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Silicone based polyurethane materials: a promising biocompatible elastomeric formulation for cardiovascular applications

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Abstract The biocompatibility of a new material for cardiovascular applications constituted by a poly(ether)urethane (PEtU) and a silicone [polydimethylsiloxane (PDMS)] was evaluated. The achieved material shows properties similar to both polyurethanes and silicones. The material was transformed into porous membranes by a spray-deposition technique. Since any material preparation and manufacturing procedure may introduce some toxicity, *in vitro* cytotoxicity screening tests were carried out. Human umbilical vein endothelial cells (HUVECs) and a mouse fibroblasts cell line (L929) were cultivated with extracts obtained from materials containing 10, 40 and 100% (w/w) of PDMS. The commercially available Estane 5714-F1® and Cardiothane 51® were used as controls. Extracts were incubated up to 72 hours with HUVECs and L929 cells. The cytotoxic effect was evaluated by light microscopy, cell viability (MTT reduction and neutral red uptake) and proliferation (5-bromo-2′-deoxyuridine incorporation) tests. *In vivo* studies were carried out using materials containing the same PDMS percentages as for *in vitro* experiments. The same commercial controls were used. Results obtained with cell culture studies agreed with those obtained in the *in vivo* experiments and showed that the material preparation and manufacturing procedure do not introduce any toxicity in the products at each PDMS concentration investigated.

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1. Introduction

Polymers are widely used in medicine for different applications, but only a limited number is suitable for human implantation. A class of interesting compounds for medical use is the elastomers, both polyurethanes (PUs) and silicones [polydimethylsiloxane (PDMS)]. The attributes that make PUs attractive as materials for biomedical applications are their good biocompatibility and excellent physical-mechanical properties [1], however, they show the phenomenon of biodegradation that occurs in long-term implantation [2, 3]. Silicones, on the other end, have shown good blood compatibility, low toxicity and a long-term biostability [4].

In this study a standard aromatic poly(ether)urethane (PEtU) was modified with the addition of increasing quantities of a reactive PDMS, to realise a new PEtU-PDMS material which, eventually, combines in one step the good properties of both PUs and silicones. This type of elastomeric formulation could find an application as biocompatible material in many different areas of medical devices and in particular in the field of cardiovascular prostheses where blood-compatibility and elasticity are primary features. Compatibility with living tissue plays an important role in any implantable device and, therefore, this property must be addressed thoroughly when a new material formulation is proposed for implant in the human body. Biocompatibility has been previously defined as "the ability of a material to perform, with an appropriate host response in a specific application" [5]. Since the purpose of this study was to design a new PEtU-PDMS material for cardiovascular application,

appropriate experiments were carried out for screening its biocompatibility in relation to its end-use behaviour. A series of three PEtU-PDMS elastomers containing 10, 40 and 100% of PDMS were investigated. The Cardiothane 51® and the Estane 5714 F1® were used as negative control materials in this study as previously done by others [6].

In vitro cell culture assays are commonly used for the preliminary biocompatibility evaluation of materials for medical implant. These studies have the advantage of being not expensive, having relatively well-controlled variables and giving quantitative results in a limited time, moreover, they are accepted as a very sensitive method for biocompatibility testing [7–9]. Several *in vitro* tests have been described, in which either the material to be tested comes into direct contact with the cells in monolayer culture or is separated from them by a thin layer of agar [7, 10]. More recently, a toxicity assay in which the material is placed in an insert submerged in the cell culture medium in close proximity to the cell monolayer, but without direct contact with it, was developed [6].

An alternative to these methods includes the effect of extracts prepared from the materials on the cell cultures. In this way, low molecular weight substances that could leach-out from the materials come into direct contact with the cells. This method could overcome possible damage to the cells due to the mechanical interaction with the material or the contact with agar and increase the sensitivity of the testing procedure.

In the present study, the extraction method was chosen to evaluate the *in vitro* biocompatibility of PEtU-PDMS materials containing increasing amount of PDMS. In particular material extracts, prepared according to ISO 10993-5 ("Tests for cytotoxicity: *in vitro* methods"), were incubated with the cells up to 72 hours and then the effect on cell cultures was evaluated.

Phase-contrast microscopy was used to assess changes in cell morphology caused by the products leached-out from the material under testing; moreover, some parameters of cell viability and growth were selected to quantitatively evaluate the eventual cytotoxicity. Cell viability was indirectly measured by means of two colorimetric tests: one is based on the reduction of the tetrazolium salt (MTT) by metabolically active cells and the other one is based on the incorporation of the neutral red dye only by living cells. Cell growth was indirectly assessed by measuring the amount of bromodeoxyuridine (BrdU) incorporation into cellular nucleic acid of replicating cells.

To establish the biological effects of the new material on living tissues, animal implant procedures were conducted. PEtU-PDMS strips were implanted into the paravertebral muscle of rabbit for seven days and subsequently the surrounding strips tissue was evaluated for evidence of irritation or toxicity according to ISO 10993-6 ("Tests for Local Effects after Implantation").

2. Materials and methods

2.1. PEtU-PDMS material preparation

The aromatic PEtU used for the PEtU-PDMS preparation was subjected to purification by a soxhlet apparatus in a 1:1 (v/v) methanol-acetone mixture, whereas the reaction solvent, a mixture of 1:1 (v/v) tetrahydrofurane (THF) and 1.4-dioxane (DX), was purified by a rotating evaporator.

The reaction between PEtU and PDMS was carried out in a three neck reactor flask equipped with a water condenser, at 90°C, under stirring and nitrogen flow for 6 hours. In this way PEtU-PDMS materials containing different percentages of PDMS with respect to the PEtU were obtained. In particular, PEtU-PDMS material containing 10, 40 and 100% of PDMS were prepared (i.e. 100% corresponding to a ratio 1:1 between PEtU and PDMS).

2.2. PEtU-PDMS and reference materials processing

After preparation, the PEtU-PDMS material was stored as a solution of 3% (w/v) in THF-DX 1:1 (v/v), protected from light. The Estane 5714 F1® was supplied in grain form (Noveon, Brussels, Belgium) and was dissolved in THF-DX 1:1 (v/v) to obtain a 3% (w/v) final concentration solution. Cardiothane 51® (Arrow-International, Everett, MA, USA) is commercially available as a solution of 13% (w/v) in THF-DX 2:1 (v/v), that was diluted with the same ratio of solvents to obtain a 3% (w/v) solution.

The working solutions of PEtU-PDMS and reference materials were brought near to their precipitation point by adding 17% (v/v) of distilled water. Then, using an instrument named "spray-machine" [11, 12], the unstable solutions were processed by a spray-deposition technique, associated with phase inversion of the material, to realize microporous membranes. Briefly, unstable materials solutions and distilled water were simultaneously sprayed to intersect on a rotating mandrel through modified spray-guns which were mounted onto a sliding carriage. The interaction of material solution and water induces a sudden phase inversion of the polymer from the solution and results in the deposition of a microfibrillar material structure onto the mandrel.

2.3. Materials extract preparation

The test materials in form of microporous membranes were cut to obtain 10 × 15 mm pieces that were sonicated for 20 min in 0.4N hydrochloric acid at room temperature and rinsed in sterile distilled water.

For the extract preparation, samples of each test material were immersed in the appropriate culture medium containing 10% fetal bovine serum (FBS) that was chosen as extraction vehicle. The ratio of sample surface area to extracting

medium volume was 3 cm^2/mL, according to ISO 10993-5. The surface area was calculated on the basis of the overall sample dimensions, not taking into account surface porosity. The extraction was performed in chemically inert containers at 37°C for 24, 48 and 72 hours with agitation (60 rpm). An inert container with the same extractant and no added material was processed according to the same conditions providing the negative control for the testing procedure. The extract of copper obtained after 24 hours of incubation in the conditions described above, diluted 1:10 with complete culture medium, provided the positive control of toxicity.

At the end of each extraction period, samples were aseptically removed and the extracts were stored at 4°C and used within 48 hours.

2.4. Cell cultures

Human umbilical vein endothelial cells (HUVECs) were obtained by treatment with 0,1% collagenase type II, according to the Jaffe method [13]. HUVECs were maintained in Medium 199, supplemented with 10% FBS, 100 μg/ml streptomycin, 100 U/ml penicillin, 2 mM L-Glutamine, 100 μg/ml heparin from porcine intestinal mucosa and 50 μg/ml epithelial growth factor (EGF). HUVECs were used for experiments within the third passage.

Mouse fibroblasts (L929) from Interlab Cell Line Collection (ICLC ATL 95001) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 2 mM L-Glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin.

Cells were subcultured when at confluence (split ratio 1:3) by trypsinization (0.5% trypsin/0.02% EDTA). The medium was changed every two days and cell viability was routinely checked by vital staining with trypan blue. The cell cultures were kept at 37°C in a humidified atmosphere of 5% CO_2 in air.

Culture media were supplied by BioWhittaker Europe, sera and all culture reagents were from Sigma Chemical Co., St. Louis, MO, USA.

2.5. MTT assay

The MTT assay is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), a water-soluble yellow dye by the mitochondrial succinate dehydrogenase to form a water-insoluble dark blue formazan product. Only viable cells with active mitochondria reduce significant amounts of MTT to formazan. The assay has been developed as a quick effective method for testing mitochondrial impairment by drugs correlating well with cell proliferation [14]. Then it was modified as a preliminary screening test for the evaluation of *in vitro* cytotoxicity of soluble synthetic polymers [8, 15].

In the present experiment, HUVECs and L929 cells were seeded into 96-well plates at a density of 8×10^3 cells per well and 1×10^3 cells per well, respectively. After 24 hours of incubation, when cells were in the logarithmic phase of growth, the medium was carefully decanted and replaced with 200 μl per well of test material extracts and controls. After an exposure time of 72 hours, the surviving cells number was determined by MTT dye reduction. MTT salt (20 μl of a 5 mg/ml MTT solution, filter-sterilized, Sigma) were added to each well and incubated for 3 hours at 37°C. At the end of this time, the MTT reaction medium was removed and formazan crystals were solubilized with the addition of 100 μl per well of dimethylsulfoxide (DMSO). The optical densities were measured in a microplate reader (Spectrafluor Plus; TECAN, Austria GmbH) at 550 nm.

2.6. Neutral red assay

The neutral red assay is a mean of measuring living cells via the uptake of the vital dye neutral red by active transport and incorporation into lysosomes [16, 17].

In the test, HUVECs and L929 cells were seeded into 96-well plates at 8×10^3 cells per well and 2×10^3 cells per well, respectively. After 24 hours of cell seeding, the medium was replaced with the material extracts and controls, following an exposure time of 72 hours, neutral red solution were added in each well to obtain final concentrations of 0.33 mg/ml for L929 cells and 0,05 mg/ml for HUVECs. After 3 hours of incubation, the medium was carefully removed, the cells were rapidly washed with phosphate-buffered saline and fixed with 0.1% $CaCl_2$ in 0.5% formaldehyde. Then, the incorporated dye was liberated by adding 100 μl per well of a 1% acetic acid in 50% ethanol solution. Finally, the culture plates were agitated on a platform shaker (60 rpm) for 10 minutes and the absorbance values were determined at 550 nm.

2.7. BrdU incorporation assay

The pyrimidine analogue 5-bromo-2′-deoxyuridine (BrdU) is incorporated instead of thymidine into the DNA of new synthesis, therefore the BrdU incorporation is a suitable method for the quantitative measurement of cell proliferation [18, 19].

In the present study a commercially available BrdU-based enzyme-linked immunoassay was used (Roche Molecular Biochemicals, Mannheim, Germany). HUVECs (8×10^3 cells per well) and L929 fibroblasts (2×10^3 cells per well) were plated into 96-well plates and incubated for 24 hours. After that, the medium was replaced by 200 μl per well of extracts and controls and the cells were exposed to them for 48 hours. Finally, BrdU reagent was added on the cells and after 4 hours of incubation the assay was performed following the procedure prescription. The optical densities were measured at 450 nm using the microplate reader.

2.8. Statistical analysis

Each test was conducted at three separate times, the results are expressed as mean ± S.D. of six replicate wells for each samples and for the positive and negative controls. Data were evaluated statistically by StatView™ 5.0 software (SAS Institute, Cary, NC, USA). The various means were statistically compared by the independent Student's t-test. $p < 0{,}01$ was considered statistically significant.

2.9. Preparation of samples for implant

The *in vivo* study was carried out using PEtU-PDMS materials containing the same percentages of PDMS of those used for the *in vitro* experiments and, as reference material, the Estane 5714 F1®.

All test materials were processed by the same spray-deposition procedure used for the *in vitro* studies, and were cut in strips of 10 mm in length and 1 mm in diameter for implantation. The edges of the strips were made as smooth as possible to avoid additional mechanical trauma upon implantation. The strips were sterilized using the same procedure as used for the *in vitro* tests, and then were inserted into the lumen of 19 - gauge hypodermic needle. As negative control, some needles were filled with similarly sized samples of high density polyethylene (HDPE, USP-grade Negative Control Plastic).

2.10. Rabbit preparation and strips implant

Twelve adult New Zealand white rabbits weighting 2.5–3 kg were used in this study. All the animals were caged and kept according to EEC (28871-22a) normative.

Anesthesia was induced with a mixture of 60 mg/kg ketamine and 30 mg/kg metedomidine injected intramuscularly; some rabbits required an additional intraperitoneal dose of anesthetic.

Before implants, the fur of the animals was clipped on both sides of the spinal column and loose hair removed. The skin was swabbed lightly with diluted alcohol and dried prior to sample injection.

Each rabbit received eight samples (four test materials and four HDPE controls) implanted in the lumbar paravertebral muscles; the test materials on the right and the controls on the left side of the spinal column. Each material was tested in three rabbits.

The strips were implanted 2.5 to 5 cm from the midline and parallel to the spine, and about 2.5 cm apart from each other. The loaded needle was inserted into the muscle at a 45° angle, a sterile stylet was fitted in the needle to hold the implant strip in the tissue while withdrawing the needle.

2.11. Tissue response evaluation

The animals were kept for seven days after implantation and then they were sacrificed by CO_2 inhalation. The seven days implantation period is commonly employed for acute response evaluation and it is longer than the minimum requirement of 72 hours according to USP reference standards.

Table 1 Scoring system for estimating the encapsulation in the implantation test (USP "Biological Reactivity Tests, In Vivo").

Capsule width	Score
None	0
Up to 0.5 mm	1
0.6–1.0 mm	2
1.1–2.0 mm	3
Greater than 2.0 mm	4

The tissue around each implant, both for test materials and controls, was macroscopically observed for necrosis or discolorations and graded for degree of capsule formation according to the scoring system in Table 1. Samples excised from implantation sites were fixed in 10% buffered formalin and stained with hematoxylin and eosin (H & E). The sections were examined using light microscopy to evaluate the extent of tissue damage.

3. Results

3.1. Phase-contrast microscope observation

Cell cultures were observed and photographed using a phase contrast microscope (Zeiss Milano, Italy). HUVECs and L929 cells cultured in presence of all PEtU-PDMS material extracts did not show any morphological change, they were very spread out and they appeared identical to those cultured with reference materials extracts and control medium. As an example, in Fig. 1 micrographs of HUVECs and L929 cells incubated for 72 hours with extract of PEtU-PDMS material (containing 100% PDMS), obtained after 72 hours of extraction are shown. The cell density of both cell types cultured with all tested material extracts was similar to those of cells cultured with the control medium, moreover, at 72 hours after seeding the cell density had increased for all samples compared to 24 hours. In contrast, after 72 hours of incubation with copper extract the few adhered HUVECs and L929 cells were spherical and had a dramatically different morphology (not shown).

3.2. MTT assay

The results of the MTT assay for HUVECs and L929 cells are reported in Table 2 and Table 3 respectively, where the absorbance values obtained following HUVECs and L929 cells incubation with extracts at different time of extraction are given. PEtU-PDMS material extracts with increasing percentages of PDMS revealed similar values to the material reference extracts and the control medium after 24, 48, 72 hours of extraction. In Fig. 2 the percentage of HUVECs and L929 cells viability related to the control

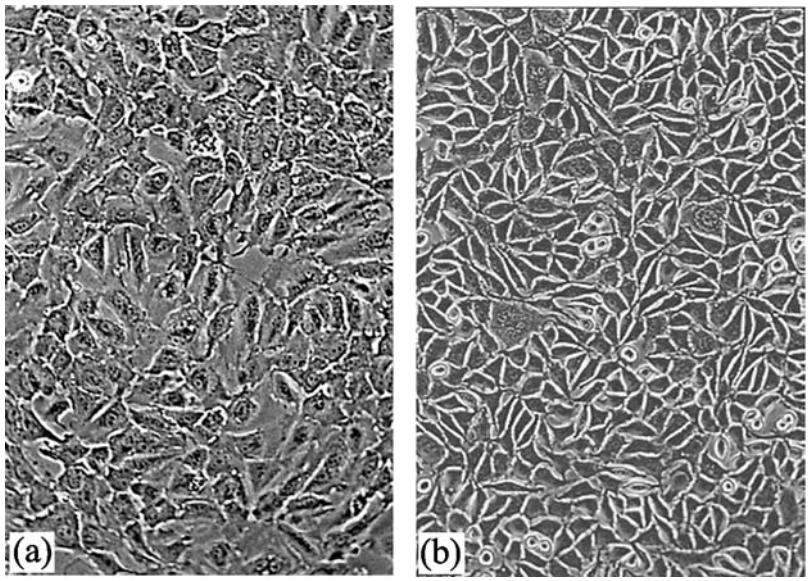

Fig. 1 Phase-contrast light micrographs of (a) HUVECs and (b) L929 fibroblasts cultured for 72 hours with PEtU/PDMS (100%) extract of 72 hours (original magnification: x 20).

Table 2 Absorbance values following 72 hours of HUVECs incubation with material extracts at different time of extraction obtained with the MTT assay. Each value represents the mean of six replicates ± standard deviation.

	Time of extraction (hours)		
Extract from	24	48	72
PEtU-PDMS (10%)	0.31 ± 0.03	0.34 ± 0.02	0.44 ± 0.02
PEtU-PDMS (10%)	0.29 ± 0.01	0.31 ± 0.02	0.41 ± 0.01
PEtU-PDMS (100%)	0.31 ± 0.02	0.34 ± 0.02	0.42 ± 0.03
Estane 5714F1®	0.28 ± 0.01	0.33 ± 0.03	0.42 ± 0.03
Cardiothane 51®	0.27 ± 0.01	0.36 ± 0.01	0.44 ± 0.03
Culture medium	0.30 ± 0.02	0.35 ± 0.02	0.43 ± 0.04
Copper	0.06 ± 0.01		

Table 3 Absorbance values following 72 hours of L929 fibroblasts incubation with material extracts obtained at different time of extraction with the MTT assay. Each value represents the mean of six replicates ± standard deviation.

	Time of extraction (hours)		
Extract from	24	48	72
PEtU-PDMS (10%)	0.60 ± 0.04	0.65 ± 0.03	0.62 ± 0.03
PEtU-PDMS (40%)	0.58 ± 0.04	0.66 ± 0.03	0.64 ± 0.02
PEtU-PDMS (100%)	0.57 ± 0.03	0.63 ± 0.05	0.63 ± 0.03
Estane 5714F1®	0.60 ± 0.02	0.61 ± 0.04	0.62 ± 0.03
Cardiothane 51®	0.62 ± 0.02	0.60 ± 0.03	0.62 ± 0.05
Culture medium	0.60 ± 0.03	0.67 ± 0.02	0.63 ± 0.03
Copper	0.07 ± 0.01		

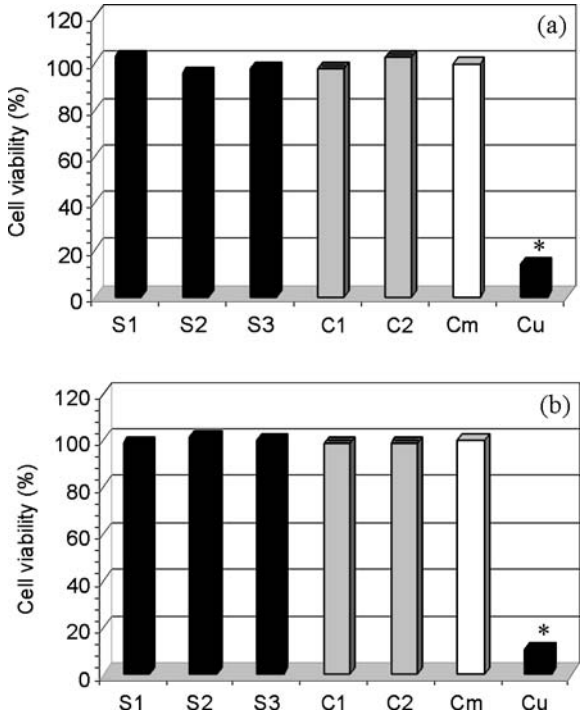

Fig. 2 Results from the MTT assay for (a) HUVECs and (b) L929 fibroblasts incubated with material extracts of 72 hours. The percentage of cell viability of all samples was calculated versus the control medium (taken as 100%). S1 → PEtU/PDMS (10%); S2 → PEtU/PDMS (40%); S3 → PEtU/PDMS (100%); C1 → Estane 5714 F1®; C2 → Cardiothane 51®; Cm → Culture medium; Cu → copper. The Cu value was statistically different from each other by Student's t-test ($^*p < 0.01$).

medium (assumed as 100%) following exposition at different material extracts of 72 hours is shown graphically. Extracts prepared from PEtU-PDMS material containing different percentages of PDMS (10, 40 and 100%) and from reference materials (Cardiothane 51® and Estane 5714 F1®) did not depress mitochondrial activity with respect to the control medium in both cell types tested. On the contrary, cells treated with copper extract revealed a considerable decrease of formazan production; in fact copper extract inhibited mitochondrial activity to about 13.9 % for HUVECs and 11.1 % for L929 of the control medium after 72 hours of incubation.

Table 4 Absorbance values following 72 hours of incubation of HUVECs with material extracts obtained at different time of extraction with the Neutral red assay. The data in the table are presented as the mean of six replicates ± standard deviation.

	Time of extraction (hours)		
Extract from	24	48	72
PEtU-PDMS (10%)	0.55 ± 0.04	0.56 ± 0.05	0.60 ± 0.02
PEtU-PDMS (40%)	0.52 ± 0.05	0.52 ± 0.03	0.62 ± 0.02
PEtU-PDMS (100%)	0.53 ± 0.02	0.53 ± 0.03	0.64 ± 0.03
Estane 5714F1®	0.53 ± 0.03	0.46 ± 0.02	0.62 ± 0.01
Cardiothane 51®	0.58 ± 0.04	0.54 ± 0.05	0.60 ± 0.04
Culture medium	0.56 ± 0.03	0.57 ± 0.03	0.63 ± 0.03
Copper	0.06 ± 0.01		

Table 5 Absorbance values following 72 hours of incubation of L929 fibroblasts with material extracts obtained at different time of extraction with the Neutral red assay. The data in the table are presented as the mean of six replicates ± standard deviation

	Time of extraction (hours)		
Extract from	24	48	72
PEtU-PDMS (10%)	0.55 ± 0.04	0.56 ± 0.05	0.60 ± 0.02
PEtU-PDMS (40%)	0.52 ± 0.05	0.52 ± 0.03	0.62 ± 0.02
PEtU-PDMS (100%)	0.53 ± 0.02	0.53 ± 0.03	0.64 ± 0.03
Estane 5714F1®	0.53 ± 0.03	0.46 ± 0.02	0.62 ± 0.01
Cardiothane 51®	0.58 ± 0.04	0.54 ± 0.05	0.60 ± 0.04
Culture medium	0.56 ± 0.03	0.57 ± 0.03	0.63 ± 0.03
Copper	0.06 ± 0.01		

3.3. Neutral red assay

The neutral red uptake revealed, with both cell types tested, similar absorbance values for PEtU-PDMS and reference materials extracts of each time of extraction, these values were similar to those obtained with the control medium (Table 4 for HUVECs and Table 5 for L929 cells).

In Fig. 3 the results of the neutral red assay for HUVECs and L929 cells, following exposition at different material extracts of 72 hours, are shown graphically as percentage of cell viability respect to the culture medium. The copper extract reduced the neutral red uptake to about 9.5% and 14.3% for HUVECs and L929 respectively.

3.4. Incorporation of BrdU

The absorbance values, measured by the incorporation of BrdU into replicating DNA, with PEtU-PDMS material extracts were similar, for both cell types, to those measured with reference material extracts and culture medium at each time point (Table 6 and Table 7).

The DNA synthesis for HUVECs and L929 cells, after incubation with different material extracts of 72 hours, is shown in Fig. 4 as percentage respect to the culture medium assumed as 100%.

The obtained values did not decrease significantly after treatment of both cell types with PEtU-PDMS material extracts of 72 hours respect to that obtained with reference material extracts and culture medium. On the contrary, copper extract caused a decrease of BrdU incorporation into DNA to about 15.9% and 16.1% for HUVECs and L929 respectively.

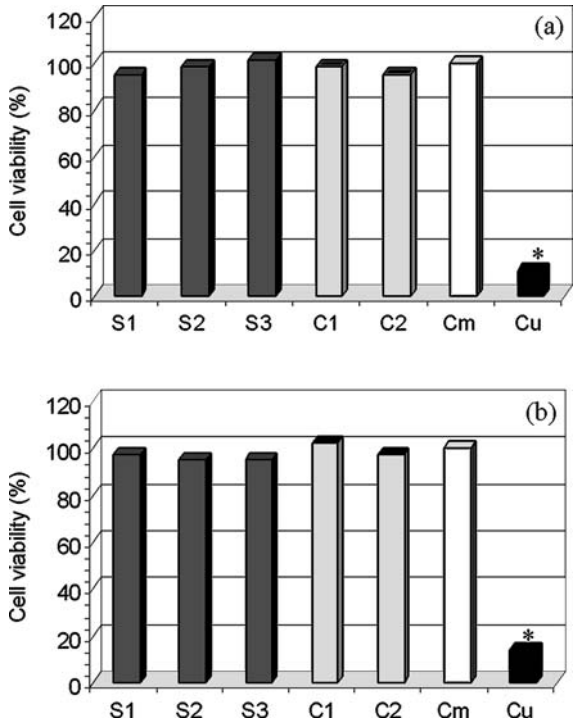

Fig. 3 Cell viability checked by the Neutral red assay for (a) HUVECs and (b) L929 fibroblats after incubation with material extracts of 72 hours. The percentage of cell viability of all samples was calculated versus the control medium (taken as 100%). S1 → PEtU/PDMS (10%); S2 → PEtU/PDMS (40%); S3 → PEtU/PDMS (100%); C1 → Estane 5714 F1®; C2 → Cardiothane 51®; Cm → Culture medium; Cu → copper. The Cu value was statistically different from each other by Student's t test ($^*p < 0.01$).

Table 6 Absorbance values following 72 hours of incubation of HUVECs with material extracts obtained at different time of extraction with the BrdU incorporation assay. The data in the table are presented as the mean of six replicates ± standard deviation.

	Time of extraction (hours)		
Extract from	24	48	72
PEtU-PDMS (10%)	0.38 ± 0.01	0.38 ± 0.01	0.41 ± 0.01
PEtU-PDMS (40%)	0.38 ± 0.01	0.37 ± 0.02	0.40 ± 0.01
PEtU-PDMS (100%)	0.38 ± 0.01	0.39 ± 0.01	0.40 ± 0.02
Estane 5714F1®	0.40 ± 0.01	0.39 ± 0.01	0.41 ± 0.01
Cardiothane 51®	0.37 ± 0.01	0.38 ± 0.01	0.41 ± 0.01
Culture medium	0.38 ± 0.01	0.39 ± 0.01	0.42 ± 0.02
Copper	0.06 ± 0.01		

Table 7 Absorbance values following 72 hours of incubation of L929 fibroblasts with material extracts obtained at different time of extraction with the BrdU incorporation assay. The data in the table are presented as the mean of six replicates ± standard deviation.

	Time of extraction (hours)		
Extract from	24	48	72
PEtU-PDMS (10%)	0.42 ± 0.03	0.42 ± 0.04	0.47 ± 0.04
PEtU-PDMS (40%)	0.44 ± 0.04	0.45 ± 0.04	0.46 ± 0.02
PEtU-PDMS (100%)	0.40 ± 0.04	0.42 ± 0.04	0.45 ± 0.03
Estane 5714F1®	0.40 ± 0.03	0.40 ± 0.03	0.47 ± 0.01
Cardiothane 51®	0.41 ± 0.04	0.45 ± 0.03	0.44 ± 0.02
Culture medium	0.42 ± 0.01	0.42 ± 0.02	0.44 ± 0.02
Copper	0.07 ± 0.01		

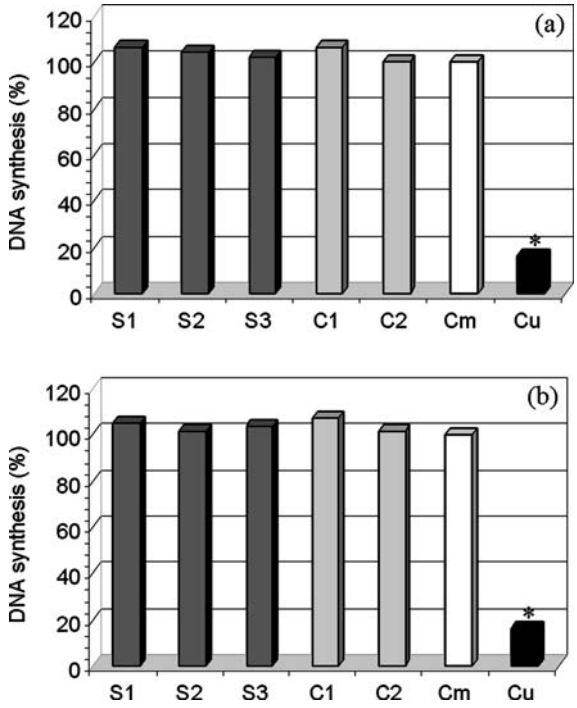

Fig. 4 DNA synthesis measured as 5-bromo-2′-deoxyuridine incorporation in (a) HUVECs and (b) L929 cells incubated with different material extracts of 72 hours. Extracts were exposed to the cells for 72 hours. S1 → PEtU/PDMS (10%); S2 → PEtU/PDMS (40%); S3 → PEtU/PDMS (100%); C1 → Estane 5714 F1®; C2 → Cardiothane 51®; Cm → Culture medium; Cu → copper. *, value significant ($p < 0.01$) when tested against each other by means of the Student's t-test.

3.5. Rabbit implant response

The animals did not show any infection or implant rejection during the experimental period. In Fig. 5 a strip of PEtU-PDMS material containing 100% PDMS retrieved after seven days of implantation is shown. No necrosis, degeneration and discolorations were observed in the area of the tissue surrounding the central portion of each strip, both for test materials and controls. Moreover, all implanted rabbits did not develop signs of encapsulation, so the average score was 0 according to Table 1.

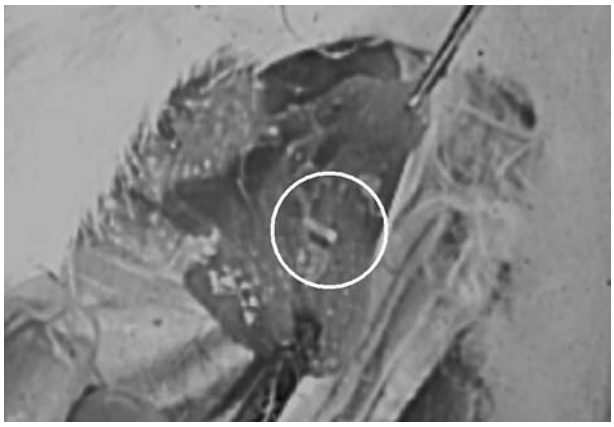

Fig. 5 Photograph of PEtU/PDMS (100%) strip explanted after 7-days of rabbit implantation in the paravertebral muscle.

The histopathological analysis of H&E stained tissue sections was performed on the implant surrounding area. The light microscopy observations of all implant sites, both of materials under testing and controls, showed a normal tissue surrounding the implants with the absence of inflammatory cells and morphological alterations of smooth muscle cells (results not shown). Thus, the 7 days implantation study showed that all tested materials were biologically accepted by the subcutaneous tissue.

4. Discussion

Although specifically prepared for medical use, polymeric materials may contain in addition to the relatively inert high molecular weight polymer, other components such as residual monomers, oligomers, catalysts and processing aids. These components are present at varying levels depending on the raw material sources and manufacturing processes. Besides that, additional chemical species (ACS) may be generated during the material preparation procedure, such as a reaction carried out for long time at high temperature; and manufacturing processes, such as heat sealing, welding, spraying or sterilization of the device. All these ACS can migrate from the device into the human body and should be the subject of risk assessments.

In the present study the biocompatibility of a new silicone (PDMS) based PEtU elastomeric formulation was investigated by cell culture methods and *in vivo* implant in accordance with ISO requirements. For the *in vitro* biocompatibility testing, appropriate cell types need to be used in respect to the use and site of implantation of a given biomaterial. For a material potentially usable for cardiovascular applications, freshly isolated human endothelial cells, which

are most likely to come in contact with the material or with substances released from it, were selected. The behaviour of these cells was compared to that of an established mouse cell line of fibroblasts (L929), frequently used to evaluate materials cytotoxicity [20, 21].

The PEtU-PDMS material cytotoxicity was studied with an indirect method evaluating the effect of possible toxic substances leached from material toward cells. These substances were obtained under defined conditions according to ISO 10993-5.

The cellular viability after incubation with materials extracts was measured by evaluation of structural and functional integrity of specific intracellular organelles, which are of fundamental importance for the survival of the cell. In particular, the functional state of mitochondria and lysosomes was evaluated respectively by MTT test and neutral red uptake. The proliferative capacity of the cells was evaluated by incorporation of BrdU during cell replication. All these cell culture methods proved to be highly reproducible (with small standard errors in the mean), quantitative and gave in a short period of time important information on cytotoxicity of the new material.

The results obtained with *in vitro* tests showed that the PEtU-PDMS materials with varying concentrations of PDMS are devoid of any cytotoxic effects on human endothelial cells and mouse fibroblasts, because no decrease in cell viability and growth was observed in comparison with controls.

The rabbit intramuscular implantation method has proved to be valuable in the past and remains the most widely used mean of assessing the potential toxicity of biomaterials before clinical trials, therefore in addition to *in vitro* tests, *in vivo* experiments to determine the biological response of animals to PEtU-PDMS material by direct contact with materials were performed. These experiments were found to be in agreement with *in vitro* toxicity tests.

In conclusion, the work presented in this study demonstrates that the chemical procedure used to react a standard PEtU with a functional silicone and, subsequently, its spray manufacturing into porous membranes do not introduce any toxicity in the resulting PEtU-PDMS products at each PDMS concentration investigated. However, further studies related to biostability and mechanical properties of the new material are necessary and remain to be investigated before that it could be employed for the realisation of a human implantable cardiovascular device.

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