraise the loss of plasma membrane integrity (i.e., necrotic and secondary necrotic cells).

After an incubation of 72 h with the film extracts, cells were stained according to the manufacturer's instructions.

Monocytes incubated for the same period in complete culture medium were considered as negative control. Cells incubated 24 h with a mixture of the topoisomerase II inhibitor etoposide (VP16, 500 µg/ml, Sigma Chemical Co.) and the potent mitogen activator of lymphocyte, the phytohemagglutinin lectin from *Phaseolus vulgaris* (PHA-P, 5 µg/ml, Sigma Chemical Co.), were considered as positive control of early apoptosis. Heat-shock treated (30 min at 70°C) cells were used as positive control of late apoptosis/necrosis. During the assay cells were kept on ice to arrest further progress through the stages of life towards death. In the end, 20 × 10³ cells were analysed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA) and running CellQuest[®] software (Becton Dickinson). Flow data analysis was based on measurement of positive events (percentage) quantification, as previously described [15].

2.8 Assessment of cytokine release

The release of human pro- and anti-inflammatory cytokines (IL-1β, IL-6, TNF-α and IL-10) in culture supernatants and PPP was measured by a fluorescent bead-based immunoassay (Flow cytomix, Bender Medsystem, Vienna, Austria) according to manufacturer instructions.

In brief, samples were transferred to a 96 wells filter plate in which, fluorescent beads coated by cytokine-specific primary antibodies were mixed with a biotinylated secondary antibody and the mixture has been incubated for 2 h at r. t. in the dark on a microplate shaker at 500 rpm. After a buffer washing, phycoerythrin-conjugated streptavidine was added to the wells and incubated for 1 h in the above mentioned conditions. At the end of the incubation period, the wells were washed and samples analysed using the FACScan flow cytometer. A minimum of 100 events was collected for each cytokine and the median fluorescence intensities were estimated. Cytokine concentrations were automatically calculated based on standard curve data using BMS FlowCytomix Software (Bender Medsystem). The minimum detectable concentrations of IL-1β, IL-6, TNFα and IL-10 are 4.5, 4.7, 7.9 and 6.9 pg/ml, respectively.

2.9 Statistical analysis

Each assay was performed in six independent experiments, the results are expressed as mean ± SD of three replicate wells for each sample and for the positive and negative controls. Data were statistically analysed by StatView[™] 5.0 software (SAS Institute, Cary, NC, USA). The various averages were statistically compared by the independent Student's *t* test. *P* < 0.05 was considered statistically significant.

3 Results

3.1 Morphological analysis

The morphological analysis of film surface showed an entirely smooth surface (Fig. 1a) for the solvent casting-manufactured films and a rough (Fig. 1b) and porous surface (mean pore diameter $\sim 100 \mu\text{m}$) (Fig. 1c) for the spray phase inversion-manufactured films obtained from 2 to 0.2% polymeric solutions respectively. All PEtU-PDMS films owned the same wall thickness ($\sim 500 \mu\text{m}$).

3.2 Assessment of leukocyte adhesion

In the static culture experiments, the number of THP-1 monocytes adherent to PEtU-PDMS films, featuring different surface topography, was compared with those adherent to pure PEtU and pure PDMS films, and LDPE ones.

The results, expressed in terms of monocyte adherent number for mm^2 of sample surface \pm SD, are showed in Fig. 2. The adhesion degree was obtained automatically counting, through the KS300 image acquisition and elaboration software, the number of adherent cells obtained from four microscopical fields randomly chosen for each determination, after Giemsa staining.

The results demonstrated that the adhesion rates were remarkably low ($\sim 5 \text{ cells}/\text{mm}^2$) for all smooth films and comparable to those obtained for the reference material, otherwise, both all rough and porous films showed a significant increase of the adhesion rate when compared with the smooth ones and the reference material. In particular, all porous films determined the greatest adhesion rates ($\sim 500 \text{ cells}/\text{mm}^2$), even significantly higher than those observed for rough films ($\sim 80 \text{ cells}/\text{mm}^2$).

All the adhesion values within each surface topography pattern were not significantly affected by the PDMS content.

As far as the perfusion experiments, all PEtU-PDMS grafts exhibited levels of monocyte, lymphocyte and PMNs adhesion comparable with those induced by pure PEtU grafts after 2 h of human blood perfusion (Table 1). The adhesion degree was about 27% for monocyte, 11% for lymphocyte and 13% for polymorphonuclear cells. In the end, the adhesion levels demonstrated to be independent from PDMS content in PEtU-PDMS grafts.

3.3 Assessment of apoptosis induction

The apoptosis induction was evaluated by flow cytometric analysis through Annexin V/PI staining of THP-1 monocytes after an incubation of 72 h with film extracts. Positive cells only for annexin V (FITC^+) were considered early

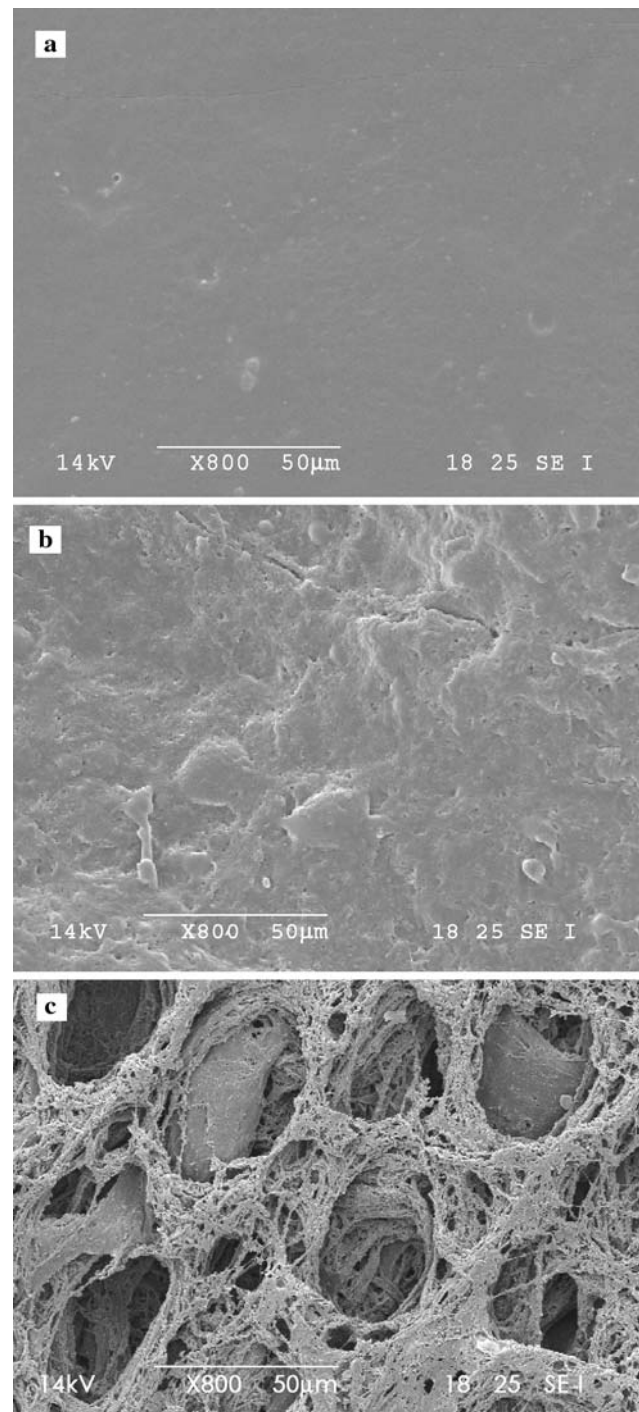


Fig. 1 SEM micro-photographs of PEtU-PDMS samples surfaces. The sample surface microgeometry does not change as regards as PDMS content. The solvent casting-manufactured films showed a smooth surface (a), while, the spray phase inversion-manufactured films showed a rough (b) and a porous surface (c)

apoptotic, while double-stained cells (FITC^+ , PI^+) were considered late apoptotic/necrotic. The results of apoptosis induction are shown in Table 2.

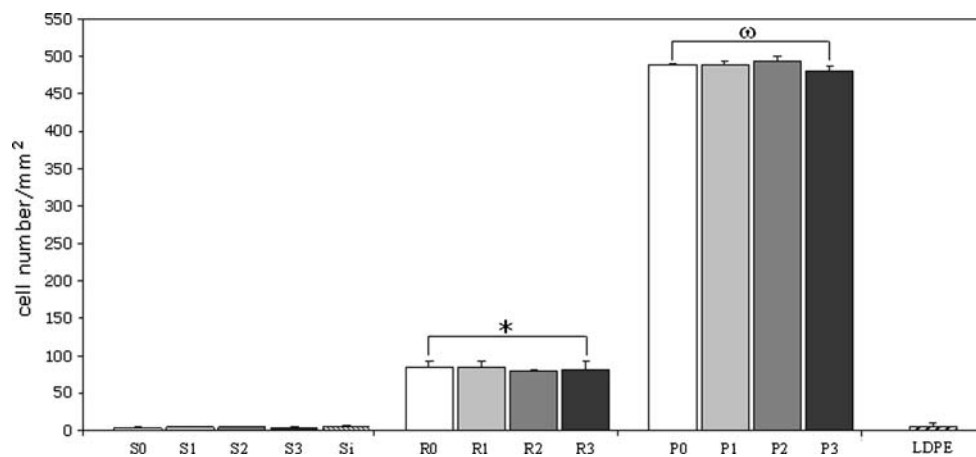


Fig. 2 Adhesion degree after a 72 h incubation of THP-1 monocytes with PEtU-PDMS films. The results are expressed as number of adherent cells automatically counted by KS300 software, after Giemsa staining of nuclei. Each value represents the mean \pm SD of six independent experiments. PEtU-PDMS smooth, rough and porous

films are named as S, R and P respectively. PDMS content of 0 (pure PEtU), 10, 30, and 40%, used to synthesize all the samples, is indicated with 0, 1, 2, 3, respectively. Si stands for the pure PDMS films. Statistical significance ($P < 0.05$, Student's *t* test) is indicated by * versus S0-3 and ω versus S0-3 and R0-3

Table 1 Leukocytes adhesion degree to PEtU-PDMS grafts after 2 h of human blood circulation in the perfusion system

Samples	Leukocyte adhesion (%)		
	Monocytes	Lymphocytes	Polymorphonuclear cells
G0	30 \pm 4	8 \pm 11	12 \pm 10
G1	25 \pm 6	10 \pm 4	16 \pm 8
G2	29 \pm 9	11 \pm 8	13 \pm 15
G3	27 \pm 7	14 \pm 8	9 \pm 9

The results are expressed as percentage of adherent cells that was indirectly obtained by the number of circulating cells counted in the blood samples at the end of the circulation time reported to the number of cells counted in the basal time point (considered as 100% of circulating cells). Each value represents the mean \pm SD of six independent experiments. G0, G1, G2, G3 stand for the PEtU-PDMS vascular grafts containing 0 (pure PEtU), 10, 30, and 40% of PDMS content respectively

Cells after 72 h of incubation in complete culture medium (negative control) showed a slight level of early and late apoptosis respectively of about 15 \pm 2% and 11 \pm 1%. Cells incubated for 24 h with the VP16-PHA-P mixture (positive control of early apoptosis) were Annexin V positive at 94%. Heat shock treated cells (positive control of late apoptosis/necrosis) showed about 80% FITC/PI positivity.

Human monocytes, after incubation with all PEtU-PDMS film extracts, exhibited low levels of early and late apoptosis comparable with those induced by pure PEtU, pure PDMS, and LDPE extracts and negative control. In the end, the apoptosis levels demonstrated to be independent from film surface micro-topography and PDMS content.

Table 2 Apoptosis induction of PEtU-PDMS film extracts on human THP-1 monocytes

Samples extracts	Apoptotic cells (%)			
	Early phase		Late phase	
	Mean	Range	Mean	Range
S0	9	4–12	7	3–13
S1	9	0–15	1	0–7
S2	8	1–13	2	0–16
S3	6	0–14	9	4–11
Si	14	7–22	5	0–15
R0	6	0–11	8	4–13
R1	13	0–22	1	0–4
R2	15	9–24	4	0–15
R3	13	0–20	4	0–16
P0	1	0–15	5	0–12
P1	10	1–15	2	0–14
P2	5	0–15	1	0–15
P3	8	0–14	1	0–6
LDPE	2	0–18	3	0–17

The results are expressed as percentage of dying cells measured by flow cytometric analysis after 72 h of incubation with extracts subtracted from that measured in the negative control (monocytes cultured in complete medium). Values are reported as mean and related range of six independent experiments, negative values are assumed as zero. PEtU-PDMS smooth, rough and porous films are named as S, R, and P, respectively. PDMS content of 0 (pure PEtU), 10, 30, and 40%, used to synthesize all the samples, is indicated with 0, 1, 2, 3, respectively. Si stands for the pure PDMS films

3.4 Assessment of cytokine release

The results are expressed in terms of fold increase mean \pm SD For the static culture experiments, the fold

increase was defined as the ratio of cytokine release after monocyte incubation with the films to that obtained without them (negative control). For the dynamic experiments, on the contrary, the fold increase was defined as the ratio of cytokine release after 2 h of blood perfusion into the grafts to that obtained before the perfusion (basal time point). All the cytokine release values measured were within 5–15 pg/ml for the negative control and basal time point, and within 100–300 pg/ml for the positive control (cells incubated for 24 h with LPS, 10 µg/ml).

Both in the static and dynamic experiments, all the PEtU-PDMS samples determined slight levels of IL-6, TNF-α and IL-10 release independently from surface topography and PDMS content: these values were not significantly different from those induced by pure PEtU, pure PDMS and LDPE films, and also by the negative control/basal time point (data not shown). As concerning IL1-β release, otherwise, the results demonstrated that all films with the highest PDMS contents (30 and 40%) induced a significant decrease in cytokine secretion when compared with the pure PEtU and with the other PEtU-PDMS films (Fig. 3).

The same behavior was noticed for PEtU-PDMS vascular grafts with the highest PDMS contents (30 and 40%), after 2 h of blood perfusion (Fig. 4).

4 Discussion

Several materials are presently used as components of biomedical implants for the treatment of damaged or lost tissue structure and function. Macrophages interact with the surface of implanted materials and are suggested to be the cells which orchestrate the inflammatory and repair

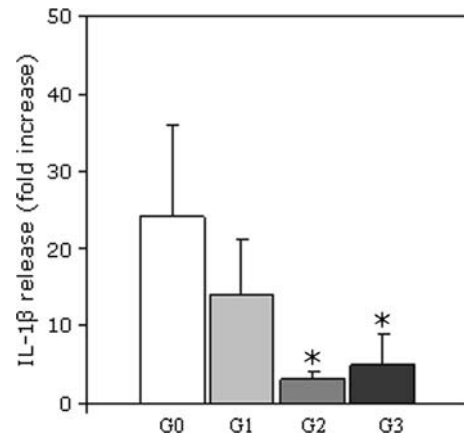


Fig. 4 IL1-β levels released in human blood after 2 h of circulation into PEtU-PDMS grafts. The results are expressed as fold increase in IL-1β release compared to the basal time point. Each value represents the mean ± SD of six independent experiments. G0, G1, G2, G3 stand for the PEtU-PDMS vascular grafts containing 0 (pure PEtU), 10, 30, and 40% of PDMS content respectively. Statistical significance ($P < 0.05$, Student's *t* test) is indicated by * versus G0-1

phase of tissue healing around implants, irrespective of material type [1].

Implantation and blood-material interaction studies over the past three decades have indicated that material surface chemistry and micro-topography influence cell responses in terms of adhesion, apoptosis and activation; these events may affect the ultimate fate of implanted biomedical devices [16, 17].

The PEtU-PDMS materials manufactured by spray phase inversion showed in vitro and in vivo good blood- and cyto-compatibility and mechanical resistance suggesting their potential application for cardiovascular reconstructions [7–10, 18]. However, the interaction

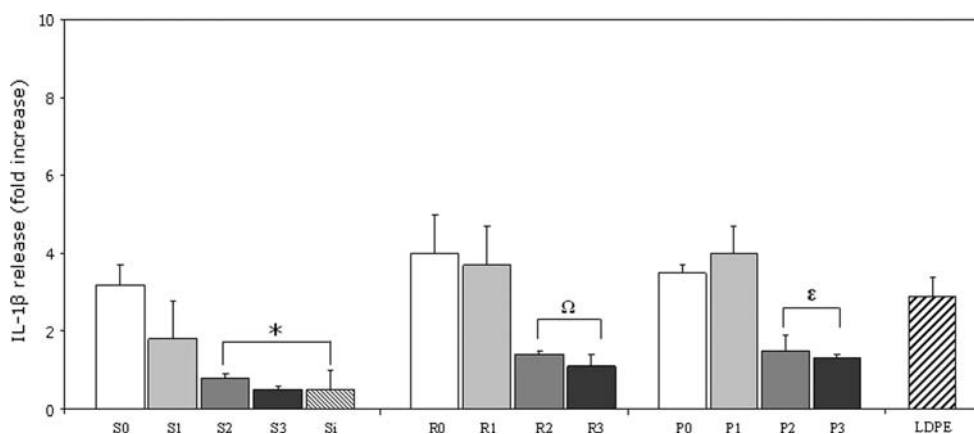


Fig. 3 IL1-β levels secreted from THP-1 monocytes after an incubation of 72 h with PEtU-PDMS films. The results are expressed as fold increase in IL-1β release compared to the negative control. Each value represents the mean ± SD of six independent experiments. PEtU-PDMS smooth, rough and porous films are named as S,

R, and P, respectively. PDMS content of 0 (pure PEtU), 10, 30, and 40%, used to synthesize all the samples, is indicated with 0, 1, 2, 3, respectively. Si stands for the pure PDMS films. Statistical significance ($P < 0.05$, Student's *t* test) is indicated by * versus S0-1, ^Ω versus R0-1 and ^ε versus P0-1

between PEtU-PDMS devices with the immune cells has not been previously investigated; to gain further insight into the biocompatibility of the PEtU-PDMS material, in this study monocyte adhesion, apoptosis and cytokine release were evaluated in relation to surface topography and PDMS content.

The adhesion process of leukocytes, in particular polymorphonuclear cells and monocytes, is one of the most important features of the foreign body inflammatory response. This process can trigger leukocyte activation and phagocytosis, macrophage fusion into giant cells, and extracellular release of leukocyte products and lead to material degradation and loss of functionality [19, 20]; the molecular basis of leukocyte binding on organic polymers is poorly elucidated, but the participation of integrins has been suggested [21, 22].

The adhesion of inflammatory cells, especially monocytes/macrophages, is dictated by the types of adsorbed proteins that covered the material surface immediately after implant; this especially depends on material wettability [23], surface texture and roughness [24], and chemistry [25, 26]. In particular, macrophages *in vitro* showed to accumulate preferentially on high wettable materials [27], and on rough and hydrophobic surfaces [5, 28, 29].

The quantitative analysis of monocyte adhesion was carried out through cell count after 72 h of incubation with PEtU-PDMS films. This analysis demonstrated that monocytes preferentially adhere to porous films rather than the others, apart from PDMS content. Moreover, our data indicated a greater number of adherent monocytes to rough films than to the smooth ones. Based on these results, in our experimental conditions only surface micro-topography, in terms of texture/roughness, showed to modulate the degree of cell adhesion. The absence of a PDMS effect on monocyte adhesion was also observed after 2 h of human blood perfusion into the PEtU-PDMS vascular grafts, furthermore, this behavior was confirmed also referring to the adhesion rate of lymphocytes and PMNs.

Moreover, surface roughness and porosity could increase the PEtU-PDMS film wettability and consequently cell adhesion, since it is well known that topographical changes influence the wettability degree [30]. To confirm our hypothesis, further investigations will be addressed to evaluate and compare the water contact angle of PEtU-PDMS films with different surface micro-topography.

Therefore, the above results indicate that PDMS content does not influence macrophage adhesion, in accordance with Jones et al. who demonstrated that silicon-modified surface of commercial polyurethanes (PUs) induced macrophage adhesion rates similar to pure PUs and pure PDMS [31].

The evaluation of cytokine secretion is one of the most important indicators of the foreign body response since this event may affect the implant outcome over time [32].

In the present work, the release of IL-1 β , IL-6, TNF- α , and IL-10, the major monocyte pro- and anti-inflammatory cytokines with pleiotropic biological activities [2, 33], was measured in static and dynamic conditions. These cytokines were selected for this *in vitro* study because they demonstrated to have a stimulatory effect after cell adhesion to different biomedical polymers such as polystyrene, PDMS, polyethylene, polyethylene terephthalate and expanded poly-tetrafluoroethylene [1].

The cytokine release was quantified using a fluorescent bead-based immunoassay. This new generation of tests, that allows the simultaneous quantification of different analytes by the use of different specific bead sets, reduces the time and cost of traditional assays, preserving high speed and sensitivity.

In this study, the evaluation of monocyte cytokine secretion induced by smooth, rough and porous PEtU-PDMS films was performed using a static culture model. The secretion of these mediators was also estimated in human anticoagulated blood after direct contact with vascular grafts featuring a porous luminal surface in a clinically-reflective perfusion system; the only porous topographical feature was examined in dynamic conditions since it confers good biocompatibility properties to the grafts, as elsewhere demonstrated [10].

The overall results demonstrated a good immunocompatibility of all PEtU-PDMS samples tested both in static and dynamic experiments since they showed a slight release of all the tested cytokines. In particular, only the films and vascular grafts containing high PDMS contents (30 and 40%) significantly restricted IL-1 β release. These findings appear to be in conflict with the adhesion rates observed both in static and dynamic experiments, however a recent leading opinion in literature backs a possible phenotypic dichotomy in the foreign body reaction and suggests that macrophage adhesion cannot correlate with or indicate a level of macrophage activation [5]. This phenomenon may be related to the surface-induced apoptosis of adherent cells. Further future investigations on cell population adherent to PEtU-PDMS devices would bear out this hypothesis.

In the present work, the assessment of apoptosis was performed on monocytes incubated for 72 h with film extracts; the choice of an extracting method allowed to avoid a wrong estimation of their functional state: decreases in cytokine secretion, in fact, could also be attributed to toxic effects of films.

All extracts did not induce either early apoptosis or cell death, independently from PDMS content demonstrating absence of toxicity on human monocytes. Therefore, it can be argued that film effects, in terms of a cytokine release decrement, do not depend on a lack in cell viability.

5 Conclusion

Our data suggest that the modification of surface topography significantly influenced monocyte adhesion, on the contrary, surface chemistry showed to restrict cell activation, the lowest cytokine secretion, in fact, was obtained with high PDMS contents. However, the molecular mechanism, by which PEtU-PDMS surfaces affect the macrophage response, has to still be carefully defined.

In the end, all PEtU-PDMS films and vascular grafts demonstrated an overall excellent immune compatibility when their surface were directly exposed to human monocytes and to human blood respectively.

Interestingly, the possibility to vary siloxane content in the PEtU matrix during the synthesis process could allow to obtain elastomeric materials with an acceptable inflammatory response, besides the low thrombogenicity, the cytotoxicity and the good mechanical resistance, that have already been demonstrated. These overall properties make PEtU-PDMS films and grafts ideal candidates for cardiovascular reconstruction, in particular for the treatment of myocardial ischemia and peripheral artery disease.

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