

1991

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### Recommended Citation

Busscher, H. J.; Stokroos, I.; Golverdingen, J. G.; and Shakenraad, J. M. (1991) "Adhesion and Spreading of Human Fibroblasts on Superhydrophobic Fep-Teflon," *Cells and Materials*: Vol. 1 : No. 3 , Article 5.

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## ADHESION AND SPREADING OF HUMAN FIBROBLASTS ON SUPERHYDROPHOBIC FEP-TEFLON

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(Received for publication June 10, 1991, and in revised form August 30, 1991)

### Abstract

Adhesion and spreading of human fibroblasts was studied on hydrophobized and hydrophilized FEP-Teflon, and compared with adhesion and spreading on untreated FEP-Teflon and Tissue culture polystyrene (TCPS). Superhydrophobic FEP-Teflon was prepared by ion etching followed by oxygen glow-discharge. Hydrophilic FEP-Teflon was prepared by ion etching only. Water contact angles of the modified surfaces were 140-150° and 5-10° for the hydrophobic and the hydrophilic variant, respectively. (Untreated FEP-Teflon: 109°). Spreading of human skin fibroblasts significantly increased on hydrophilic FEP-Teflon (257  $\mu\text{m}^2$  per cell), whereas a significant decrease was observed on superhydrophobic FEP-Teflon (158  $\mu\text{m}^2$  per cell), as compared to untreated FEP-Teflon (209  $\mu\text{m}^2$  per cell). Cell spreading on TCPS was significantly higher as compared to FEP-Teflon, but it was not significantly different from spreading on hydrophilic FEP-Teflon. The number of adhering cells on TCPS however was significantly higher than on the hydrophilic FEP-Teflon, illustrating that adhesion and spreading are two different phenomena.

**Key Words:** Super-hydrophobic, FEP-Teflon, ion etching, glow discharge, cellular adhesion, cell spreading.

### Introduction

The choice of a biomaterial for *in vivo* applications is often a difficult one. Biocompatibility and mechanical demands have to be combined into an optimally functioning device. Many times, a compromise has to be made. A good example of this problem is given by the application of polytetrafluoroethylene (PTFE).

The mechanical properties of this biomaterial can often be fitted to the demand *in vivo*: PTFE is strong, flexible, it can be made elastic (e-PTFE) and it is highly bioinert (5). Upon implantation, it evokes a minor foreign body reaction resulting in encapsulation by fibrous tissue (10). Since PTFE is an extremely hydrophobic material (surface free energy approximately 20 ergs/cm<sup>2</sup>; water contact angle 109°, (11)) its application is limited to situations where no or minor adhesion to body tissues is needed or anticipated (e.g., cardiovascular prostheses, periodontological membranes) (2, 6, 8). If PTFE is applied in situations where good interaction with body tissue is needed (e.g., abdominal wall patches), failure often results (7).

It is therefore that we tried to combine the optimal mechanical properties of PTFE with surface properties needed in specific clinical applications: ranging sometimes from very hydrophobic (e.g., vascular prostheses) to hydrophilic (e.g., abdominal wall patches).

In this study we describe a recently developed method (Busscher *et al.*, submitted for publication) to create so-called superhydrophobic FEP-Teflon, and how this method can be adapted to create hydrophilic FEP-Teflon. The possible potential of these new materials for biomedical application was tested by studying the *in vitro* adhesion and spreading of human skin fibroblasts on these materials.

### Materials and Methods

#### Substrate modification procedure

FEP-Teflon, obtained from Fluorplast b.v., The Netherlands, thickness 1 mm, was used as the basic material. Samples of 1 x 2 cm were cut, washed in

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## Fibroblasts on Super-Hydrophobic FEP-Teflon

**Table 1.** Contact angles (degrees) of sessile droplets of water, formamide, diiodomethane and  $\alpha$ -bromonaphthalene on variously treated FEP-Teflon surfaces and corresponding stylus surface roughnesses  $R_a$  ( $\mu\text{m}$ ).

Material	water <sup>a</sup>	formamide	diiodomethane	$\alpha$ -bromo-naphthalene	$R_a$
FEP-Teflon	109	90	77	73	0.4
Hydrophobized FEP-Teflon	> 140	123	111	97	0.5
Hydrophilized FEP-Teflon	6	10	26	16	0.5

<sup>a</sup> water contact angles on TCPS amount 60-68 degrees.

**Table 2.** Adhesion (density,  $10^4$  cells. $\text{cm}^{-2}$ ) and Spreading (area,  $\mu\text{m}^2$  per cell) of human skin fibroblasts on Tissue culture Polystyrene (TCPS), FEP-Teflon, Hydrophobized FEP-Teflon and Hydrophilized FEP-Teflon. A total number of 400 cells per material were measured, SEM is Standard Error of the Mean.

Material	TCPS	FEP-Teflon	Superhydrophobic	Hydrophilic-Teflon
Density $\pm$ SEM	4.5 $\pm$ 0.3	2.8 $\pm$ 0.5	2.8 $\pm$ 0.3	2.3 $\pm$ 0.4
Significance <sup>a</sup>	#	*	*	*
Area $\pm$ SEM	270 $\pm$ 16	209 $\pm$ 13	158 $\pm$ 22	257 $\pm$ 23
Significance <sup>a</sup>	#	*	* #	#

<sup>a</sup> The asterisk (\*) indicates a significant difference from TCPS ( $p < 0.01$ , Student's t-test).

The # indicates a significant difference ( $p < 0.01$ ) from FEP-Teflon.

acetone and dried. Hydrophobic modification consisted of a 5 hours ion etching, using an Ion Tech saddle field ion source (Teddington, UK) at  $4 \times 10^{-4}$  torr argon pressure, 8 mA and 6 kV, with rotating sample disk, followed by an oxygen glow discharge, 5 minutes at 15 mbar oxygen pressure and a radio frequency power of 50 watts, using a PLASMOD, Tegal Corporation, Richmond, CA, USA. The PLASMOD is a commercially available, inductively coupled (13.56 MHz RF) instrument equipped with a cylindrical, quartz-made reaction chamber (8 cm inner diameter, 15 cm length). A Balzers 320 lit/min rotary pump, in combination with a liquid nitrogen cold trap was used to reach the necessary vacuum. The entire procedure as well as a full physico-chemical characterization of the material is described in detail by Busscher *et al.* (submitted for publication).

The hydrophilic modification consisted of a 45 minutes ion etching at 8 mA, 6 kV and at  $4 \times 10^{-4}$  torr argon pressure with a rotating sample disk. These samples were subsequently stored in water.

Table 1 summarizes the contact angles of water, formamide, diiodomethane and  $\alpha$ -bromonaphthalene, as well as the stylus surface roughness values of the vari-

ously treated FEP-Teflon surfaces (See Busscher *et al.*, submitted for publication, for details). We note, however, that a full physico-chemical characterization of the hydrophilically modified material is not available at present.

### Surface topography

The modified FEP-Teflon surfaces were studied by scanning electron microscopy. To this end, the samples were sputter-coated with gold (10 nm) and examined in an ISI DS 130 scanning electron microscope.

### Cellular adhesion and spreading

An established cell line of human skin fibroblasts (PK 84; passage number 16) was cultured in TCPS flasks (Greiner, 75  $\text{cm}^2$ ) using RPMI 1640 medium (Gibco) supplemented with 15% foetal calf serum (Gibco) and 100 U.I./ml penicillin/streptomycin (Gibco) at 37°C in humidified air with 5%  $\text{CO}_2$ . Every other day cells were subdivided by trypsinization (by addition of 2ml of a 1:250 trypsin stock solution) in  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free Hanks balanced salt solution. Trypsin was inactivated by adding RPMI 1640 medium containing 15% foetal calf serum.

**Fig. 1 (at right).** Scanning electron micrographs (tilt angle 30°; accelerating voltage 5 kV) of the various modified FEP-Teflon surfaces: (a) untreated FEP-Teflon, (b) hydrophobized FEP-Teflon and (c) hydrophilized FEP-Teflon. Bar = 2.4  $\mu\text{m}$ .

After trypsinization  $10^4$  cells per  $\text{cm}^2$  were seeded in 6-well plates (Greiner) on the bottom of which the different substrata ( $n = 6$ ) were positioned. After 120 minutes, photographs were taken of the spread cells through an inverted phase contrast microscope and the number of adhered cells per unit area as well as the cell spreading area per cell were determined by morphometric image analysis (Cambridge Instruments, Quantimet 520), while manually outlining the cell borders with a mouse. Tissue culture polystyrene (TCPS) was used as a reference material. The entire experiment was performed in triplicate with different cell cultures.

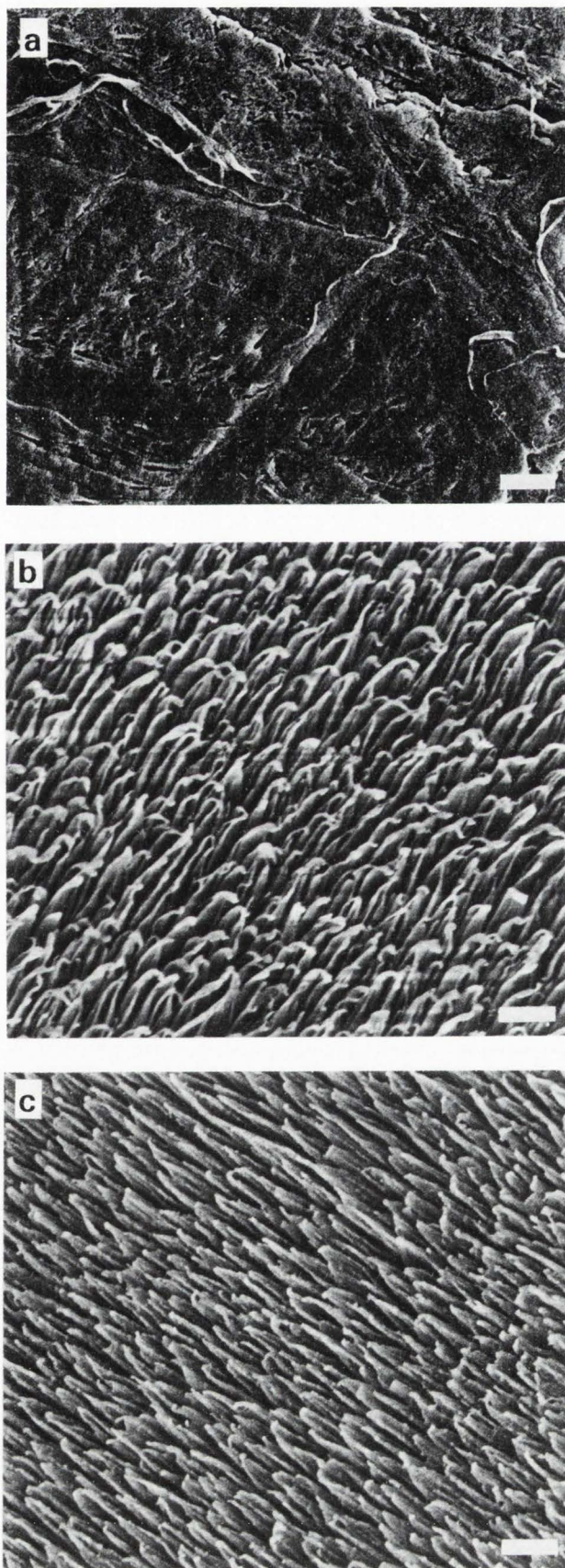
### Results

Scanning electron micrographs (Figs. 1a-c) revealed that the surfaces of the superhydrophobic FEP-Teflon (Fig. 1b) and the hydrophilic FEP-Teflon (Fig. 1c) are roughened with hair like structures of approximately 40 nm in diameter, as compared to the surface of untreated FEP-Teflon (Fig. 1a). Clearly, one can see the melted ends of the hair like structures on the hydrophobized FEP-Teflon (Fig. 1b), as compared with the hydrophilized FEP-Teflon (Fig. 1c), due to the glow discharge treatment.

The cell spreading data, shown in Table 2, clearly demonstrate that cells on untreated FEP-Teflon spread only to 77% of their spreading area on TCPS, set to 100%. Cells on hydrophobized FEP-Teflon showed an even smaller spreading area (58%), whereas cells on hydrophilized FEP-Teflon showed an enhanced spreading area (95%) as compared to untreated FEP-Teflon. However, the optimal spreading of cells as on TCPS is not yet met.

Despite the fact that equal cell densities were seeded on the different materials, a differential loss of adhering cells occurred upon handling the substrata, resulting in the cell densities listed in Table 2. Cell densities on untreated FEP-Teflon and on the hydrophobized FEP-Teflon are clearly lower than on TCPS. Also, the cell density on the hydrophilized FEP-Teflon is lower than on untreated FEP-Teflon. Cell density is highest on TCPS. These observations illustrate that adhesion and spreading are two separate phenomena (4).

Fig. 2 shows human skin fibroblasts adhering and spreading on the different materials at the light microscopical level.



### Discussion

The results of this paper clearly demonstrate that the surface properties of FEP-Teflon can be modified from superhydrophobic to hydrophilic, and that these modifications are reflected *in vitro* by the behavior of adhering and spreading human skin fibroblasts.

Physico-chemically, we only have a poor understanding at present of the origin of the superhydrophobicity created and no understanding at all of the origin of the hydrophilicity created. Previously, the superhydrophobicity of FEP-Teflon, as created by ion etching and glow discharge, was attributed to a combination of the specific topography of the material and a defluorination of the surface, resulting in an increased amount of C-C rather than C-F bonds at the surface (Busscher *et al.*, submitted for publication). Yet, C-F groups are generally thought to be the most hydrophobic. Possibly therefore, oxygen glow discharge after ion etching, causes melting down of hair like structures created during etching, therewith simultaneously concentrating fluorine in the tips.

The superhydrophobic and hydrophilic properties of the FEP-Teflon described in this paper, are not transient and storage in ambient air has up to now (6 months) not affected the properties. This makes the modification procedures extremely useful for FEP and PTFE biomaterials.

Application of the superhydrophobic modification can be thought in all clinical and dental circumstances where bioadhesion and spreading is undesirable. For example, a vascular prosthesis can be constructed of PTFE, which is superhydrophobized on the luminal surface. It is thus expected to fully prevent adhesion or clotting on the luminal surface (Schakenraad *et al.*, in preparation). Dental prosthetic devices can possibly be coated and modified to decrease bacterial adhesion and plaque formation. Construction of superhydrophobic heart valves might result in a decrease in bacterial colonization and subsequent failure.

Application of the hydrophilic modification can be thought in all clinical and dental applications where adhesion and spreading of cells is desirable. In case of a vascular prosthesis, the outer surface could be hydrophilized offering a good matrix for cellular anchorage and ingrowth.

Abdominal wall reconstruction materials, are either hydrophobic (e-PTFE) or more hydrophilic (collagen, polyurethane, polypropylene). However, an ideal material used for abdominal wall reconstruction should have a biphasic character: hydrophobic on the visceral side to prevent adhesion of the bowel and hydrophilic on the dermal side to promote adhesion and ingrowth of fibrocollagenous tissue. Modification of e-PTFE abdominal

wall reconstruction material could result in the above described properties.

As a last example, clinical application can be thought in the hydrophobic modification of vocal assist devices (tracheal-esophageal shunts), which are apt to undergo colonization by e.g., *Candidae* within a few weeks, and therefore have to be replaced regularly (9).

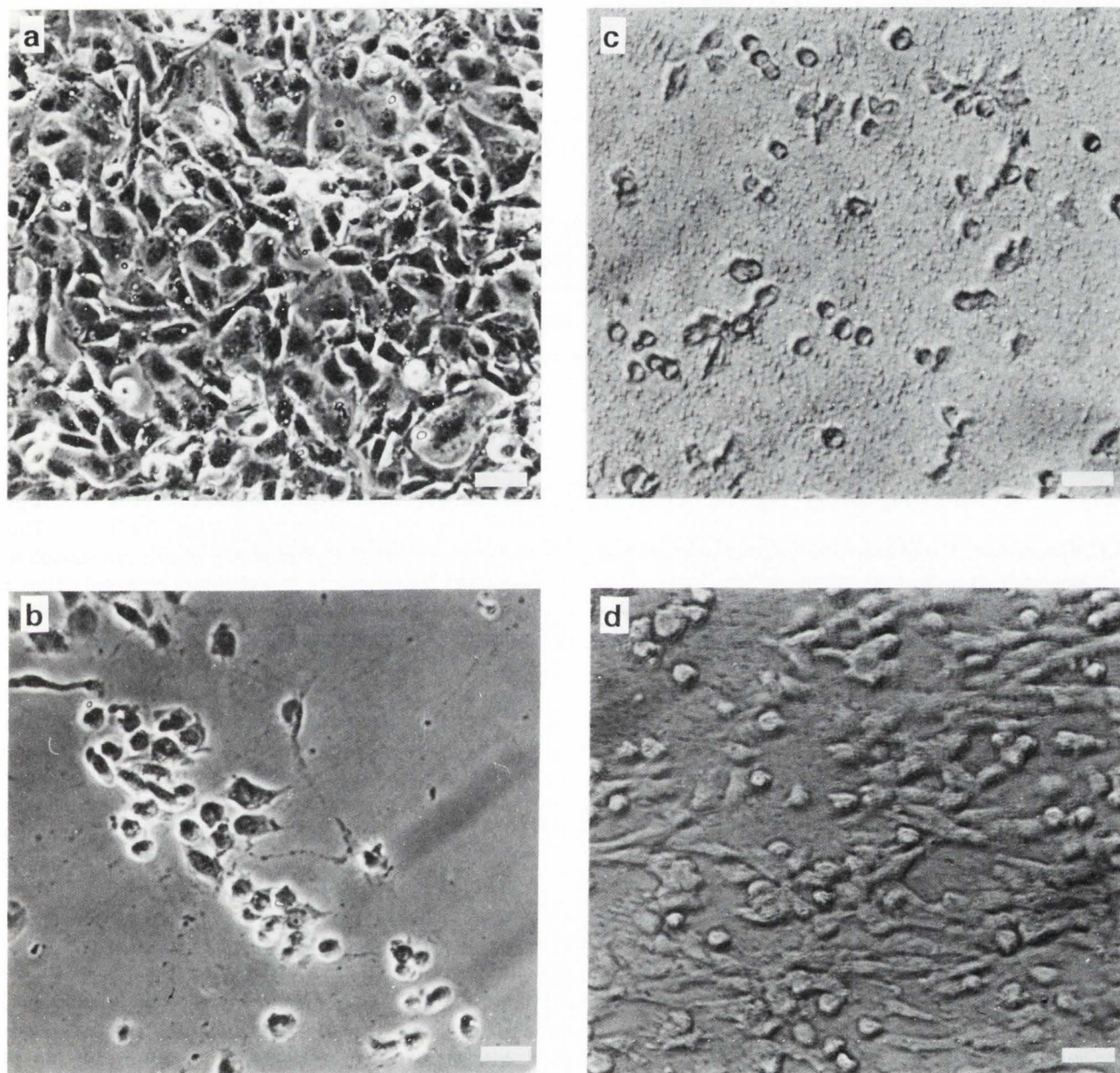
Apart from clinical applications, especially the superhydrophobic modification of FEP-Teflon has many potential industrial applications; we only mention the prevention of adhesion of proteins and other bioparticles on heat exchanger plates in e.g., food industry (12), or on ship hulls and other water contacting surfaces (1, 3).

### Acknowledgement

The authors are greatly indebted to Mrs. M.P. Schakenraad-Dolfing for the preparation of this manuscript.

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**Fig. 2.** Light micrographs of human skin fibroblasts adhering and spreading on (a) Tissue Culture Polystyrene, (b) untreated FEP-Teflon, (c) hydrophobized FEP-Teflon, (d) hydrophilized FEP-Teflon. The bar denotes 37  $\mu\text{m}$ .

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#### Discussion with Reviewers

**C.J. Kirkpatrick:** Have the authors an explanation for the decreased adhesion of cells on the hydrophilic Teflon, compared to the unmodified FEP-Teflon?

**Authors:** We do not have a rigorous explanation for this, but would like to note that the difference in density is small and not really significant. In the experimental set-up used here, differences in density arise due to

differential cell-substrate interactions, but also due to differential shear/forces during sample handling. Presumably, differences in area are therefore a better reflection and parameter of consideration of the interactions forces.

**K. Park:** Is two hours enough for cell spreading?

**Authors:** Yes, cells being brought in contact with a substratum by sedimentation as in this study, reach an equilibrium spreading in approximately 2 hours.

**K. Park:** Isn't it remarkable that cells reached a confluent stage on the tissue culture polystyrene only after 2 hours of incubation?

**Authors:** We do not agree. The time in which confluency is reached by sedimentation depends only on - concentration, - gravity and - time. Obviously 2 hours suffices under the conditions used.

**S.L. Goodman:** The observed stylus surface roughness is considerably greater than the size of the "hairs", thus the stylus does not detect these shapes. Is the stylus roughness measure therefore appropriate to the current investigation?

**Authors:** We fully agree. However, from the literature a criterium is known, stating that contact angles remain unaffected by surfaces roughness as long as the stylus surface roughness is within the submicron range. For this reason we did the profilometry in addition to the scanning electron microscopy.

**S.L. Goodman:** Does "a differential loss of adhering cells upon handling" mean that attached cells fell off? Do you have any evidence?

**Authors:** Yes, this is what we mean. The evidence is in the data: Sedimentation under identical conditions should eventually yield the same number of cells on all substrata. If not, cells are lost during handling. This is why we are currently developing flow cell systems for *in situ* observations of cell adhesion and spreading.

**S.L. Goodman:** A key element influencing cell behaviors on the different materials may be the great differences in surface topography. Since the surface roughness, hence the surface area of the treated materials (both the hydrophilic and the hydrophobic materials) are very much greater than the untreated FEP-Teflon, the actual surface area of cell-material contact may actually be much greater than that with light microscopy. In fact, considering the significant extent of spreading on the superhydrophilic material, it is possible that the total surface area of some cells equals or exceeds the total surface area of fibroblasts on TCPS. Thus, it would be desirable to image the interaction between the surface

"hair like structures" and the cells, especially to determine if cellular processes extend into the "hairs". Do you have any insight into this question and/or have you done any SEM (or other microscopies) to examine the extent of inter-penetration between these materials and adherent cells?

**Authors:** We agree that this may be an important point, but we do not have any insight to this question at the moment.

**S.L. Goodman:** In the discussion you suggested that the increased hydrophobicity was partially attributed to the topography of the ion-etched and glow discharged material. Since both the superhydrophobic and hydrophilic materials have a similar topography this suggestion appears untenable. Please comment.

**Authors:** Crucial in the statement mentioned is the word "partially". We note that subsequent glow discharge after ion-etching seems to "melt-down" the "hairs". This is a small change in topography, probably not enough to cause the big difference in wettability observed. Thus a chemical effect is likely to be present as well. This is more fully discussed in Busscher *et al.* (submitted for publication).

**M.F. Sigot-Luizard:** Citation of reference (4) in Results is not correct since we used a different culture technique and we measured quite different mechanisms. In our experiments, we compared cell adhesion which implies an extracellular matrix organization, and cell migration which implies a cell movement, both mechanisms quite different from cell attachment and cell spreading. Taking into account the experimental conditions described in the paper, shall we speak of cell attachment or cell adhesion?

**Authors:** The authors agree that for cellular adhesion, extracellular components are required. These components can be either provided by the surface or can be produced by cells themselves. It is quite likely that this indeed occurs (see also, van Wachem *et al.*, Biomed. Mat. Res. **21**, 1317-1327, 1987). We therefore believe that "adhesion" is the correct word; "attachment can be better reserved for the transport process eventually leading to adhesion.

The reference was not selected to compare our methods but merely to demonstrate that your group as well has done extensive work to demonstrate the differences between adhesion, attachment, spreading, migration, and proliferation.

**J.L. Duval:** Is the adhesion a morphological criterion assessed by image analysis or determined by another method? Cell adhesion is a phenomenon requiring an extracellular matrix assembly and happens after a long

time in a cell culture. This study seems to be an attachment assessment implicating physico-chemical properties of the substratum only. If the adhesion assessment is calculated as a function of the rounded shape of the cells, you have to make a cell viability test with Trypan Blue staining.

**Authors:** Please see previous answer also. Adhesion is assessed by measuring the surface area of cells on the different substrata. We perform the Trypsin Blue exclusion test as a routine procedure. We only measured viable and adhered cells.

**O. Johari:** Please provide more information about the papers mentioned in the text as submitted or in preparation.

**Authors:** The two papers are as follows:

Busscher HJ, Stokroos I, van der Mei HC, Rouxhet PG, Schakenraad JM. Preparation and characterization of superhydrophobic FEP-Teflon surfaces, submitted to J. Adhesion Science and Technology, 1991.

Schakenraad JM, Stokroos I, Busscher HJ. Application of superhydrophobic-PTFE-Teflon as a vascular graft: A pilot study in rabbits, in preparation for submission to Cells & Materials.

We shall be happy to provide a reprint on request.