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Evaluation of cell behaviour on atmospheric plasma deposited siloxane and fluorosiloxane coatings

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Abstract: For developing functional biomaterials, an understanding of the biological response at material surfaces is of key importance. In particular, surface chemistry, roughness and cell type influence this response. Many previous reports in the literature have involved the study of single cell types and their adhesion to surfaces with a limited range of water contact angles. The objective of this study was to investigate the adhesion of five cell lines on surfaces with contact angles in the range of 20° to 115°. This range of water contact angles was obtained using siloxane and fluorosiloxane coatings deposited using atmospheric plasma deposition. These nm thick coatings were deposited by nebulizing liquid precursors consisting of poly(dimethylsiloxane) (PDMS) and a mixture of perfluorodecyl acrylate/ tetraethylorthosilicate (PPFDA/TEOS) into the atmospheric plasmas. Cell adhesion studies were carried out with the following cell types: Osteoblast, Human Embryonic Kidney (HEK), Chinese hamster ovary (CHO), Hepatocytes (HepZ) and THP1 leukemic cells. The study demonstrated that cell adhesion was significantly influenced by the type of cell line, water contact angle and coating chemistry. For example the sensitivity of cell lines to changes in contact angle was found to decrease in the following order: Osteoblasts >Hepatocytes> CHO. The HEK and THP-1 inflammatory cells in contrast were not found to be sensitive to changes in water contact angle.

1. INTRODUCTION

Cell adhesion is involved in various natural phenomena such as embryogenesis, maintenance of tissue structure, wound healing, immune response, metastasis as well as tissue integration of biomaterials. It has previously been established that the surface properties of biomaterials strongly influence cellular behaviour. In particular, parameters such as surface energy, roughness and chemical composition significantly influence these interactions [1-3].

Many research groups have studied the effect of water contact angle on the interactions of biological species with surfaces. A large number of studies have concluded that cells tend to attach onto hydrophilic, rather than onto hydrophobic surfaces [4-9]. In contrast, other reports demonstrated that fibroblast cells adhered and proliferated at the highest rate when cultured on hydrophobic surfaces [10]. Others observed that cells adhere optimally on moderately wettable surfaces and cell adhesion and proliferation rates were lower on more hydrophilic or more hydrophobic surfaces [11-13]. These conflicting results may be explained by the fact that the tests were performed on different substrates (metals, ceramics, polymers, etc) and surface topographies [10, 12, 14-18]. Moreover, many of these studies were carried out on surfaces with a relatively narrow range of water contact angle. It has also been demonstrated that different cell lines exhibit different levels of sensitivity to material surfaces. For example, Jansen *et al.* [19] observed that human fibroblasts were more sensitive to surface wettability than epithelial cells. Johann et al. [20] also showed that HEK cells were not sensitive to changes in the surface wettability of PDMS.

The aim of this work was to investigate the effect of cell type and water contact angle on the cell-surface interactions. In order to obtain a wide contact angle range, siloxane and fluorosiloxane coatings were deposited using the atmospheric plasma processing technique. This involved

nebulising precursors of the liquid monomers into a helium or helium / oxygen plasmas. The chemical functionality of the precursor is retained in the deposited coating [21]. The level of coating oxidation is controlled by modifying the exposure of the precursor to the plasma. Siloxane coatings with water contact angles in the range 20° to 97° were obtained. Similarly, fluorosiloxane coatings with water contact angles ranging from 63° to 115° were also deposited. In order to investigate the effect of this wide range of water contact angles on cell adhesion, the following cell types were chosen: Osteoblast, Human Embryonic Kidney (HEK), Chinese Hamster Ovary (CHO), Hepatocyte (HepZ) and Human Monocytic Leukemia (THP-1) cells. For the THP-1 inflammatory cells, monocytes/macrophages adhesion and CD14 expression were also evaluated.

2. MATERIALS AND METHODS

2.1 Surface preparation and characterization

The siloxane and fluorosiloxane coatings were deposited on polystyrene (PS) cut from Petri dishes. Uncoated PS and tissue culture grade polystyrene (TCPS) were used as references. PS is an example of a hydrophobic surface with a low surface energy and a correspondingly relatively high contact angle of approximately 90° [22-23]. The surface of TCPS has been modified to impart an oxygen-containing surface chemistry that has a water contact angle in the range of 60°–70° [22-24].

2.1.1 Atmospheric plasma deposition of siloxane coatings

Siloxane coatings were deposited on the PS substrates using the Labline[TM] (Dow Corning Plasma Solutions, Midleton, Co. Cork) atmospheric pressure plasma system [25]. This reel-to-reel web system combines the liquid delivery of precursors with an atmospheric pressure dielectric barrier discharge plasma. It comprises two vertical plasma chambers made up of 30×32 cm^2 electrodes

consisting of a conductive liquid housed in a dielectric perimeter. The PS samples were mounted onto the poly(ethylene terephthalate) (PET) web with a double sided tape and passed through the plasma chamber at speeds of approximately 1 m/min. A helium (He) flow rate of 40 L/min and oxygen (O_2) flow rate of 0.25 L/min were used. The input power to the electrodes was maintained at 1000 W. The liquid PDMS precursor was nebulized into the helium (He) plasma. The precursor flow rate and the number of depositions (passes through the deposition chamber) were varied in order to build up layers of coating using the same total flow rate (Table 1). This effectively increased the exposure to the plasma during deposition, while maintaining the same volume of precursor.

2.1.2 Atmospheric plasma deposition of fluorosiloxane coatings

The deposition of fluorosiloxane coatings onto PS substrate was carried out using an atmospheric plasma jet system known as PlasmaStream^{TM} (Dow Corning Plasma Solutions, Midleton, Co. Cork) [26]. The atmospheric pressure discharge is formed from a modified PTI 100 W rf power supply between two metallic electrodes. The plasma operates at a frequency of approximately 15-25 kHz, with maximum output voltages of between 11.8 and 14.9 kV. A Teflon tube is mounted at the orifice of the jet and under the flow of He gas, the plasma extends out from the base of this tube. This Teflon tube is 75 mm long and has a diameter of 15 mm. The substrate to nozzle distance was maintained at 2 mm in this study. The jet is moved over the substrates using a computer numerical control (CNC) system with speed of 25 mm/s. A manual valve rotameter is used to control the He gas flow (10 µL/min) and a syringe pump supplies reactive precursor liquids to an atomiser positioned between the electrodes. The fluorosiloxane coatings were deposited from a mixture of two precursors 1H,1H,2H,2H-perfluorodecyl acrylate (PPFDA) and tetraethylorthosilicate (TEOS) mixed in equal volumes. The flow rate of this precursor mixture

into the nebulizer was 5 µL/min. The TEOS was added to PPFDA because this alkoxysilane acts as a crosslinking agent and improves the adhesion and crosslinking of the coating [27]. As detailed in Table 2 the number of passes and the CNC line speed were varied in order to deposit fluorosiloxane coatings with a range of water contact angles. In a previous study we demonstrated that broadly similar siloxane coating chemistries are deposited in both the PlasmaStream and Labline systems [28].

2.1.3 Surface characterisation

Contact angle measurements were carried out using a Dataphysics OCA 20 Video-Based Contact Angle Meter. A 1 µL drop of de-ionised water was allowed to sit on the surface for approximately 10 seconds before the water contact angle was measured. Measurements were made at three different locations on the coating and averaged. Three samples of each coating type were studied. All contact angle measurements were carried out 10 days after the coating was deposited. This was carried out to minimize the influence of any activation effect that the plasma has on the polymer substrate [29]. It has been reported that significant hydrophobic recovery of siloxane materials can occur. This hydrophobicity recovery is due to a reorientation of hydrophilic groups from the surface into the bulk by torsion about sigma bonds thereby replacing hydroxyl groups by methyl groups in the surface region.

Coating thickness was determined by a Woolam M2000 (J.A. Woolam Co., Inc., USA) variable wavelength ellipsometer on silicon wafer samples passed through the plasma along with the PS samples. Measurements were made at three different locations on three silicon wafer samples. Siloxane coating thicknesses was maintained at approximately 5 nm, while the thickness of the plasma jet deposited fluorosiloxane coatings varied between 40 and 150 nm.

The surface roughness of the coated surfaces was measured using a Wyko NT1100 optical profilometer (Veeco, USA). Three measurements were obtained on each of the PS surfaces, each over an area of 256 μm by 290 μm. Measurements were carried out on three randomly selected fields on each sample and the arithmetic mean surface roughness (Ra) averaged. Three coatings of each type were examined.

The FT-IR analysis was carried out using a Bruker Vertex-70 (Bruker Optik GmbH, Germany) system with a liquid nitrogen cooled MCT detector and a KBr beam splitter. Spectra were collected in the range of 4400-400 cm^{-1} using a spectral resolution of 4 cm^{-1} and an overlay of 64 scans per sample cycle.

2.2 Cells culture assay

The cell adhesion studies were carried out approximately 2 weeks after coating deposition. This ensured that there was no influence of plasma activation on the chemistry of the coated samples as detailed earlier

. **2.2.1 *Mammalian cells adhesion***

The adhesion of four different mammalian cells (Osteoblasts, HEK, CHO and HepZ) was evaluated on the siloxane surfaces. The MG63 cells were supported in Minimum Essential Medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), penicillin:streptomycin (100U: 100μg/mL) (Gibco), L-glutamine (1% v/v) (Gibco) and non-essential amino acids (1% v/v) (Sigma). The HEK and HepZ cells were cultured in Dulbecco's Modified Eagle's Medium (D5671, Sigma) supplemented with 5% fetal bovine serum (FBS; Lonza) and L-glutamine (4mM) (Sigma). For CHO cells non-essential amino acids (NEAA, M7145, Sigma) were added. All cells were maintained at 37 ºC, in 5% CO_2, at 100% humidity in

incubators. The cells were seeded onto prepared siloxane surfaces at a density of 2.5×10^5 cells/well. Osteoblast cell adhesion was evaluated after 4 hour's incubation; however, for HEK, CHO and HepZ cells, adhesion was evaluated after 2 hours and 30 min. After this time had elapsed, non-adherent cells were removed by washing the surface gently with warm phosphate buffered saline (PBS). Cells were then detached using Trypsin for Osteoblast cells and Accutase[TM] for the other mammalian cells and counted to determine the extent of adhesion.

2.2.2 THP-1 human cells adhesion and inflammatory response

The adhesion and the inflammatory response of Human acute monocytic leukemia cell line (THP-1) and macrophage-like cells were also evaluated. The monocytic cells were a kind gift from Dr Paola Maderna of the Mater Misericordiae Hospital, Dublin. The haematopoietic cell line was maintained in suspension culture in RPMI complete media (Promocell, UK) with 10% (v/v) heat inactivated fetal bovine serum (FBS) (Gibco, UK), 2mM L-glutamine, 100 U penicillin, and 100μg/mL streptomycin. The cells were grown in a humidified atmosphere with 5% CO_2. THP-1 cells were differentiated into macrophage-like phenotype by incubation with the phorbol ester, phorbal-12-myristate-13-acetate (PMA). The THP-1 cells were seeded at a density of 7.5×10^5 cells/well per coated disc and incubated for 24 and 48h. At the end of the incubation period the cells were rinsed with PBS to remove non-adherent cells and treated with glucosaminidase solution [7.5mM p-nitro-phenyl-N-acetyl-β-D-glucosaminide, 0.1M sodium citrate and 5 % (v/v) Triton X-100 (pH5)] for two hours at 37°C in a humidified atmosphere. The reaction was stopped by the addition of 80 mM glycine containing 5 mM EDTA (pH 10.4). An aliquot of supernatant was transferred to a 96-well plate and the optical density was measured at 405 nm in a multi-well plate reader. The number of cells adhered to the polymeric surface

(cells/cm^2) were calculated by standard curve of known cell amounts and the adhesion level was then determined.

Enzyme-linked immunosorbent assays (ELISAs) were used to evaluate the inflammatory response of the THP-1 human acute monocytic cell line on the tested surfaces. A Human s-CD14 Immunoassay (Quatikine® Cat No. DC140) was used for the determination of soluble CD14 (sCD14) concentrations in cell culture serum. All samples were assayed for the presence of sCD14 by ELISA, according to the manufacturer's instructions (R&D Systems Europe Ltd., Abingdon, UK).

The cell adhesion values are presented as the mean ± standard error. Statistical significance was evaluated using the Student's t test for paired comparison; $p < 0.05$ was considered significant.

3. RESULTS AND DISCUSSIONS

3.1 Influence of deposition conditions on siloxane and fluorosiloxane water contact angle

The volume of the precursor used to deposit the PDMS coating on the PS and silicon wafer substrates in the Labline system was kept constant at 50 µL. By varying the flow rate and the duration of exposure to the He/O$_2$ plasma, the chemistry of the deposited coatings could be systematically altered (Table 1). With the increased exposure, a higher level of siloxane coating oxidation occurs [30]. This change in chemistry facilitated the systematic change in water contact angle given in Table 1 and Figure 1. In the absence of oxygen, a hydrophobic siloxane coating is deposited; in contrast, with increased exposure of the precursor to the He/O$_2$ plasma, the coating becomes more hydrophilic.

As shown in Table 2, fluorosiloxane coatings deposited using the PlasmaStream system from the PPFDA/TEOS precursor mixture exhibited an increase in water contact angle from 63 to 115°,

with higher levels of plasma exposure (increased number of passes). This indicates higher levels of fluorine at the film surface for coatings deposited with higher plasma exposure. The coating chemistry was assessed by FTIR and compared to the TEOS coating. The resulting spectrum (Figure 2) included peaks at 1180 cm^{-1} and 1135 cm^{-1} corresponding to -CF$_3$ and -CF$_2$ bonds.

Examination of the PS surfaces before and after the application of both the siloxane and fluorosiloxane coatings by optical profilometry demonstrated that there was no significant change in the surface roughness (Ra). The Ra value of the siloxane coated coated surfaces remaining at approximately 8 ± 2 nm, while that of the fluorosiloxane coating surfaces roughness varies between 10 and 20 ± 2 nm. Thus, any changes in cell adhesion are associated with only changes in surface chemistry and not topography.

3.2 Mammalian cell adhesion to siloxane coatings

3.2.1 *Osteoblast cell adhesion*

The effect of water contact angle on Osteoblast cell adhesion to PS, TCPS and Labline deposited PDMS coatings is given in Figure 3. While cell adhesion was observed on all the siloxane surfaces, optimal adhesion of Osteoblast cells was observed for the siloxane coating with water contact angle of approximately 65°. Cell adhesion was found to progressively decrease on more hydrophobic or hydrophilic surfaces. As demonstrated in Figure 3 also significantly lower Osteoblast cell adhesion is observed on PS compared with the TCPS surfaces. This as outlined earlier is associated with the enhanced oxygen-containing surface chemistry of the TCPS.

3.2.2 HEK cell adhesion

The adhesion of HEK cells on the different siloxane coatings is summarized in Figure 4. Similar to the other cell types, HEK cells adhere well on all the siloxane coatings. This is in agreement with the observation in the literature that oxidized PDMS and other hydroxyl containing surfaces are very favorable for cell attachment [31-33]. In contrast, Johann *et al*. [20] report that no HEK cell adhesion was observed on PDMS surfaces, although relatively good adhesion was observed on the hydrophobic PDMS coating (θ= 97°) examined in this study.

The surface wettability and chemistry appear to have a minor influence on the ability of these cells to adhere. This is in agreement with the observation in the literature by Johann et al. [20] who observed that HEK cells were not sensitive to variations in the water contact angle of functionalised PDMS surfaces.

3.2.3 CHO cell adhesion

As shown in Figure 5, CHO cells exhibit better adhesion on the more hydrophilic compared with the more hydrophobic siloxane surfaces. This behaviour is similar to results previously reported for these type of cells [34-35]. An interesting observation with respect to Figure 5 is that comparing the adhesion of the cells on TCPS and a siloxane coating with similar water contact angle (θ= 65°), the adhesion was higher on the TCPS. This would indicate that the type of oxygenated chemistry of the TCPS surfaces is more favourable to the adhesion for these cells. It is interesting to note that in a previous study by Lee et al. [36] the adhesion of CHO cells was found to be higher on H_2O plasma treated PS (θ= 61°), compared with that of the O_2 plasma treated polymer (θ= 53°). The authors indicated that hydroxyl groups positively influence the adhesion and spreading of the CHO cells.

3.2.4 Hepatocyte cell adhesion

Figure 6 demonstrates that the Hepatocyte cells are more adherent on hydrophilic siloxane surfaces. For the siloxane surface with water contact angle of 20°, for example, a relative adhesion of approximately 80% is obtained. In contrast, only 20% adhesion is observed on hydrophobic siloxane surface with water contact angle of 97°. Similar observations were reported recently by Nakazawa et al. [37]. They demonstrated that the Hepatocytes strongly adhered to the hydrophilic siloxane surface (contact angle 28±3°); however, cell adhesion to the hydrophobic siloxane surface (contact angle 120±3°) was strikingly inhibited. Optimal Hepatocytes adhesion was observed on TCPS (96%).

Comparing the adhesion of the four mammalian cell types investigated, adhesion is, in general, better on the more hydrophilic siloxane surfaces. This behaviour can be attributed to the presence of hydroxyl/carboxylic groups generated during the plasma polymerization of PDMS in the presence of oxygen. This is in agreement with previous reports that oxidized PDMS and other hydroxyl containing surfaces are favorable for cell attachment [20, 31-33]. This may be explained as due to the variation in the integrin-fibronectin bonding affinities on these surfaces, which decrease in the order $-OH > -CH_3$ [38]. Fibronectin is able to adsorb onto surfaces with a wide range of physicochemical properties and plays a dominant role in the adhesion of most cultured cell lines [32, 38-39].

Comparing the level of cell adhesion on the different siloxane surfaces, the following results are highlighted. In general, the level of adhesion decreases in the following order: HEK> CHO>Hepatocytes>Osteoblasts. This demonstrates that the adhesion of cells depends not only on the biomaterial surface properties but also on the cell line examined. Osteoblast and Hepatocytes

cells are more sensitive to surface wettability than CHO cells. On the siloxane coatings tested, the percentage of the adhering Osteoblast cells varies from 13% to 50%. However, it varies only between 73% and 95% in the case of CHO cells. HEK cells are however not sensitive to the water contact angle, adhering similarly on all the siloxane coatings. Lee et al. [40] have previously demonstrated higher level of adhesion of CHO cells on corona treated polyethylene surfaces, compared to endothelial cells and fibroblasts. These differences in cell adhesion can be explained as due to the differences in type, quantity, conformation and activity of the adhesive proteins synthesized by each cell on the substrates during adhesion and proliferation. Moreover some cells secrete growth factors and some other molecules that promote cell adhesion [41].

3.2.5 THP-1 cell adhesion and inflammatory response

Monocyte and macrophage cells adhesion and foreign body giant cell (FBGC) formation are vital processes in the inflammatory and wound-healing responses to implanted biomaterials [42]. To investigate the influence of the surface wettability on the adhesion of THP-1 monocyte and macrophage, the adhesion and inflammatory response of these cells were tested on siloxane and fluorosiloxane coatings. As detailed in Figure 7, THP-1 monocytic cells adhesion is not significantly influenced by the siloxane wettability variation. The PMA-differentiated THP-1 macrophage cells exhibited enhanced adhesion compared with the THP-1 monocytic cells. Figure 8, showing optical microscopy images of THP-1 monocytic cells and PMA-differentiated THP-1 macrophage cells grown on siloxane coated PS, confirm these results. No effect of wettability on macrophage cells adhesion was observed.

THP1 monocytic cell adhesion studies were also carried out on fluorosiloxane surfaces (Figure 9). The level of adhesion was found to be three times higher on moderately hydrophilic fluorosiloxane coating (θ= 63°) than that on the more hydrophobic coating surface (θ= 103°). The slightly higher adhesion on more hydrophobic fluorosiloxane surface (θ= 115°) could be due to the relatively rougher surface of the coating (Ra= 60nm ±3) compared with that of the other fluorosiloxane coating surfaces (Ra= 10- 20 nm). In a previous study, Eloy et al. [43] reported that the development of inflammatory cells was prevented when the substrate was treated in CF_4 plasma. This was attributed to the hydrophobic property of these surfaces.

The immune system provides the first response to an infection by initiating an inflammatory response and the monocyte surface molecule CD14 is a key element in this response system. In this work, the effect of the surface chemistry on the inflammatory response of THP-1 cells was investigated by evaluating CD14 secretion on TCPS, PS, siloxane (55°) and fluorosiloxane (103°) surfaces (Figure 10). Higher CD14 secretion was observed on fluorosiloxane and untreated PS surfaces. Low CD14 secretion was observed on the more moderately hydrophilic siloxane surface. These observations confirm the major effect of the surface chemistry on the inflammatory response of the THP-1 cells [44-45].

4. CONCLUSIONS

In this study, the effect of siloxane and fluorosiloxane coating water contact angles on the adhesion of five cells line was investigated. The cells studied were: Osteoblasts, Human Embryonic Kidney (HEK), Chinese hamster ovary (CHO), Hepatocytes (HepZ) and THP1 inflammatory cells. The study has demonstrated the following:

1- All the cells tested adhered to the siloxane surfaces; however, the sensitivity of the cells to contact angle variation depends on the cell line. For three cell lines the sensitivity to changes in contact angle decreased in the following order: Osteoblasts >Hepatocytes> CHO. The percentage of the adhering Osteoblast cells on the siloxane surfaces varies from 13 to 50% but only from 73 to 95% in the case of CHO cells. The HEK and THP-1 inflammatory cells, in contrast, were found to not be sensitive to changes in water contact angle.

2- The level of adhesion is cell type, contact angle and chemistry dependent. Osteoblast cells adhere better on moderately hydrophilic surfaces ($\theta= 64°$). However, CHO and HepZ cells were found more adhesive on hydrophilic siloxane surfaces ($\theta= 20°$). HEK and THP-1 monocytic cell adhesion was found to be relatively insensitive to the water contact angle of the siloxane coatings tested.

3- The adhesion of the THP-1 monocytic cells was found to be sensitive to surface chemistry. For example, higher levels of adhesion were observed on fluorosiloxane compared with siloxane surfaces with similar water contact angles.

4- The adhesion of PMA-differentiated THP-1 macrophage cells on PS, TCPS and siloxane surfaces is higher than that observed for monocyte cells.

5- The inflammatory response of THP-1 cells is surface chemistry dependent. The CD14 expression is four times higher on fluorosiloxane and untreated PS surfaces than on siloxane and TCPS surfaces.

These results demonstrate the importance of the biomaterial surface wettability and chemistry for cell attachment and confirm the major and complex role of the cell-surface interaction in

determining cell adhesion. The results are significant for the assessment and design of new surfaces for implant device applications.

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Table 1: Influence of the number of passes through the LabLine^{TM} deposition chamber and addition of O_2 into the plasma on siloxane (PDMS) coating water contact angles

PDMS Flow rate [µL/min]	O_2	No. of passes	Water Contact Angle (degree)
50	No	1	97
50	Yes	1	73
25	Yes	2	65
17	Yes	3	40
10	Yes	5	20
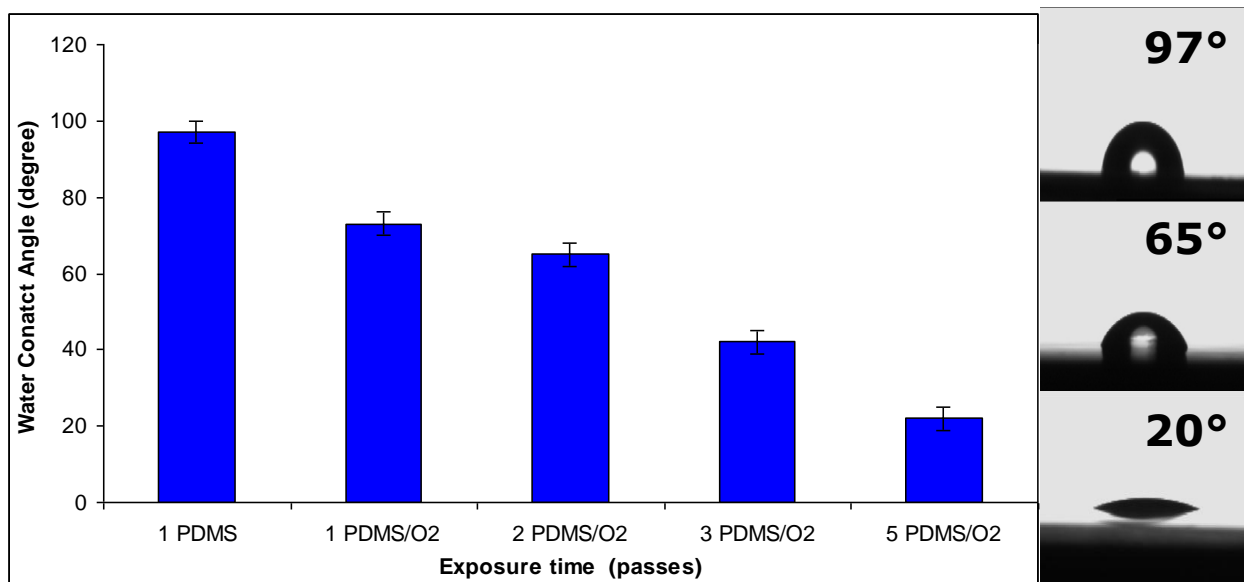
Figure 1: The effect of increasing PDMS precursor plasma exposure time on the coating water contact angle. The number of passes through the chamber is shown i.e. 1 to 5
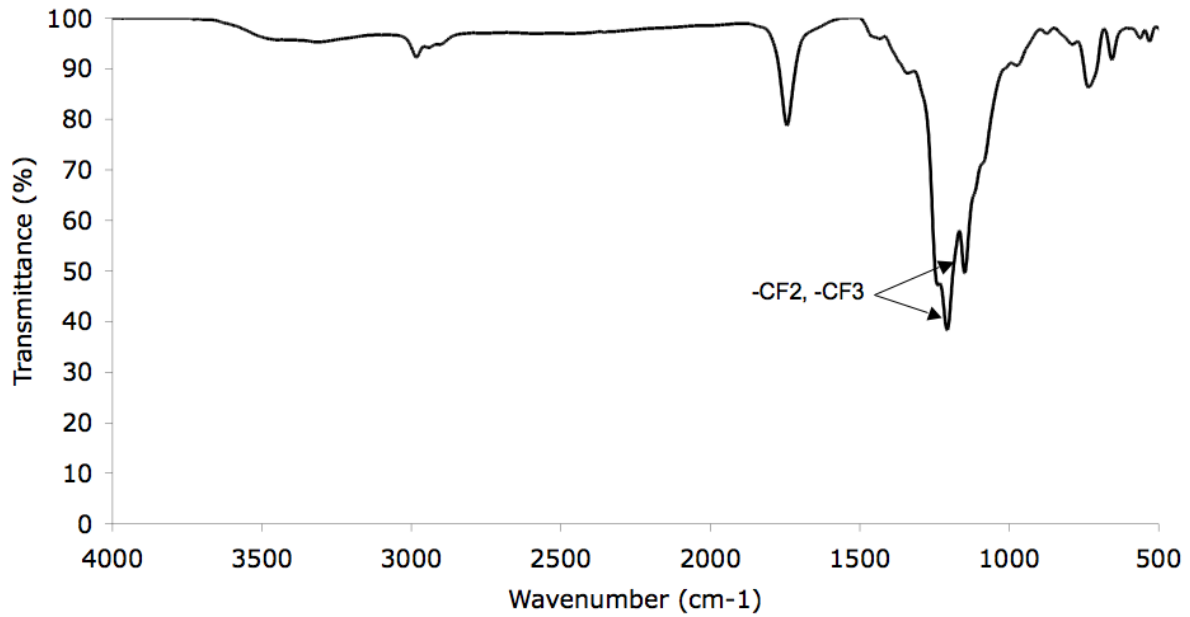
Figure 2: FTIR spectrum of PPFDA/TEOS coating.

Table 2: Influence of the number of plasma jet passes over the surface using the PlasmaStream[TM] system on the fluorosiloxane coatings water contact angles

Computer Numerical Control (CNC) speed (mm/s)	No. of passes	Water contact angle (degree)
150	3	63
10	3	76
10	6	78
50	9	103
10	15	115
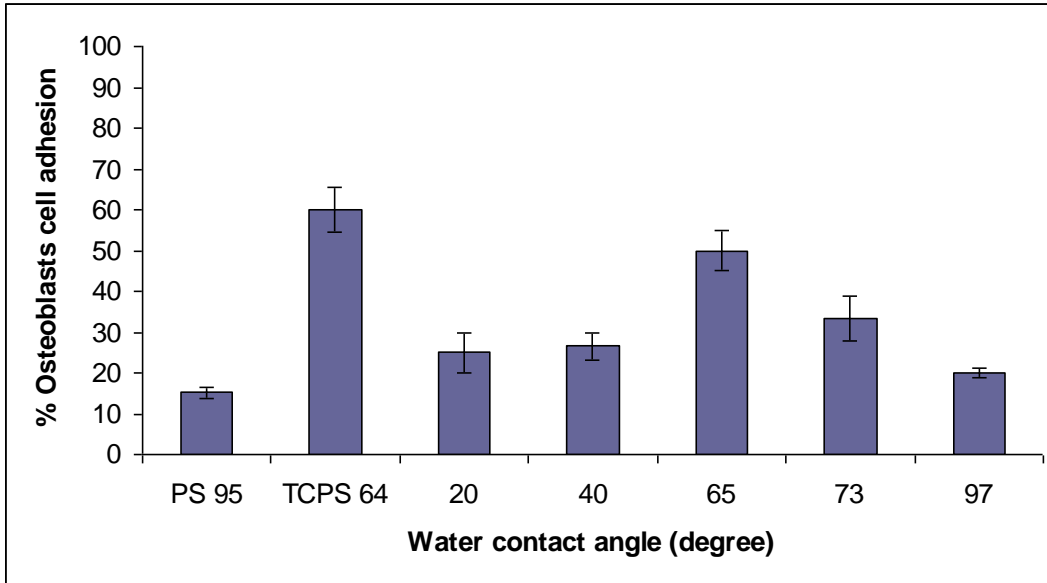
Figure 3: Influence of siloxane water contact angle on Osteoblast cell adhesion
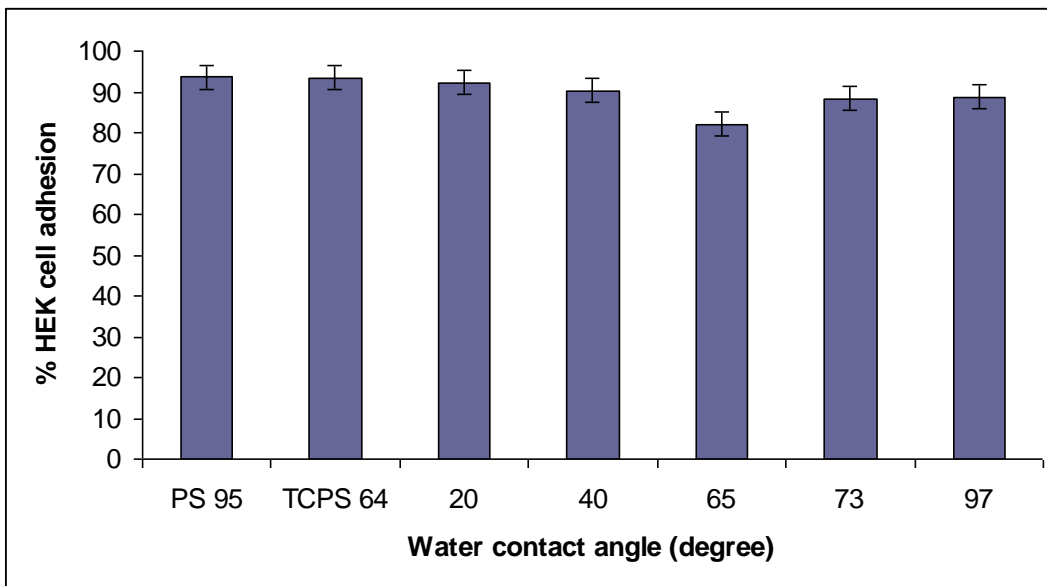
Figure 4: Influence of siloxane water contact angle on HEK cell adhesion
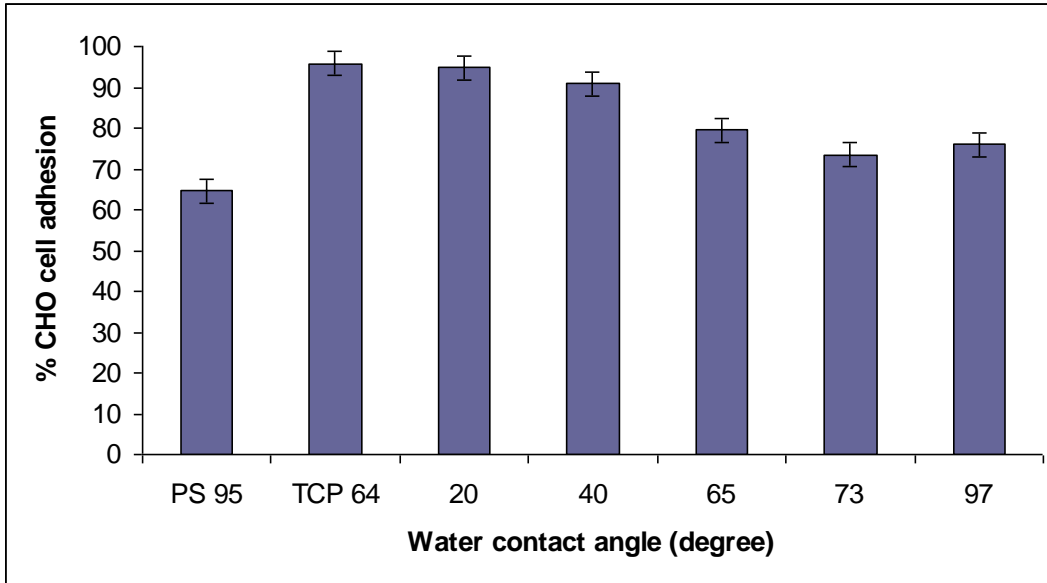
Figure 5: Influence of siloxane water contact angle on CHO cell adhesion
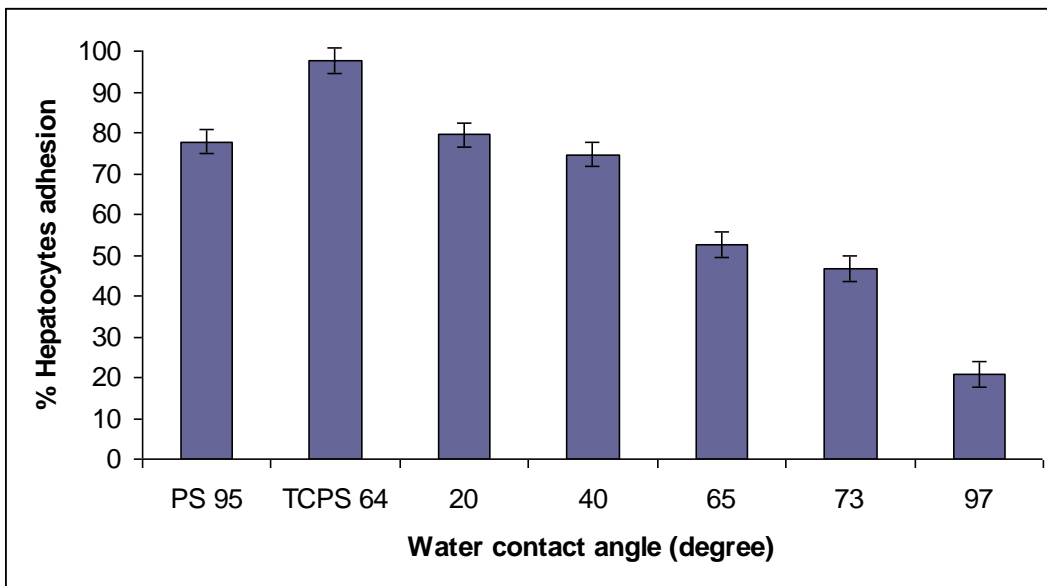

Figure 6: Influence of siloxane water contact angle on Hepatocytes (HepZ) cell adhesion
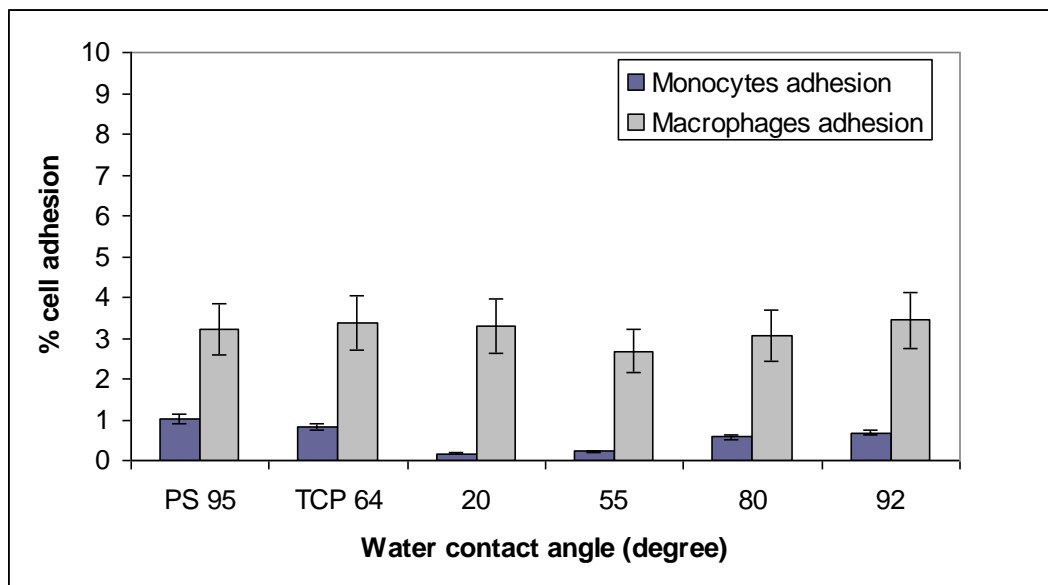
Figure 7: Influence of siloxane coating water contact angle on the adhesion of THP-1 monocytic
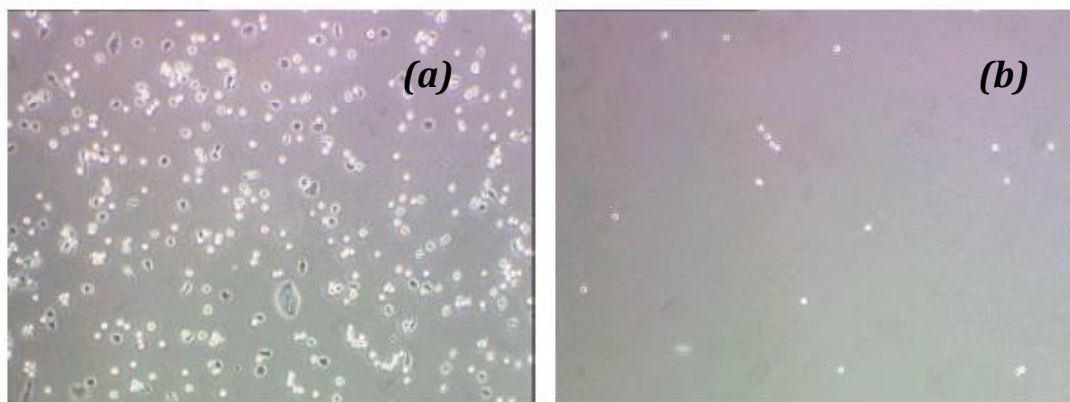
and macrophages cells (24h incubation)

Figure 8: Representative data comparing the adhesion of (a) PMA-differentiated THP-1

macrophage cells and (b) THP-1 monocytic cells on PDMS coated tissue culture polystyrene (θ

= 92°) after 4 hours incubation. The images were captured on Nikon TMS microscope (area of
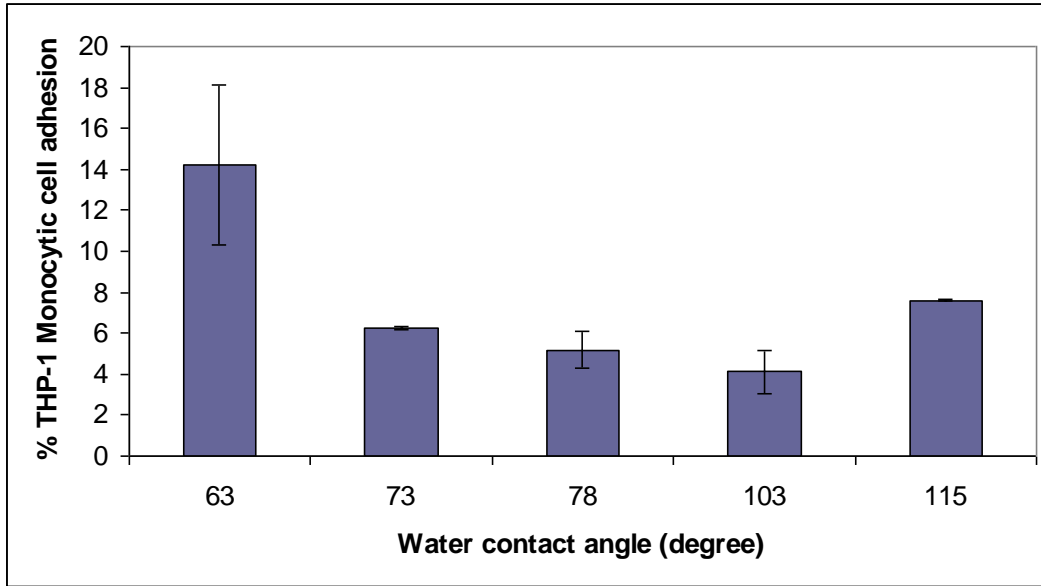
1.289 mm^2)

Figure 9: Influence of water contact angle on THP-1 monocytic cells adhesion on fluorosiloxane
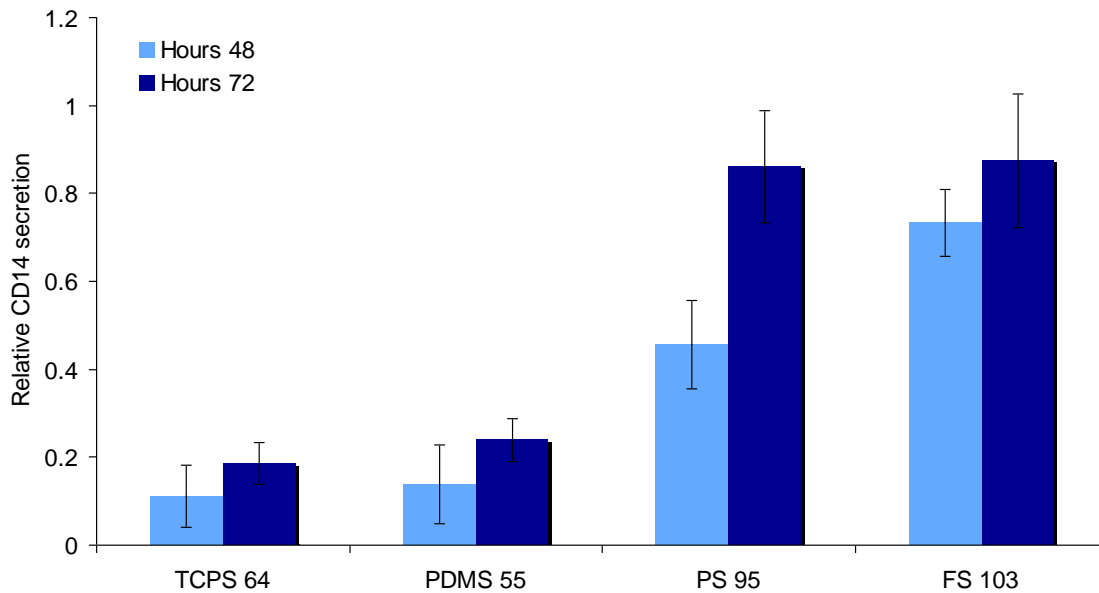
coatings

Figure 10: Effect of the surface chemistry on the CD14 secretion by THP-1 cells (48 and 72

hours incubation). Note PDMS 55 and FS103 refer to the siloxane and fluorosiloxane coatings

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