In Vitro Leukocyte Adhesion to Modified Polyurethane Surfaces

II. Effect of Wettability

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The influence of substrate wettability on leukocyte adhesion was studied using a series of polyurethane films with different surface wettabilities, prepared by a two step gas plasma modification procedure. In the first step the films were made hydrophobic by exposure to a tetrafluoromethane plasma. In the second step hydrophilicity was gradually increased by exposure to an argon plasma. XPS analysis of these films demonstrated that the first step resulted in the incorporation of fluor into the film surface (~50 At.-%). In the second step fluor was almost completely removed from the surface, while the surface oxygen concentration increased from 5 to 20 At.-%. The wettability of the modified films, as characterized by water contact angle measurements, increased as a function of the argon treatment time. The advancing/receding contact angles (Wilhelmy plate method) decreased from 128/83 to 50/2 degrees respectively. *In vitro* adhesion of granulocytes and lymphocytes to the modified surfaces was studied under static conditions as a function of time. For both types of leukocytes it was found that the number of cells which adhered to the surface within a certain incubation time increased when the surface became more hydrophilic. The number of adherent granulocytes was about 2-3 times higher than the number of adherent lymphocytes. The number of adherent cells which could be predicted from the relationship between leukocyte adhesion and the wettability of the substrates within the series of homologous surfaces corresponded with the number of adherent cells to control surfaces (untreated polyurethane and Teflon). © 1994 Academic Press, Inc.

INTRODUCTION

Leukocyte adhesion plays a predominant role in leukocyte filtration, which is applied clinically to remove these cells from blood (1). In order to optimize filters for this purpose, e.g., leukocyte filters, a relation between the physicochemical properties of material surfaces and the adhesion of leukocytes should be known. Relevant reports in the literature mostly

deal with the *in vivo* adhesion of leukocytes, which usually accompanies the inflammatory response following implantation of artificial materials (2-4). More specific studies on *in vitro* leukocyte adhesion are often limited to either one type of substrate (5-8) or one particular subtype of leukocytes, mostly granulocytes (7-11). Therefore, more systematic studies on the reactivity of leukocytes toward artificial surfaces are required.

Previously we have reported the effect of ionizable functional groups, incorporated into polyurethane (PU) surfaces, on leukocyte adhesion (12). It appeared that both amine groups and carboxylic acid groups enhance the adhesion of leukocytes. However, the mechanism of the cell-substrate interaction was not fully clear. Surface wettability, which is associated with the surface free energy (13), has often been related to cell adhesion phenomena (14-18), but since different types of materials were compared, the effect of chemical composition may have interfered with the effect of wettability. In order to study leukocyte adhesion as a function of surface wettability it is preferable to compare a chemically homologous series of substrates in order to minimize the contribution of specific interactions between adherent cells and chemical groups at the solid surface.

Since surface wettability can be independently changed from the bulk properties of the material, surface modification of a given material is a preferred method to obtain such homologous series. Gas plasma, or glow discharge, treatment is currently used for surface modification of materials, particularly to improve either the hydrophobicity (19, 20) or the hydrophilicity (21, 22) of substrates. Gas plasma modification of polymer surfaces has been used to gradually increase the wettability of hydrophobic substrates in order to study the effect of wettability on the adhesion of endothelial cells (23, 24). However, this strategy is not applicable for moderately wettable substrates such as PU, since the wettabilities will then range from moderate to complete.

In the present study a combination of two plasma modification techniques has been used to vary the wettability of

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PU substrates over a relatively wide range. For this purpose PU films were first made hydrophobic by pretreatment with a tetrafluoromethane (CF_4) plasma, followed by treatment with an argon (Ar) plasma to achieve a defined wettability. PU was taken as the polymer substrate, since it was previously used in related studies (1, 12). By changing the Ar treatment time, a series of PU substrates with different surface wettabilities were prepared. These substrates were subjected to leukocyte adhesion studies. To exclude effects like complement activation (25, 26), platelet adhesion (11, 27), and interference of various plasma proteins (8, 28), we used an *in vitro* adhesion assay in which film surfaces were exposed under static conditions to purified leukocyte populations, suspended in phosphate buffered synthetic medium.

Polyurethane Films

Polyurethane (PU) films with a thickness of 0.3 mm were prepared from a solution of medical grade Pellethane 2363-80AE (Dow Chemical Nederland BV, Delfzijl, the Netherlands). To remove low molecular weight fractions, the polymer was first purified. A 5% (w/v) solution of PU in dimethylformamide was slowly added to a 10-fold excess of water while stirring at high speed. The precipitate was dried *in vacuo* at 60°C. Films were prepared by pouring a 10% (w/v) solution of the purified PU in tetrahydrofuran into large petri dishes, after which the solvent was allowed to slowly evaporate at room temperature. The smooth side of the PU film, originating from contact of the solution with the glass surface of the dishes, was used for leukocyte adhesion studies. The films were washed with organic solvents (analytical grade) to remove contaminating surface active agents. This washing procedure involved twofold sonication for 15 min in cyclohexane, followed by twofold sonication for 15 min in ethanol. The washed films were then dried *in vacuo* and stored dry in the dark at room temperature until use.

Surface Modification

Two successive gas plasma treatments were used to alter the wettability of the PU films. These treatments were performed in a system which consisted of a glass reactor tube (volume approximately 3 liters) connected to a vacuum system (Balzers) and three externally capacitively coupled electrodes to generate plasmas by a 13.56 MHz radiofrequency generator (ENI Power Systems). The set-up is schematically depicted in Fig. 1.

In the first step, PU films were made hydrophobic by treatment with a tetrafluoromethane (CF_4) plasma. PU films (4 pieces, 2.5×7.5 cm each) were centered in the reactor, which was subsequently rinsed (0.2 mbar, 10 ml/min) with

FIG. 1. Schematic representation of the gas plasma modification system; CF_4 = tetrafluoromethane gas supply, Ar = argon gas supply, MN = matching network, P = pressure sensor, and FC1/FC2 = mass flow controllers. The PU films are placed in the center of the reactor.

CF_4 gas (Freon 14, $\text{CF}_4 > 99.95\%$, Hoekloos, Schiedam, the Netherlands). After 10 min a CF_4 plasma (75 W) was generated for typically 35 min. After rinsing with CF_4 for 2 min, the films were removed from the reactor and stored dry until further treatment.

In the second step, the wettability of the hydrophobic films was gradually improved by exposure to an argon (Ar) plasma, analogous to the method used for CF_4 treatment. After placement of the CF_4 pretreated PU films in the reactor, the reactor was subsequently rinsed (0.2 mbar, 10 ml/min) for 10 min with Ar gas (Ar $> 99.999\%$, Hoekloos, Schiedam, The Netherlands). An Ar plasma (75 W) was then applied for varying periods of time up to 100 s. Relatively short Ar treatment, typically less than 10 s, was achieved by using a pulse generator (Wavetek, model 183), connected to the radiofrequency generator. After rinsing with Ar for 2 min, the films were removed from the reactor and stored dry until use.

Surface Characterization

Wettability. To characterize both sides of the films separately, water contact angles were determined at room temperature using the captive bubble method (29) and the sessile drop method (30). Drops of ultrapure water and air bubbles in ultrapure water, both with an approximate diameter of 2–3 mm, were photographed within 30 s after contact with the film surface at room temperature. The contact angles were directly measured on the photographs using a goniometer. Contact angles were also determined with a dynamic method, the Wilhelmy plate method (31). Samples both sides

of which were identically modified were immersed in ultrapure water at a rate of 1.1 cm/min. The apparent sample weight as a function of immersion depth was measured with an electrobalance. From the wetting curves obtained, the advancing and receding contact angles were calculated (31).

Surface composition. X-ray photo electron spectroscopy (XPS) (32) was used to analyze the elemental composition of the film surfaces. XPS measurements were performed with a Kratos XSAM 800 (Kratos analytical, Manchester, UK), using X-rays from a $MgK\alpha$ source (15 kV/15 mA) at a takeoff angle of 90° between the film surface and the analyzer. Under these conditions, the detection depth was about 70 Å. For quantitative analysis, detailed scans were made of C1s, F1s, O1s, N1s, and Si2p peaks (diameter of the spot size 3 mm). Empirically derived sensitivity factors (supplied by Kratos) were used to convert the peak areas in surface elemental composition. Fluor concentrations were corrected using cleaned Teflon (Fluoroplast, Raamsdonkveen, the Netherlands) as a reference. Each surface was characterized at least *in duplo*.

Leukocytes

Leukocytes were isolated from freshly collected citrated human blood (33). One unit (500 ml) of blood was centrifuged at 400g for 5 min, followed by separation of the leukocyte-rich interface (buffy coat) from the plasma layer and the packed red cells. The buffy coat was diluted with phosphate-buffered sodium chloride solution (PBS, pH 7.4) containing 13 mM trisodium citrate and 0.5% (w/v) human albumin ("medium-G," 290–295 mOsm, pH 7.2–7.4, and surface tension 50.7 mN/m at room temperature) to a final volume of 200 ml, and layered on a Percoll suspension (Pharmacia, Uppsala, Sweden) with a specific gravity of 1.077 g/ml. Leukocytes were fractionated at room temperature by centrifugation for 20 min at 1000g.

The lymphocyte rich layer present on top of the Percoll suspension was collected. To remove residual platelets, medium-G was added to the cells, and the resulting suspension was centrifuged at room temperature for 5 min at 400g. The pellet was resuspended in medium-G. This procedure was repeated three times. Contaminating monocytes were removed by putting the cell suspension in polystyrene tissue culture flasks for 1 h at 37°C . The supernatant was decanted and centrifuged at 400g. The lymphocyte-containing pellet was resuspended in medium-G (see below).

To prepare granulocyte suspensions, cells from the pellet formed below the Percoll layer were collected. Red cells were lysed by resuspension in ice-cold ammonium chloride medium (155 mM NH_4Cl , 10 mM KHCO_3 , and 0.1 mM EDTA, 290–295 mOsm, pH 7.2–7.4), which was gently shaken for 20 min while kept on ice. Red cell stroma and hemoglobin were removed by centrifugation of the cell suspension at 4°C for 5 min at 400g. The granulocyte containing

pellet was resuspended in medium-G. This procedure was repeated three times.

All cell suspensions were adjusted to 1.0×10^6 cells/ml in medium-G. Leukocyte counts and the purity of cell suspensions were determined using a flow cytometer (Technicon, type H1). Lymphocyte suspensions had a purity of at least 91%; monocytes were the main contaminants. Granulocyte suspensions were 92–95% pure. The viability of the cell suspensions was more than 97%, as determined by trypan blue exclusion (34). Similar results were obtained by using the acridine orange/ethidium bromide method (35). Before being used in adhesion experiments, leukocyte suspensions were kept at room temperature for no longer than 1 h. During storage, the cell viability did not significantly change.

Leukocyte Adhesion

Leukocyte adhesion experiments were performed with PU films, Teflon, and a series of modified PU films. Films were mounted in Teflon test devices (four wells, with a test surface of 1.2 cm^2 per well), essentially as previously described (14). Each well was first rinsed with 1.0 ml of PBS, and subsequently filled with 1.0 ml of leukocyte suspension (1.0×10^6 cells/ml). Cell suspensions were incubated with the surfaces for 5, 15, and 30 min at 37°C . After incubation, non-adherent cells were removed by washing five times with PBS (0.8 ml/wash). Excessive shear stresses on adherent cells were avoided by leaving 0.2 ml of supernatant in the well after each wash. After the final wash adherent cells were fixed by replacing 0.8 ml of the supernatant with 0.8 ml of 2% glutaraldehyde in PBS. After 10 min the glutaraldehyde solution was removed. Thereafter the films were detached from the test devices and dried in air.

Adherent leukocytes were stained by immersion in a hematoxylin solution (Delafield; Merck, Darmstadt, Germany) for 10 min, followed by drying in air. Using a light microscope, two areas of 0.1 to 0.4 mm^2 were chosen at random and cell counts were performed. It was previously demonstrated that the number of adherent cells did not significantly depend on the exact location of these spots. Consequently, in each experiment, the individual countings in four wells were averaged and expressed as the number of adherent cells per unit surface area. Each experiment was repeated four times.

Scanning Electron Microscopy (SEM)

After adhesion experiments, small samples of the films were prepared for scanning electron microscopy (SEM). Immediately after fixation with glutaraldehyde, the samples were dehydrated by 10 min immersion in successively 25, 50, and 75% ethanol in water, and twice in 98% ethanol. After drying in a vacuum desiccator the samples were sputter-coated with gold and examined by means of a JSM-35 CF

FIG. 2. Effect of tetrafluoromethane (CF_4) plasma treatment time on the wettability of PU films. Receding (open circles) and advancing water contact angles (closed circles) were measured using the Wilhelmy plate method (mean $\pm$ SD, $n = 4$).

scanning electron microscope (Japan Electron Optics Laboratory) using a 15 kV accelerating voltage.

RESULTS

Dynamic contact angle measurements revealed that the hydrophobicity of PU films increased during CF_4 plasma treatment (Fig. 2). XPS analysis proved that this increased hydrophobicity is related to the incorporation of fluor and loss of oxygen atoms in the PU surface layer (Table 1). After

35 min of exposure to the CF_4 plasma, a level of hydrophobicity was reached which approached the hydrophobicity of a Teflon control surface, suggesting a completely fluorinated surface (see Table 1). Analysis of the C1s peak in the XPS spectrum of the CF_4 plasma pretreated film revealed that CF, CF_2 , and CF_3 groups were present in the film surface in a ratio of approximately 2:2:1.

The surface wettability of the films increased during Ar plasma treatment of the CF_4 plasma pretreated PU surfaces. Figure 3 shows a decrease of contact angles as a function of the Ar plasma treatment time. Although the absolute data of the different contact angle measurements differ significantly, each method demonstrates the same tendency. The reproducibility of the modification procedure was good for separately prepared film samples, which is demonstrated by the relatively small standard deviation of the contact angles (Table 1). Static contact angle measurements on both sides of identically modified films confirmed that contact angles on both sides were comparable. This means that the Wilhelmy plate method can be used to measure contact angles, since this method yields an average contact angle of both sides of the tested film. Measured with the Wilhelmy plate method, the receding contact angle of the substrates gradually decreased from 80° to almost 0° after 100 s, indicating complete wettability. Within this wide range, a moderate wettability of untreated PU films was found. During the Ar plasma treatment, the chemical composition of the film surface gradually changed (Table 1). Figure 4 shows that after 100 s of exposure to the Ar plasma, almost no fluor could be detected in the CF_4 plasma pretreated PU film surfaces.

TABLE 1
Surface Characterization of Modified and Control Film Surfaces

Sample ^a	Water contact angle ^b [deg]				Relative surface elemental composition ^c [at.%]				
	θ_a (WP)	θ_r (WP)	SD	CB	C	F	O	N	Others
PU	92 $\pm$ 1	42 $\pm$ 1	77 $\pm$ 5	64 $\pm$ 3	78 $\pm$ 1	n.d.	19 $\pm$ 1	3 $\pm$ 1	0.3 (Si)
Teflon	130 $\pm$ 1	87 $\pm$ 3	108 $\pm$ 4	96 $\pm$ 5	33 $\pm$ 1	67 $\pm$ 1	n.d.	n.d.	n.d.
Ar0	128 $\pm$ 6	83 $\pm$ 4	104 $\pm$ 3	89 $\pm$ 2	43 $\pm$ 2	50 $\pm$ 3	5 $\pm$ 1	2 $\pm$ 1	n.d.
Ar0.005	131 $\pm$ 5	81 $\pm$ 3	106 $\pm$ 2	88 $\pm$ 5	50 $\pm$ 3	39 $\pm$ 2	9 $\pm$ 1	2 $\pm$ 1	n.d.
Ar0.01	133 $\pm$ 3	72 $\pm$ 5	105 $\pm$ 2	87 $\pm$ 7	45 $\pm$ 2	47 $\pm$ 4	6 $\pm$ 2	2 $\pm$ 1	n.d.
Ar0.05	127 $\pm$ 4	80 $\pm$ 2	105 $\pm$ 3	86 $\pm$ 2	44 $\pm$ 2	47 $\pm$ 5	7 $\pm$ 3	2 $\pm$ 1	n.d.
Ar0.5	126 $\pm$ 6	53 $\pm$ 6	102 $\pm$ 3	57 $\pm$ 9	44 $\pm$ 2	46 $\pm$ 2	7 $\pm$ 1	2 $\pm$ 1	n.d.
Ar2.5	109 $\pm$ 13	15 $\pm$ 9	83 $\pm$ 4	45 $\pm$ 3	52 $\pm$ 5	34 $\pm$ 9	12 $\pm$ 1	3 $\pm$ 1	n.d.
Ar5	96 $\pm$ 8	14 $\pm$ 11	59 $\pm$ 2	34 $\pm$ 5	46 $\pm$ 7	42 $\pm$ 9	9 $\pm$ 1	2 $\pm$ 1	n.d.
Ar25	61 $\pm$ 7	1 $\pm$ 1	58 $\pm$ 4	36 $\pm$ 3	56 $\pm$ 1	26 $\pm$ 1	14 $\pm$ 1	4 $\pm$ 1	n.d.
Ar50	56 $\pm$ 4	2 $\pm$ 3	47 $\pm$ 5	32 $\pm$ 5	68 $\pm$ 1	7 $\pm$ 2	21 $\pm$ 2	5 $\pm$ 1	n.d.
Ar100	50 $\pm$ 1	2 $\pm$ 3	49 $\pm$ 2	32 $\pm$ 3	74 $\pm$ 1	1 $\pm$ 1	20 $\pm$ 1	5 $\pm$ 1	n.d.

^a Sample codes refer to the Argon (Ar) treatment time in the second plasma modification step, see methods. Thus, the Ar0 sample has only been exposed for 35 min to the CF_4 plasma, and not to the Ar plasma. Consequently, the Ar0.005 sample is exposed for 0.005 s to the Ar plasma, after pretreatment for 35 min with the CF_4 plasma, etc.

^b Contact angles (mean $\pm$ SD) were measured using Wilhelmy plate (WP, $n = 4$), sessile drop (SD, $n = 10$), and captive bubble (CB, $n = 10$) methods.

^c Relative elemental composition according to XPS measurements (mean $\pm$ SD, $n \geq 2$). In some cases, specific elements were not identified in a wide scan spectrum, and were therefore not determined (n.d.) by detail scanning. Detection limits are generally less than 0.3%.

FIG. 3. Effect of argon (Ar) plasma treatment time (log scale) on the wettability of CF_4 plasma pretreated PU films. Receding contact angles (open circles) and advancing contact angles (closed circles) were determined by the Wilhelmy plate method. Contact angles were also measured by the captive bubble method (open squares) and sessile drop method (closed squares). The separated data points on the left show corresponding control values for untreated PU and Teflon.

Simultaneously, the surface concentration of carbon, oxygen, and nitrogen increased. Significant traces of other elements could not be detected on the modified films. In a separate experiment, the wettability and the surface composition of the modified films were also determined after approximately 8 weeks of storage and subsequent washing with PBS and water. It appeared that the physicochemical properties of the modified films had not significantly changed during this period, suggesting that the surface properties of the modified surfaces did not change during the experiments.

From the entire range of substrates with different wettability, five substrates were tested with respect to leukocyte adhesion. Untreated PU and Teflon were taken as control substrates. In Table 2 the extent of leukocyte adhesion to each substrate as a function of exposure time is summarized. In Figs. 5 and 6 these results are presented as a function of substrate wettability. In general, the number of adherent cells increased with increasing substrate wettability, measured with the sessile drop method. It should be emphasized that untreated PU and Teflon qualitatively fit into this relation, although they do not belong to the homologous series of modified films. Although many more granulocytes (Fig. 5) adhered to the substrates than lymphocytes (Fig. 6), the tendency with respect to substrate wettability was similar for both cell types.

Scanning electron microscopic examination of adherent leukocytes (Fig. 7) revealed that adherent granulocytes tended to spread on the substrate surface when this surface becomes more hydrophilic. Lymphocytes did not demonstrate this tendency. In general they maintained their spherical native shape, although they sometimes formed small pseudopods (Fig. 7.II.c).

DISCUSSION

The present study was undertaken to investigate the effect of gas plasma modification of PU film surfaces on the extent of leukocyte adhesion to these films. Since our aim was to relate leukocyte adhesion to the wettability of the modified substrates, the effect of the gas plasma treatment on the physicochemical properties of the film surfaces formed an essential part of this study.

Surface Modification

Using the two-step gas plasma modification procedure, we prepared a homologous series of PU films with a gradual increase of surface wettability (Fig. 3). Plasma derivatization of PU surfaces is generally complicated by polymer mobility and surface rearrangements (36, 37). Rearrangements may annul the effect of plasma modification, as derivatized PU chains may slowly disappear from the surface into the bulk material (36). For this reason the modified film surfaces were characterized before and after two months of storage. We found that the surface composition and the wettability of the modified films did not change during this period. The interpretation of contact angle measurements to quantify surface wettability was, however, complicated by substantial contact angle hysteresis. Rearrangements at PU surfaces generally lead to large differences between the advancing and the receding contact angle (38). Since the process of surface rearrangement is time-dependent, contact angle hysteresis will be smaller when static instead of dynamic methods are used to measure contact angles (31). This explains the differences between statically and dynamically determined contact angles of the surfaces in our study (Fig. 3). Also the influence of surface roughness on contact angle hysteresis

FIG. 4. Effect of argon (Ar) plasma treatment time (log scale) on the relative surface elemental composition of CF_4 plasma pretreated PU films, according to XPS; fluor (closed circles), oxygen (closed squares), and nitrogen (open circles). The separated data points on the left show corresponding control values for untreated PU and Teflon respectively.

TABLE 2
In Vitro Adhesion of Purified Leukocytes after Exposure for 5, 15, and 30 min to a Series of Substrates, which Differ in Wettability

Substrate ^a	Adherent granulocytes ^b [mm ⁻²]			Adherent lymphocytes ^b [mm ⁻²]		
	5 min	15 min	30 min	5 min	15 min	30 min
PU	281 ± 65	804 ± 452	1720 ± 597	103 ± 49	231 ± 86	466 ± 154
Teflon	117 ± 39	273 ± 87	525 ± 322	44 ± 10	133 ± 48	216 ± 83
Ar0	155 ± 95	350 ± 104	484 ± 138	73 ± 12	186 ± 81	270 ± 62
Ar0.01	133 ± 36	394 ± 103	504 ± 121	48 ± 27	141 ± 38	234 ± 97
Ar2.5	289 ± 58	574 ± 177	703 ± 187	96 ± 59	243 ± 49	386 ± 116
Ar25	476 ± 111	1001 ± 132	2565 ± 559	223 ± 122	456 ± 132	780 ± 203
Ar100	586 ± 22	1135 ± 289	3240 ± 638	163 ± 71	340 ± 151	891 ± 283

^a For sample codes see Table 1.

^b Mean number of adherent cells ± SD (*n* = 4).

(39) should be taken into account, although SEM of the films demonstrated that the surfaces were relatively smooth (Fig. 7). Using SEM, we observed substantial surface roughness in the case of Teflon films. This finding probably explains the occurrence of contact angle hysteresis for these surfaces. It is not clear which angle has to be used when cell adhesion is related to surface wettability. Considering that the cells adhere to a surface in an aqueous environment, it seems adequate to use the captive bubble or the receding contact angle. However, during measurement of these angles the solid surface becomes exposed to air and rearrangements at the surface may occur. It is unlikely that such rearrangements will also occur during cell adhesion, as the leukocyte membrane contains many polar groups and the surface tension of leukocytes (granulocytes, 69.0 mN/m (16)) is comparable to water (72.1 mN/m (31)). From this viewpoint,

the sessile drop and the advancing contact angle are also meaningful. In the present study it turned out that the relation between leukocyte adhesion and surface wettability was well described by the sessile drop angle.

Not only the surface wettability but also the surface composition of the modified films changed as a result of the plasma treatment (Fig. 4). Fluor was incorporated into the PU films during CF₄ plasma pretreatment. XPS analysis of the CF₄ plasma pretreated films revealed that CF, CF₂, and CF₃ groups were incorporated into the PU surface at a ratio of approximately 2:2:1. The total C/F atomic ratio was about 1:1. These results agree with those obtained by Strobel *et al.* for CF₄ plasma modified polypropylene surfaces (40). They reported that CF₄ plasma reacts with the polymer surface according to a grafting mechanism, thus forming a completely fluorinated surface layer with a thickness of several

FIG. 5. *In vitro* adhesion of granulocytes, after exposure for 5, 15, and 30 min at 37°C to modified as well as unmodified PU and teflon, arranged in sequences with increasing wettability, according to the sessile drop measurement (sample codes see Table 1). Bars show the mean number of adherent cells from four independent measurements, for SD see Table 2.

FIG. 6. *In vitro* adhesion of lymphocytes, after exposure for 5, 15, and 30 min at 37°C to modified as well as unmodified PU and Teflon, arranged in sequences with increasing wettability, according to the sessile drop measurement (sample codes see Table 1). Bars show the mean number of adherent cells from four independent measurements, for SD see Table 2.

FIG. 7. Typical SEM micrographs of adherent granulocytes (I) and lymphocytes (II) after 30 min exposure at 37°C to modified and unmodified PU, differing in wettability. In each series hydrophobic PU-Ar0 (a), untreated PU (b), and hydrophilic PU-Ar100 (c), are shown, respectively, from top to bottom. Stage tilt 60°, original magnification $\times 2000$. For sample codes see Table 1.

monolayers. It is likely that the same surface structure has been formed on the films used in our study. We have removed the fluorinated layer by surface etching using an Ar plasma. Surface etching by the Ar plasma is clearly dem-

onstrated by the surface elemental composition (Fig. 4) which shows a gradual decrease of the surface fluor concentration with increasing Ar plasma treatment time. The simultaneous increase of the surface oxygen concentration may

either be due to the disclosure of the underlying PU layer or to the incorporation of oxygen during post-plasma reactions in the plasma treated films. The incorporation of oxygen into Ar plasma treated surfaces is generally explained by the formation of long living free radicals at the film surface due to UV emission of the Ar plasma (41). When the films are subsequently exposed to air, these radicals react with atmospheric oxygen and form peroxides which may further decompose to a variety of oxidation products ranging from alcohols to carboxylic acids (42). Although the chemical composition of such oxidized surfaces is often unknown, most of the incorporated oxygen has been ascribed to carbonyl groups (43, 44). Youxian *et al.* studied the effect of Ar plasmas on the wettability of Teflon films (22). Although they reported a significant improvement of the surface wettability, they did not detect a marked increase of surface oxygen content, which was explained by the occurrence of rapid intramolecular radical reactions preventing radical reactions with air molecules. If such a mechanism also governs the present surface modification, the number of oxygen containing functional groups introduced after Ar plasma treatment may be small. If, however, postplasma reactions of surface radicals with atmospheric species do occur, a variety of oxygen containing groups may have been incorporated into the film surfaces. In this case it is precarious to explain leukocyte adhesion to the modified surfaces only in terms of wettability.

Leukocyte Adhesion

In order to study leukocyte adhesion to the modified surfaces without the interference of various plasma proteins, leukocytes were isolated from blood and resuspended in a plasma-free synthetic medium. It was, however, inevitable to add a small amount of albumin to the medium because a protein is necessary to protect cells against mechanical damage (33). During adhesion experiments the albumin will immediately adsorb to the film surface (45, 46), thereby altering the surface to which leukocytes will subsequently adhere. Strictly taken, the characteristics of the albumin-coated surface rather than the well-characterized bare polymer surface determine the extent of leukocyte adhesion. Since the concentration of albumin in the cell suspension medium is as high as 5 mg/ml and the experiments were performed under static conditions, monolayer coverage may be established within a few minutes (47, 48). It is very difficult, however, to predict the exact amount and the conformational state of the adsorbed protein molecules in the course of the leukocyte adhesion process. The characterization of protein coated surfaces is not a trivial task since standard techniques such as XPS and the Wilhelmy plate method employ the exposure of the protein coated films to an air surface or require other protein denaturing pretreatments (49). In this manner these techniques would lead to mis-

leading results which do not represent the *in situ* conditions during cell adhesion experiments. Since the present study was undertaken in the context of leukocyte filter optimization we have left the specific effects of albumin adsorption out of consideration. Absolom *et al.* have reported that for a series of polymer surfaces the most hydrophobic surfaces remain relatively more hydrophobic than the initially more hydrophilic surfaces (50, 51). It is thus very likely that the sequential series of substrates with increasing wettability used in our study will not be altered after albumin adsorption. The albumin can be regarded as an intermediate between the solid surface and the adhering leukocytes. Preadsorbed albumin is known to reduce the extent of leukocyte adhesion to solid surfaces (8, 28). Moreover, albumin generally adsorbs faster and stronger to more hydrophobic surfaces (45, 46). Thus, overall, cell adhesion is directly influenced by the hydrophilicity of the substrate, albeit through a layer of adsorbed albumin.

When leukocyte adhesion to the modified surfaces is studied in the context of leukocyte filtration, it is important to distinguish between the adhesion behavior of different subpopulations of leukocytes since their adhesiveness to polymer surfaces may differ substantially (12). In the present study we investigated the adhesion of purified granulocytes and lymphocytes. It was found that the adhesion of both cell types to the modified PU surfaces increased when the surface became more hydrophilic (Figs. 5 and 6). However, on each tested surface the number of adherent lymphocytes was substantially lower than the number of adherent granulocytes. This low adhesiveness of lymphocytes with respect to polymer surfaces as compared to the adhesion of granulocytes is also mentioned in literature (52, 53). Differences between the adhesiveness of granulocytes and lymphocytes also appeared from the morphology of adherent cells (Fig. 7). Granulocytes tended to spread at hydrophilic surfaces whereas this tendency was less pronounced for lymphocytes. Cell spreading is generally associated with the extent of cell adhesion (53), although the exact relation between these phenomena is not fully clear (54).

Our results confirm the widely accepted concept that hydrophilic surfaces enhance the adhesion of leukocytes (10, 52, 53). Contradictory results were recently reported by Lim and Cooper, who studied leukocyte adhesion onto surfaces of a series of PUs differing in wettability (55). They reported that the highest number of adherent granulocytes was found on the most hydrophobic surface. In their study, different surface wettabilities were achieved by changing the soft segment composition of the PU. Changing the polymer composition may, however, also change the phase separated nature of the PU surface, which may influence the extent of leukocyte adhesion. Besides, their strategy limits the feasible range of surface wettabilities, which was small as compared to the range used in the present study.

To explain the increase of leukocyte adhesion to surfaces

with increasing wettability, Neumann *et al.* have used a thermodynamic model, in which the free energy resulting from adhesion is related to the surface free energy of the solid surface (16, 17). Although our results qualitatively correspond with the results of their model, this model cannot be applied here since it requires a state of thermodynamical equilibrium, which is clearly not observed in the present study. As we studied leukocyte adhesion in the context of leukocyte filtration, our particular strategy was to count adherent cells after relatively short exposure times, i.e., up to 30 min. Within this period of time the number of adherent cells to the surface had not yet reached a plateau value. Using Stokes law (56), it was calculated that leukocyte adhesion was not restricted by the sedimentation rate of leukocytes in the test device. The different rates of leukocyte adhesion to the film surfaces should therefore be explained in terms of kinetics.

The DLVO theory, which generally describes the free energy of a colloidal particle as a function of the interparticle distance, has often been used as a qualitative guide to predict the rate of cell deposition to different surfaces (18, 57–60). In this theory the deposition rate of cells to a solid surface is defined by the presence and the height of an energy barrier, which principally depends on the balance of electrostatic interactions and van der Waals–London interactions between the surface and the cell. When it is assumed that the zeta potential of the PU surface is not significantly changed by the modification procedure, the electrostatic interactions between leukocytes and the film surface will be practically the same for each surface tested. We have previously demonstrated that the zeta potential of PU films, i.e., -32 mV, did not significantly change when ionizable groups such as carboxylic acid and amine groups were incorporated into the film surface (12). Dekker reported that modification of Teflon surfaces using oxygen and nitrogen gas plasmas did not significantly change the zeta potential of the substrates (24). It is reasonable to assume that van der Waals–London interactions between the cells and the film surface are also not significantly changed by the modified surface layer (58). The energy barrier for cells to overcome will then be practically the same for all surfaces tested, and in this case the DLVO theory cannot explain our results. However, the DLVO theory was originally derived using boundary conditions which presume long range interactions, i.e., distances of separation above ~ 20 Å (58). In order to explain the reapeptization behavior of flocculated sols, Frens and Overbeek showed that the DLVO theory needs modification when two colloidal particles approach to a closer distance, and their charged double layers start to overlap (61). During the collision, the charge in the double layers cannot generally be adjusted fast enough to maintain electrochemical equilibrium at the particle surface. As a consequence the surface potential increases until equilibrium is restored by charge transfer. Until this

time the increased surface potential attributes to an increased repulsion between the particles, thus facilitating the redispersion of the particles. It is likely that cell adhesion to solid surfaces is governed by a similar process. As charge transfer will principally be faster in the case of hydrophilic surfaces compared to hydrophobic surfaces, relatively few cells will desorb from the more hydrophilic surfaces during the initial stage of the adhesion process. This may explain that the rate of leukocyte adhesion increases with the substrate wettability.

Also the effect of short range interactions such as hydrogen bonding and dipole interactions should be taken into account. Specific interaction between the cells and the substrates may be due to presence of functional groups, introduced by the surface modification procedure. In this respect it should be noted that surfaces with oxygen-containing groups like hydroxyl groups (9, 62), carbonyl groups (63), and carboxylic acid groups (12, 64) have been reported to enhance cell adhesion. There is evidence, however, that the presence of such groups at the modified surfaces do not practically interfere with the effect of wettability on leukocyte adhesion. Unmodified PU and Teflon, which served as control surfaces in this study, do not belong to the homologous series of substrates used to study leukocyte adhesion as a function of substrate wettability. The composition of these control surfaces is therefore chemically different as compared to the gas plasma modified surfaces. Nevertheless, the extent of leukocyte adhesion to the control surfaces clearly followed the trend with respect to surface wettability, which was found for leukocyte adhesion to the gas plasma modified surfaces (Figs. 5 and 6). This finding supports the assumption that leukocyte adhesion to solid surfaces can generally be related to the wettability of the solid surface.

SUMMARY

In conclusion, leukocyte adhesion to modified PU surfaces was studied as a function of surface wettability. It was found that the number of cells adherent to the surface increased with increasing surface hydrophilicity. These results can be theoretically explained when it is assumed that the surface wettability of the PU surface was altered without changing the surface charge or introducing significant amounts of specific groups in the surface layer which promote leukocyte adhesion. Several practical limitations, however, complicate a quantitative explanation. Since the number of adherent cells to untreated PU is not very large, as was also predicted by the observed relation between leukocyte adhesion and surface wettability, the present modification procedures may be applied to the development of biomaterials, either to inhibit or to improve leukocyte adhesion. It should be noted, however, that the effects of plasma proteins, platelets, hematocrit, divalent ions, and flow conditions have to be further evaluated.

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