In vitro biocompatibility of fluorinated polyurethanes

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The *in vitro* biocompatibility of fluorinated polyurethanes (FPUs), labelled as FPU 42, 52, 58, and 60, was evaluated by means of thrombogenicity, cytoxicity and cytocompatibility tests. Cardiothane® was taken as control material. The thrombogenicity was tested on thin material films by measuring the activation of prekallikrein (PKK) to kallikrein (KK). Level I cytoxicity tests of the bulk materials, i.e. Neutral Red (NR) uptake, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and Kenacid Blue (KB) assays, were performed to assess the influence of the polymer extracts on, respectively, lysosomes, mitochondria and cell proliferation. The cytocompatibility was evaluated, on thin membranes made by a spraying phase-inversion process, by measuring the area of the polymer surface covered by human umbilical vein endothelial cells (HUVEC) 1 week after seeding. The results indicate that all the polymers are not thrombogenic, and not cytotoxic. The FPUs that contain polycaprolactone glycol (PCLG) (FPU 52 and 60) instead of poly(tetramethylene ether) glycol (PTMEG) (FPU 42 and 58) as soft segment show the lowest thrombogenicity and the best cytocompatibility.

1. Introduction

The polyurethanes are elastomers with a high medical grade performance, which are reported to possess good blood compatibility, hydrolytic stability, abrasion resistance, tensile strength and flexure endurance [1].

The aim of this study was to evaluate the *in vitro* biocompatibility of fluorinated polyurethanes (FPUs, patented by Ausimont, Milano, I). FPUs are of interest for several reasons. Properties in the bulk are such that thermal stability is high. Properties at the surface are such that the interfacial free energy is low [4]. This low surface energy finds applicability in many different areas such as biomaterials [4] and in particular in the field of cardiovascular prostheses. As far as we know the only information reported in the literature concerning the *in vitro* biocompatibility of FPUs deals with their haemocompatibility [5]. In the present studies four different FPUs were used, differing in their fluorine content and macroglycol portion. FPUs labelled as 42, 52, 58 and 60 were used for thrombogenicity, cytotoxicity and cytocompatibility tests.

2. Materials and methods

2.1. Membrane preparation and material composition

FPU 42, 52, 58 and 60 (test materials) and Cardiothane® (control material) membranes were made by a spraying, phase-inversion process already described elsewhere [2, 3]. In short: 1% w/v solutions were prepared in suitable solvents: 1,4 dioxane/THF 1/2 for Cardiothane® and DMF/THF 1/9 for FPUs. Porous membranes were obtained by simultaneously spraying distilled water and a thermodynamically unstable PU solution (obtained by addition of distilled water) onto a rotating mandrel. The local precipitation of the polymeric material results in a porous membrane with reproducible structural characteristics. The FPUs differ in their soft phase, with that of FPU 52 and 60 being made of polycaprolactone glycol (PCLG), and that of FPU 42 and 58 being made of poly(tetramethylene ether) glycol (PTMEG). The composition of the materials is shown in Table I.

2.2. Thrombogenicity test

The thrombogenicity of the materials was tested on thin material films by measuring the activation of prekallikrein (PKK) to kallikrein (KK), by the proteolytic reaction between KK and the chromogenic substrate H-D-Pro-Phe-Arg-pNa (S-2302 Kabi Diagnostica, Kabi Vitrum, Sweden). To evaluate the contact PKK activation, a pool of citrated plasma from nine healthy donors was dispensed in samples of 0.5 ml, and then frozen at $-20\,^{\circ}C$. Before carrying out the activation test, the plasma was thawed at $37\,^{\circ}C$

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TABLE I FPUs composition

	Components	Molecular weight MW	Molar ratio
FPU 42	ZDOLTX	2000	0.2
	PTMEG	1000	1.0
	MDI	250	2.0
	BDO	90	0.8
FPU 52	ZDOLTX	2000	0.2
	PCLG	1250	0.8
	MDI	250	2.0
	BDO	90	1.0
FPU 58	ZDOLTX	2000	0.3
	PTMEG	2000	0.7
	MDI	250	2.0
	BDO	90	1.0
FPU 60	ZDOLTX	2000	0.3
	PCLG	2000	0.7
	MDI	250	2.0
	BDO	90	1.0

ZDOLTX: $HO(CH_2CH_2)_nCH_2CF_2(OCF_2CF_2)_p(OCF_2)_qOCF_2CH_2(OCH_2CH_2)_nOH$.
$n = 1.0–1.5$; $p/q = 0.8–1.2$.
PTMEG: polytetremethilene ether glycol.
PCLG: polycaprolactone glycol.
MDI: methylene diisocyanate.
BDO: 1,4-butanediol.

and diluted 1:10 with a 0.05 M TRIS-HCL buffer (pH 7.8). Volumes of 0.52 ml diluted plasma were placed in 0.50 cm inner-radius borosilicate glass tubes both uncoated and coated with the materials to be tested. The plasma was incubated at 37 °C under a constant stirring speed (1100 rpm) for 3 min; 0.2 ml of activated plasma was added to 0.2 ml of 2 mM solution of the chromogenic substrate and to 0.6 ml of the same buffer in a spectrophotometric cell thermostated at 37 °C. The releasing of *p*-nitroaniline was followed at 405 nm (molar absortivity 9950) by means of a Shimadzu 2100 UV-visible spectrophotometer; the initial rates were obtained by "differential plots", calculated by a "tailor-made" computer program. Kallikrein-like activity (KLA) values were obtained from the initial rates by the "initial velocity" method, using the kinetic constants evaluated from the reaction between S-2302 and purified kallikrein [6, 7].

2.3. Cytotoxicity test

The cytotoxicity of the bulk materials was tested using protocols modified from FRAME (Found for the Replacement of Animals in Medical Experiments, Nottingham, GB) [6, 8]. They are level I cytotoxicity tests according to the NIH Guidelines [9]. Neutral Red (NR), 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT), and Kenacid Blue (KB) assays were used. Material samples of 300 mg each were prepared, first sterilized by ethylene oxide, then added to 15 ml phosphate-buffered saline (PBS) and left at 37 °C for 5 days. The negative control consisted of 15 ml PBS with no added material samples. Extracts were filtered with 0.2 μm cellulose acetate (Nalgene, USA) and used for the assays. 3T3 cells, from a mouse fibroblast cell line, were cultured in Dulbecco's Minimal Essential Medium with 10% fetal bovine serum, 2 mM L-glutamine, 100 U ml^{-1} penicillin-streptomycin, 5 μg ml^{-1} fungizone. The cells were seeded onto 96-well plates at two different densities: 1.2×10^4 cells cm^{-2} for the 24 h and 6×10^3 cells cm^{-2} for the 72 h exposure period assays. The extracts were added to the wells (100 μl per well) 24 h after seeding. The plates were then incubated for 24 or 72 h at 37 °C in a CO_2 atmosphere. The positive control wells had 2,4-dinitrophenol (70 μg added ml^{-1}). After the incubation period, the procedures were different for the three assays. NR assay: the culture medium was replaced with 150 μl NR medium per well. The plates were incubated for 3 h at 37 °C, the NR medium was removed, the cells were rinsed twice with PBS and 150 μl destain solution (1% glacial acetic acid + 50% ethanol + 49% distilled water) per well were added. The plates were shaken for 10 min and the absorbance was read at 540 nm against a reference well which contained no cells. MTT assay: the culture medium was replaced with 10 μl MTT solution per well. After 4 h incubation the solution was removed, 100 μl DMSO were added and, after a 5 min slow agitation, the absorbance was read at 550 nm. KB assay: the culture medium was replaced with 150 ml KB dye per well. The dye was aspirated after a 20 min slow agitation and the wells washed twice with a washing solution. The desorbing solution was then added and the plates shaken for 20 min. The absorbance was read at 570 nm.

2.4. Cytocompatibility test

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords by trypsin/EDTA (0.05% trypsin, 0.02% EDTA (w/v)) treatment following a standard protocol [10]. They were cultured in 80 cm^2 culture flasks (Greiner, FRG) precoated with fibronectin in a complete culture medium composed of culture media T199 and RPMI 1640 with 20 mM Hepes buffer (Gibco, Paisley, UK), 2 mM L-glutamine, 100 U ml^{-1} penicillin-streptomycin, 5 μg ml^{-1} fungizone, 20% human serum (Sigma, Milano, Italy). Small fragments (area of about 50 mm^2) of the materials to be tested were cut from previously prepared [2, 3] small diameter (4 mm) vascular prostheses of FPU 58, FPU 60 and Cardiothane®. After ethylene oxide sterilization the material samples were distended on the bottom of 24-well TCPS culture dishes (Greiner, Austria). The experimental scheme was as follows. Sixteen samples of each material were prepared and divided into two identical sets. The samples of the first set were glued on the well bottom with a fibrin glue (Fbg, Immuno, Austria, 1 μl mm^{-2}). The samples of the second set were fixed by means of TCPS rings. Four samples of both sets were coated with human fibronectin (Fn, 5 μg ml^{-1}) and the other four were left uncoated. HUVEC were then seeded onto the materials at a seeding density of 4×10^4 cells cm^{-2}. The Fbg compound contained fibronogen (35 mg), plasma Fn (1–4,5 mg), factor XIII (5 U), plasminogen (17.5 μg), bovine thrombin (500 UI), and aprotinin (3000 UIK).

After one week in culture the materials were fixed and processed [11] for SEM. The presence of HUVEC on the material surfaces was estimated, as area covered by HUVEC, by the point counting method [11].

3. Results

Thrombogenicity. Fig. 1 shows the plasma PKK activation induced by FPU, compared with that induced by borosilicate glass (as a high-activation reference), silicone (as a low-activation reference) and Cardiothane® (a commercial polyurethane whose good haemocompatibility is known [1]). The PKK activation induced by FPUs appeared low, even lower than that induced by silicone and Cardiothane®. Moreover FPU 52 and FPU 60 showed a PKK activation lower than FPU 58 and FPU 42. A statistical analysis (Fisher PLSD test) was carried out to check the presence of significant differences between the KLA values of the different materials. The different FPUs were compared with the positive and negative controls. All the tested materials gave, as could be expected, KLA values lower than glass ($p < 0.01$). FPU 52 and FPU 60 showed lower KLA levels than silicone and Cardiothane® ($p < 0.05$ for FPU 52, $p < 0.01$ for FPU 60). FPU 60 showed a KLA activity lower than FPU 58 ($p < 0.01$).

Cytotoxicity. The results of the cytotoxicity tests are shown in Fig. 2. After a 24 h incubation of the polymer extracts with 3T3 cells, signs of lysosomal damage (NR test, Fig. 2a) were present with polymers FPU 60, FPU 52 and FPU 58 (Fisher PLSD test, $p < 0.05$) if compared with the negative control (PBS). This result was not confirmed after 72 h when no sign of cytotoxicity was detected. The MTT and KB assays at both 24 and 72 h (Fig. 2b, c) showed no significant difference between treated and control wells, which

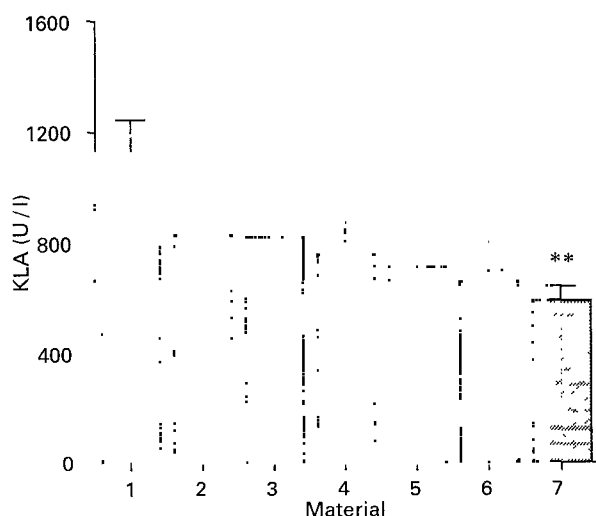


Figure 1 Plasma PKK activation induced by FPUs *in vitro*. The four FPUs are compared with positive (borosilicate glass) and negative (silicone and Cardiothane®) controls. Statistical significance is referred to both Cardiothane® and silicone (Fisher PLSD test, $*p < 0.05$, $**p < 0.01$). The results are the mean ($\pm$ SD) of six measures. 1 glass, 2 silicone, 3 cardiothane, 4 FPU 58, 5 FPU 42, 6 FPU 52, 7 FPU 60.

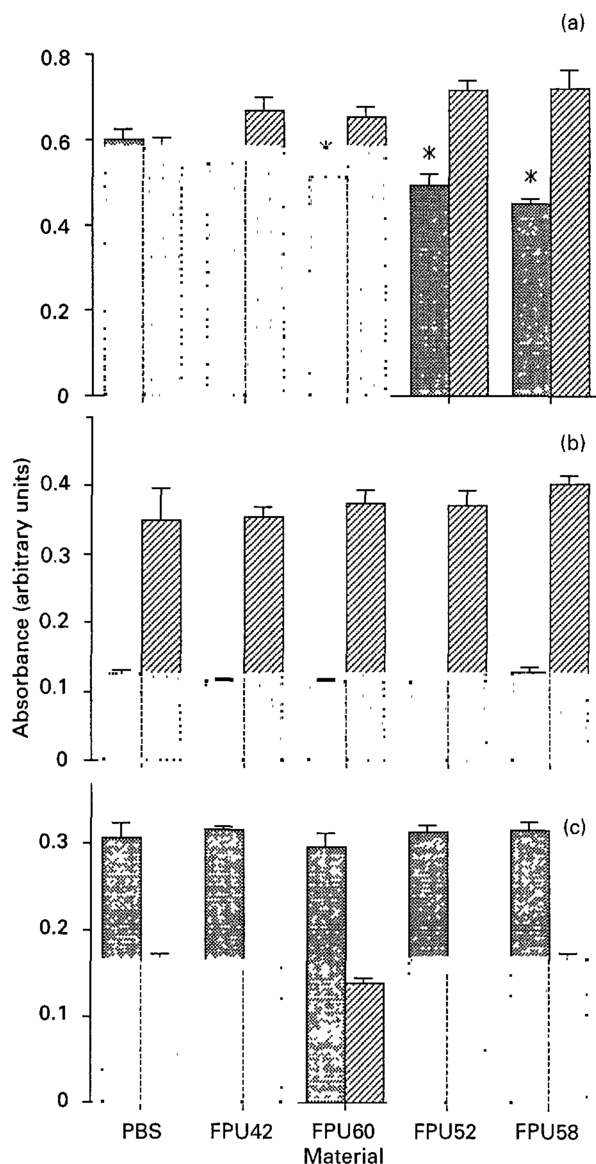


Figure 2 Cytoxicity tests of FPU 42, 60, 52 and 58 polymer extracts after 24 h (▩) and 72 h (▨) incubation with 3T3 cells: (a) NR uptake; (b) MTT assay; (c) KB assay. Negative control wells were incubated with PBS instead of polymer extracts. The statistical significance of the absorbance values is referred to the PBS values (negative control) (Fisher PLSD test, $*p < 0.05$). The results are the mean ($\pm$ SD) of five measures.

indicated that neither mitochondrial activity nor cell proliferation were affected by the extracts.

Cytocompatibility. In Fig. 4 SEM pictures of the surface patterns of respectively FPU 60, 58 and Cardiothane® precoated with Fn, before HUVEC seeding, are shown. They show a porous wall about 300 μm thick with a trabecular structure. One week after HUVEC seeding, FPU 60 showed a better endothelialization compared with FPU 58 and Cardiothane®. In particular the simultaneous presence of both Fn and Fbg in the FPU 60 samples seemed to give the best results (Fig. 3, Fig. 5a). A monolayer of distended and, in many parts, confluent HUVEC was observed (Fig 5a). The HUVEC spreading area on FPU 60 was significantly less (Fig. 3) when either Fn or Fbg alone were used, even if the cells presented a normal morphology. FPU 58 endothelialization gave satisfactory

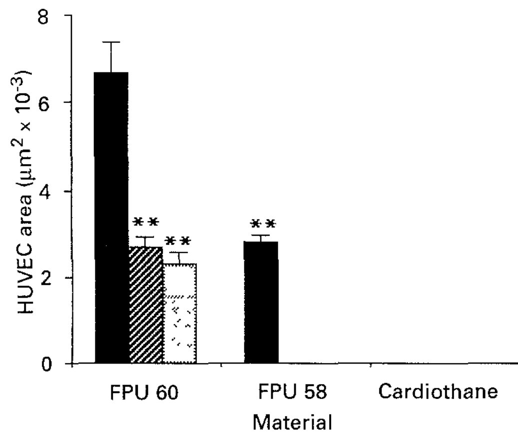


Figure 3 Endothelialization of FPU 60, 58 and Cardiothane® membranes. The three material samples were either fixed with fibrin glue on the bottom of 24-well TCPS culture dishes and coated with fibronectin (samples Fn + Fbg ■) or fixed with fibrin glue and left uncoated (samples Fbg ▨) or fixed by means of TCPS rings and coated with fibronectin (samples Fn ▩). Estimations of the HUVEC area were performed by the point counting method [11] on SEM pictures (magnification: × 750) taken at random on the material surfaces. No endothelialization was found on FPU 58 (Fn), FPU 58 (Fbg) and on Cardiothane®. Statistical significance is referred to FPU 60 (Fn + Fbg) (Fisher PLSD test, $^{**}p < 0.01$). The results are the mean (± SD) of four measures.

results only when Fn and Fbg were simultaneously used (Fig. 3, Fig. 5b). In all the other cases no appreciable endothelialization was present with the exception of rare spread round cells. Cardiothane® that was also tested gave constantly results equal to zero (Fig. 3). Rare HUVEC were found only on small flat patches, not representative of the whole material, occasionally present on the membrane surface. Significant endothelialization was not observed on any of the materials (data not shown), when they where both fixed with TCPs rings and not coated with Fn. The statistical analysis (Fisher PLSD test) showed that endothelialization was significantly higher on FPU 60 (Fn + Fbg) ($p < 0.01$) than on FPU 60 (Fn) or FPU 60 (Fbg) and FPU 58 (Fn + Fbg) (Fig. 3).

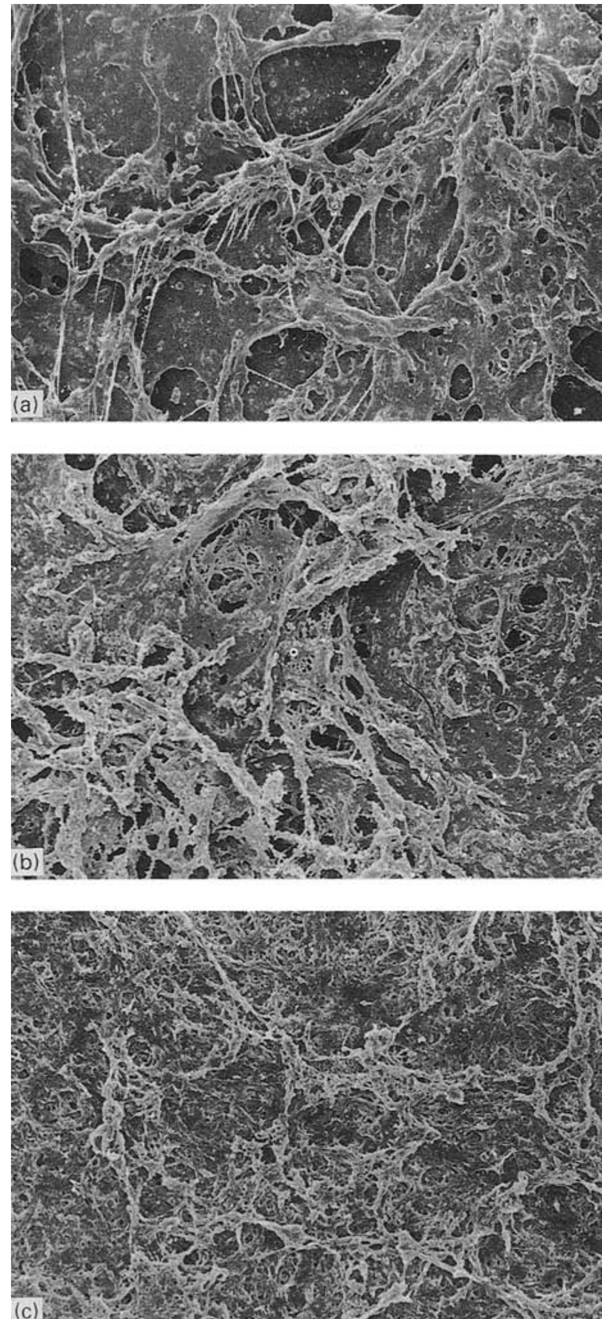
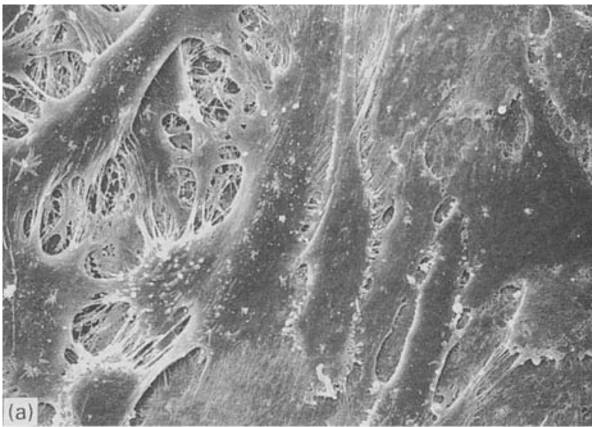


Figure 4 SEM pictures of the surfaces of (a) FPU 60, (b) FPU 58 and (c) Cardiothane® membranes before HUVEC seeding. (Original magnification: × 370)

4. Discussion

The thrombogenicity of a material candidate for vascular prostheses is of paramount importance, since it can lead to early thrombus formation and occlusion. All the tested FPUs showed a PKK activation lower than their negative controls. This result agrees well with published data [5] and is probably to be attributed to the presence of fluorine at the polymer surface, which might lower the polymer interfacial free energy with, as a consequence, a decreased thrombogenicity [4, 5]. FPU 52 and FPU 60, which contain PCLG as soft segment, showed PKK activation values lower than FPU 42 and FPU 58, whose soft segment is made of PTMEG. We can therefore infer that the nature of the macroglycol portion is important in determining the thrombogenicity of the materials. The tested FPUs demonstrated to be not cytotoxic after 72 h incubation of their extracts with 3T3 murine cells (Fig. 2). A relative instability of the cellular response at 24 h with the NR assay was detected and discussed in previous experiments [8]. The use of PUs as suitable candidates for endothelialization has been reported [12, 13] and is confirmed by our data. Endothelial cell attachment to FPUs was favoured by Fn coating (Fig. 3). Fn is an adhesive protein containing the sequence RGD that recognizes the endothelial cell adhesion receptors called integrins [14]. The binding between the RGD sequence and the integrins is an important step in the adhesion process. After this first event, the exogenous Fn adsorbed to the material is displaced by the endogenous Fn [15]. It seems that this displacement can occur only on materials that have an intermediate degree of surface hydrophilicity [15]. Too hydrophobic or too hydrophilic materials



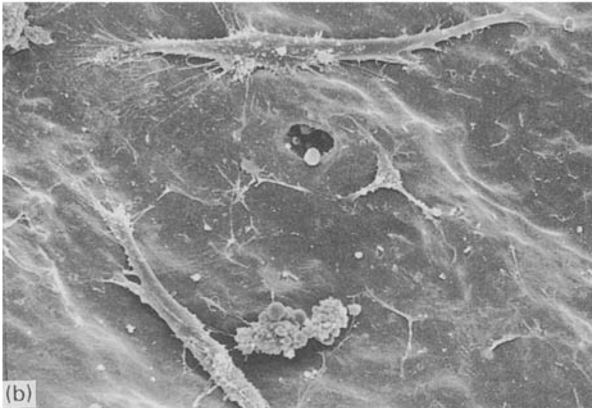


Figure 5 SEM pictures of (a) HUVEC on FPU 60 (Fn + Fbg) and (b) on FPU 58 (Fn + Fbg) surfaces one week after cell seeding. (Original magnification: (a) × 1000, (b) × 750).

do not allow displacement of the adsorbed exogenous Fn and the process of adhesion and proliferation can not further continue. It seems therefore that integrin binding with Fn is a "necessary but not sufficient" event, that needs to be coupled to appropriate surface characteristics. Fn-mediated HUVEC attachment and growth was fully successful only on FPU 60, less on FPU 58 (i.e. only in the presence of Fn + Fbg) and not at all on Cardiothane® (Fig. 3). We could not measure the water contact angles of the polymers under study due to their porous and trabecular structure (Fig. 4). We have therefore to infer from our results that the surface characteristics of FPU 60 are such as to favour HUVEC adhesion and growth compared with the other materials. Fbg, even if present only at the interface between the material sample and the TCPS well bottom, seems to exert a synergic effect with Fn on HUVEC adhesion and proliferation on FPU 60 and 58 (Fig. 3). It has already been reported that Fbg can be used as a substrate for in vitro endothelialization of PTFE vascular grafts [16]. Fbg contains human Fn and fibrinogen, which probably are partially soluble into the culture medium. Once in solution, they might adsorb onto the test materials, thus contributing to a better endothelialization process. Fibrinogen has been demonstrated to specifically bind to endothelial cells [17] and to promote not only cell attachment, stress fibres and adhesion plaque organization but also cell migration [18], events that are fundamental for the HUVEC proliferation process.

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