Initial biocompatibility of plasma polymerized hexamethyldisiloxane films with different wettability

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Abstract. Understanding the relationships between material surface properties, behaviour of adsorbed proteins and cellular responses is essential to design optimal material surfaces for tissue engineering. In this study we modify thin layers of plasma polymerized hexamethyldisiloxane (PPHMDS) by ammonia treatment in order to increase surface wettability and the corresponding biological response. The physico-chemical properties of the polymer films were characterized by contact angle (CA) measurements and Fourier Transform Infrared Spectroscopy (FTIR) analysis. Human umbilical vein endothelial cells (HUVEC) were used as model system for the initial biocompatibility studies following their behavior upon preadsorption of polymer films with three adhesive proteins: fibronectin (FN), fibrinogen (FG) and vitronectin (VN). Adhesive interaction of HUVEC was evaluated after 2 hours by analyzing the overall cell morphology, and the organization of focal adhesion contacts and actin cytoskeleton. We have found similar good cellular response on FN and FG coated polymer films, with better pronounced vinculin expression on FN samples while. Conversely, on VN coated surfaces the wettability influenced significantly initial cellular interaction spreading. The results obtained suggested that ammonia plasma treatment can modulate the biological activity of the adsorbed proteins on PPHMDS surfaces and thus to influence the interaction with endothelial cells.

1. Introduction
The initial interaction of cells with biomaterial surfaces is of fundamental for their biocompatibility and contributes to the clinical success of implants. It is well documented that cells interact with biomaterials not directly but via a layer of adsorbed plasma proteins [1, 2]. When a biomaterial comes into contact with a biological system proteins spontaneously adsorb onto the surface within a time frame of seconds to minutes [3]. The resulting surface-bound protein layer mediates the subsequent cell attachment through interactions with cell surface receptors [4]. Both, protein adsorption and cell adhesion are strongly affected by the surfaces characteristics including the surface chemistry, topography as well as surface wettability. A lot of research has been undertaken to study the effects of the surface wettability on the cellular responses and even a certain optimal wettability has been described, however, often these studies yield contradictory results [5, 6]. The reason for this is that the surface parameters cannot be considered independently; each material with defined surface wettability may have different chemistry or topography. Therefore, to better understand the effect of this single
parameter in this study we employed a wide range of wettability with a single material, thus without change in surface topography or chemistry.

Plasma polymers are attractive candidates for biomaterials modification because their surface hydrophilic can be easily modified. Plasma polymerization is a convenient and versatile solvent-free technology, providing fast and one-step synthesis. Many monomers which do not polymerized by normal (chemical) methods can be polymerized by plasma because this is a process in which the monomer is subjected to successive fragmentation and recombination, and thus forms a polymer [7]. Therefore, the structure and properties of the plasma polymers substantially differs from conventional polymers. Moreover, varying with the process parameters of plasma polymerization from one monomer could be obtained different polymers, including such with desirable structure and properties. Resulted polymers usually have highly irregular three-dimensional structure and cross-linked network which determines properties such as chemical resistance, mechanical toughness, thermal stability, and good adhesion to any substrate [8]. All these advantages have led to wide application of plasma polymer, for example as protective coatings [9], membranes [10] sensors [11-13] and biomaterial films [14, 15]. In particular, plasma polymerization using organosilicon monomers, such as hexamethyldisiloxane (HMDS), has attracted great interest because of their high deposition rates and ease ability of varying the deposition conditions to control their structure and properties, biocompatibility and low toxicity [16-18]. The relatively high hydrophobicity of HMDS has been used to prevent coagulation and inflammatory related problems in cardiovascular systems. In order to improve cell adhesion however polymer surface should be render hydrophilic. This could be realized by means of surface treatments using various gases such as Ar, O2, N2, SO2. Indeed, several studies have showed that O2-plasma treatment of polymer surfaces was able to control hydrophilicity/hydrophobicity and to introduce various functional groups [19]. We anticipate that ammonia plasma-surface modification could also be useful to influence the surface wettability, and thus affecting protein adsorption and cell behavior.

Here, we report on a simple method for modulation of surface wettability of plasma polymerized hexamethyldisiloxane (PPHMDS) films and how it affects the endothelial cells interaction. Varying with the processing parameters (current density of the plasma, monomer flow rate and time of deposition) of both, plasma polymerisation and plasma modification, we found a technological regime for deposition of ultrathin film from HMDS with lower wettability of about 96°, that could be rapidly hydrophilised by a single step of ammonia plasma treatment. To investigate the biological response of thus modified surfaces we have prepared model samples ranging significantly in their wettability and follow the initial cellular interaction of human umbilical vein endothelial cells (HUVEC).

2. Materials and methods

2.1. Preparation of polymer films
Prior to polymer films deposition, glass coverslips (CG) were cleaned by repeated washing in double distilled (DI) water, followed by ultrasonic treatment in acetone (99.99% purity) for 15 min, rinsing with boiling isopropanol (99.99% purity) for 10 min and then rinsing again with DI water (about 10 times). Finally, the samples were dried under a stream of pure nitrogen (99.9 %) and loaded into the reactor chamber. The monomer in this experiment was HMDS [(CH3)3-Si-O-Si-(CH3)3] –>99% Merck (Germany) used without further purification. Plasma deposition reactor was described in details elsewhere [20]. Briefly, thin polymer films were obtained by plasma polymerization of HMDS monomer in vacuum chamber for 10 min under following technological conditions: 0.13 mA/cm² current density and 10 l/h monomer flow rate. By using several polymerization regimes it was found that the deposition rates were proportional to the polymerization time. The thickness of the polymer layers was approximately 160 nm determined by a Dektak Stylus Profile System (Vecco Instruments Inc., Woodbury, NY, USA). In order to obtain polymer surfaces with different wettability PPHMDS films were further modified by ammonia plasma as the duration of the modification varied from 1 to
10 min. The process of ammonia plasma treatment was carried out at the same conditions as polymerization process. For cell experiments, the materials were sterilized in 70% ethanol for 10 min and then rinsed intensively with sterile PBS.

2.2. Contact angle measurement
Static contact angle was measured on treated polymer films by sessile drop method using Easy Drop Shape Analysis System. For each treatment two samples were tested and three droplets per sample were measured.

2.3. FTIR spectroscopy measurement
FTIR spectra of PPHMDS films were registered by Brucker-Vector 22 FTIR spectrometer in the range of 400 to 4000 cm$^{-1}$ wavelength. On the average of 64 scans of the polymer films were done with a resolution of 4 cm$^{-1}$ using OPUS software. All spectra were recorded at room temperature (RT) under standard instrument settings. The assignment of the absorption bands is based on experience with organic compounds and the literature data. The quoted wavelengths are believed to be within 2 or 3 cm$^{-1}$ of the true values.

2.4. Protein coating
Before cell seeding one set of polymer films had been pre-coated with fibronectin (FN) at a concentration of 20 μg/ml in phosphate-buffered saline (PBS) pH 7.4; the other set - with fibrinogen (FG) at concentration 50 μg/ml in PBS and the third set of materials - with vitronectin (VN) at concentration 6 μg/ml in PBS. Pre-coating of the proteins was conducted for 30 min at RT and then the samples were rinsed twice with PBS.

2.5. Cell culture
Human Umbilical Vein Endothelial Cells (HUVEC) purchased from PromoCell (Heidelberg, Germany) was used as a model system to examine the effects of surface hydrophility on biological activity of three types of adhesive proteins, including fibronectin (FN), fibrinogen (FG) and vitronectin (VN). Cells were cultured in Endothelial Cell Growth Medium (PromoCell) supplemented with Supplement Mix (PromoCell), containing 0.4% ECGS/H; 2% Fetal Calf Serum, 1 ng/ml Epidermal Growth Factor, 1 μg/ml hydrocortisone and 1 ng/ml basic fibroblast factor. The culture medium was exchanged every second day. Before the experiments the confluent cells were harvested with 0.05% Trypsin/EDTA (Invitrogen, USA), subsequently washed with culture medium and finally the cells were resuspended in serum-free medium and seeded at a concentration of 50 000 cells/ml onto PHMDS films, individually placed in tissue culture plates.

2.6. Immunofluorescence for actin and vinculin
To study the overall cell morphology and cytoskeleton of adhering HUVEC the cells were stained for actin. For that purpose, 10$^5$ cells/well were seeded in 24-well tissue culture (TC) plates (Costar, Corning, Lowell, MA, USA), containing the polymer films, pre-coated with adhesive proteins, as described above, for 2h in serum-free medium. At the end of incubation, the cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.5% Triton X-1000 for 5 min and saturated with 1% albumin in PBS for 15 min. Subsequently, to visualize actin cytoskeleton the samples were incubated for 30 min with 20 μg/ml AlexaFluor 488 phallolidin (Molecular Probes, Eugene, OR) in PBS, washed several times with PBS and finally mounted in mounting media (Mowiol) (Polysciences, Warrington, PA, USA).

To visualize focal adhesion contacts, fixed, permeabilized and saturated cells were incubated for 30 min with monoclonal anti-vinculin antibody (Sigma, Cat No V9131), dissolved in 1% albumin in PBS, followed by incubation with Cy 3-conjugated Affini-Pure Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch, Newmarket, Suffolk, UK) as a secondary antibody. After intensive washing the samples were finally mounted in Mowiol and were viewed and photographed on an inverted
fluorescent microscope (Nikon Eclipse E800; Nikon, Tokyo, Japan) where at least 3 representative images were acquired. As a positive control glass coverslips (Menzel, 15 mm in diameter) have been used.

3. Results and discussion

3.1. Surface contact angle measurements

In order to obtain information about the change in surface wettability of PPHMDS films with the time of ammonia plasma treatment, we measure the water contact angle (WCA). The results are shown on Figure 1. As can be seen from the graphic, the WCA of PPHMDS surfaces decreased rapidly with increasing of ammonia plasma duration up to 5th min meaning that the surface of polymers become more hydrophilic in a result of increased amount of amino groups generated on polymer surface. After 5th min however the process slowed down and from 5th to the 10th min only a slight decrease (less than 5 degrees) was observed. When ammonia plasma treatment exceeded 10 min, WCA reached a plateau (around 35º) and did not change anymore with the time, suggesting a saturation of the treated surfaces with ammonia groups. Therefore, we concluded that by controlling the duration of ammonia plasma treatment we can control the surface hydrophilicity of the materials.

![Figure 1. Kinetic of contact angle of PPHMDS films as a function of time of ammonia plasma treatment](image1)

![Figure 2. FTIR spectra of PPHMDS films, deposited on glass coverslips, untreated (in black) and treated with ammonia plasma 30 s (in red), 1 min (in blue) and 10 min (in violet)](image2)

3.2. FTIR spectroscopy measurements of PPHMD polymer films

The FTIR measurements were performed to compare the surface functional groups of PPHMDS layers before and after ammonia plasma modification (Figure 2). The FTIR spectra demonstrated significant changes in the surface functionality after ammonia plasma treatment. The comparison between the spectrum of untreated PPHMDS (black line) and the spectrum of treated with ammonia for 30 sec PPHMDS (red line) pointed to an existence of NH\(^+\) cations (new bands in the regions of 1410-1450 cm\(^{-1}\); and 1620-1635 cm\(^{-1}\) ) and NH\(_3\) molecules (new bands at around 3745, 3280, 3195 and 990 cm\(^{-1}\) ). An interaction between the obtained in the plasma ammonium groups with both, Si-OH and OH- groups in the polymer structure occurs as can be seen from the FTIR deconvoluted spectra in the characteristic for the polymer chain range 1200-800 cm\(^{-1}\). With increasing the time of ammonia plasma treatment from 1 to 10 min, ammonia molecules were grafted onto the surface of PPHMDS until the filling of all available polymer centres was achieved. A small shift and intensification of the bands around 1320, 1410-1450, 1630 and 1800 cm\(^{-1}\) was observed (Figure 2 lines blue, olive and violet). Another notable change was the strong decrease in intensity of the bands for methyl groups (around 600 cm\(^{-1}\) ) as well as broadening and intensification of the bands around 3750 cm\(^{-1}\).
determined the amount of grafted ammonia. Due to the reduction of the relative part of alkyl groups in the ammonia treated polymer, a decrease of the hydrophobic and an increase of the hydrophilic character of the surface were obtained. It is worth to note that in parallel experiments using KBr as substrates only slight differences were observed in all FTIR spectra. This observation confirms the importance of the CG substrate for the properties of plasma formed layer and its surface functionality. It also shows that by ammonia plasma modification, layers with novel functional groups and importantly with strongly hydrophilic properties could be obtained on CG.

3.3. Cellular interaction

Other main goal of this study was to evaluate the biological activity of thus modified PPHMDS polymer surfaces using three types of adhesive proteins e.g. FN, FG and VN, upon their adsorption to better understand how their activity is affected by the surface wettability modification. Previously, we have reported that ammonia plasma treatment of PPHMDS layers improve the proliferation and function of osteoblast cells [21]. In the present study we have used HUVE cells for the biological investigations because endothelial cells are more sensitive to any changes in substrate properties and because we wanted to understand if these modifications could affect endothelisation potential of PPHMDS layers upon implantation. As a first indicator of bioactivity, cell adhesion was investigated by monitoring the overall cell morphology and specific adhesive structures such as focal adhesion contacts, as well as the development of actin cytoskeleton. The experiments revealed that all studied PPHMDS layers adsorb FN and FG in a way (e.g. conformation) that support cellular interaction, judged by the high number of attached cells and their good spreading (Figure 3). Actin stress fibers also were well developed on both surfaces as found at higher magnification (data not shown).

![Figure 3](image-url)

**Figure 3.** Overall morphology of HUVEC, cultured for 2 hours on plain glass coverslips (a), untreated PPHMDS (b) and PPHMDS, treated with ammonia plasma for 1 min (c), 5 min (d) and 10 min (e); pre-coated with FN (upper panel), FG (middle panel) and VN (lower panel); bar 100µm
On VN-coated surfaces, however, the cells represented rather stelate morphology with actin distributed predominantly at cell borders, suggesting that VN is worst recognized by the cells. However, the increase of wettability did not change significantly cell spreading on FN and FG while on VN-coated polymer films cell spreading was significantly improved. Thus the observed differences in the adhesion behavior of HUVEC suggested that PPHMDS layers adsorbed the adhesive proteins in a different way, resulting on more or less availability of their adhesive sequences and thus affect the cell-material interactions.

Results from vinculin staining (Figure 4) showed that it was best expressed and organized in focal adhesion contacts on FN-coated materials in comparison to FG- and VN-coated samples. However, any significant differences between ammonia treated (up to the 5 min) and non-treated materials were not observed. With increasing time of NH3 treatment however, (more than 5 min) the focal adhesions formation decreased. They become smaller (spot-like) and weaker expressed. On FG- and VN-coated materials the optimum seems to be on 5 min treated samples.

**Figure 4.** Vinculin expression in HUVEC, cultured for 2 hours on plain glass coverslips (a), untreated PPHMDS (b) and PPHMDS, treated with ammonia plasma for 1 min (c), 5 min (d) and 10 min (e); pre-coated with FN (upper panel), FG (middle panel) and VN (lower panel); bar 100µm

**Conclusion**

Collectively, our results showed that surface wettability of PPHMDSO polymer films could be easy controlled by altering the duration of ammonia plasma treatment. Cell adhesion studies however revealed that the biological activity depends on the adhesive protein involved in the cellular interaction that should be taken in account in the design of new biomaterial surfaces.
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References
[1] Andrade J D and Hlady V 1986 Progress in Surface Science 79:1-64
[2] Groth T and Altankov G 1998 In: New Biomedical Materials, eds P I Harris and D Chapman (IOS Press, Amsterdam), p 12-23
[3] Grinnell F 1987 Ann. NY Acad. Sci. 56 280-290
[4] Hynes R O 1992 Cell 69 11-25
[5] Van Wachem P B, Beugeling T, Feijen J, Bantjes A, Detmers J P and Van Aken W G 1985 Biomaterials 6 403-408
[6] Lee J H and Lee H B 1993 J. Biomater. Sci. Polymer. Edn 4 467-481
[7] Yasuda H 1984 J. Membrane Sci. 18 273-284
[8] Hamman C and Kampfrath G 1984 Vacuum 34 (12) 1053-1059
[9] Sacher E, Klemberg-Sapieha J E, Schreiber H P and Wetheimer M R 1984 J. Appl. Polym. Sci., Appl. Polym. Symp. 38 163-171
[10] Sakata J and Yamamoto M 1988 J. Appl. Polym. Sci. 42 339-356
[11] Radeva E 1997 Sensors and Actuators B44 275-278
[12] Georgieva V, Radeva E and Spassov L 2000 Vacuum 5 315-320
[13] Avramov I D, Kurovska S, Rapp M, Krawczak P and Radeva E 2002 IEEE Sensors journal 2(3) 150-159
[14] Bouaidat S, Winther-Jensen B, Flygenring Christensen S and Jonsmann J 2004 Sensors and Actuators A 110 390-394
[15] Kase Y and Muguruma H 2004 Analytical Science 20 1143-1145
[16] Radeva E, Pramatarova L, Pcheva E, Hikov T, Iacob E, Vanzetti L, Dimitrova R, Krasteva N, Spassov T and Fingarova D 2010 AIP Conf. Proc. CP1203, 949-954
[17] Loughran M, Tsai S, Yokoyama K and Karube I 2003 Curr. Appl. Phys. 3 495-499
[18] Muguruma H, Kase Y and Matsumura K 2006 J. Phys. Chem. B 110 26033-26039
[19] Wei J, Yoshinari M, Takemoto S, Hattori M, Kawada E, Liu B and Oda Y 2004 J Biomed Mater Res. Part B, Applied Biomater 81 (1) 66-75
[20] Radeva E, Tsankov D, Bobev K and Spassov L 1993 J. Appl. Polym. Sci. 50 165-171
[21] Krasteva N et all 2010 AIP Conf. Proc. CP1203 688-693