In vitro leucocyte adhesion to modified polyurethane surfaces

I. Effect of ionizable functional groups

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To study the effect of ionizable functional groups on the adhesion of leucocytes to surfaces, both poly(ethyleneimine) and poly(acrylic acid) were immobilized on polyurethane films, resulting in the introduction of amine and carboxylic acid groups, respectively. This was confirmed by contact angle measurements and XPS analysis. *In vitro* adhesion of granulocytes and lymphocytes on untreated and modified surfaces was compared. The number of adherent cells on modified surfaces as a function of time was significantly higher than on untreated surfaces. This effect was most pronounced for the adhesion of lymphocytes to surfaces modified with amine groups. In this case, the number of adherent cells after 1 h of exposure was three times higher than on untreated surfaces. A moderate enhancement of leucocyte adhesion was observed in the case of surfaces modified with carboxylic acid groups. There is evidence that these groups were not ionized under the experimental conditions used. The modification procedures described may be used to improve polyurethane filters for the removal of leucocytes from blood.

Keywords: Cell adhesion, polyurethanes, leucocytes, surface modification

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Adhesion of leucocytes to artificial surfaces is an important topic in the biological evaluation of biomaterials. The number of leucocytes adherent to biomaterials which have been implanted is often related to the inflammatory response[1,2]. Leucocyte adhesion may, however, also improve the biocompatibility of biomaterials. For example, leucocytes adherent to arterial implants may inhibit thrombus formation[3]. Leucocyte adhesion to polymeric surfaces is essential for leucocyte filtration. This technique is clinically applied to obtain leucocyte poor cell concentrates for transfusion[4]. It has been suggested that the adhesive properties of the filter material augment the filtration process in the removal of leucocytes[5-8]. However, this hypothesis has never been rigorously tested since the observations were based on studies using only one particular type of filter. To demonstrate the relationship between leucocyte adhesion and leucocyte removal, structurally equivalent filters which have different surface properties with respect to leucocyte adhesion should be compared. Such model filters may be prepared by surface modification of leucocyte filter materials with a well-defined porous structure, such as polyurethane (PU) membranes[8].

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Several factors may be involved in the adhesion of leucocytes to solid surfaces. These include surface free energy[9], wettability or surface hydrophilicity[10], surface charge[10,11], surface chemistry[3,12,13], protein adsorption[14-17], activation of the complement system[18] and adhesion of other cells such as platelets[19]. Furthermore, extrinsic factors such as cell concentration[15], shear stress[15], the presence of divalent ions[15], anticoagulants[16], duration of incubation[17], temperature[20] and cell age[21] are involved. Since most of these factors are related, it is difficult to study the effect of a single parameter. The present study has been focused on the effect of ionizable functional groups at the PU surface on the adhesion of leucocytes.

Since leucocytes bear a negative charge, resulting from their lipoprotein membrane structure[10,22], electrostatic forces may influence cell adhesion. It has been reported that negatively ionized groups, like carboxylic acid[12,13] and sulphonate[12,23] groups reduce cell adhesion, and that positively ionized groups like quarternary ammonium groups[24,25] promote cell adhesion. In this respect, polycations such as polyammonium salts[25], polylysine[26-28], and poly(ethyleneimine)[28,29] have been used to improve the adhesion of cells to artificial surfaces. These modifications have been applied in

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chemotherapy[25], microscopy[26], cell culture[27], cell diagnostic procedures[28] and biotechnology[29], but they have not been used to improve leucocyte adhesion.

In this study, the incorporation of positively or negatively ionizable groups into PU film surfaces was achieved by the introduction of either amine or carboxylic acid groups. Amine groups were introduced by adsorption of poly(ethyleneimine) (PEI) and carboxylic acid groups were incorporated by gas plasma treatment of PU surfaces to which acrylic acid had been pre-adsorbed. Leucocyte adhesion on modified PU films was compared with leucocyte adhesion on untreated PU films. To exclude the influence of factors such as protein adsorption, flow and complement activation, an *in vitro* adhesion assay was used. In this assay film surfaces were exposed under static conditions to purified leucocyte populations suspended, in phosphate buffered synthetic medium.

MATERIALS AND METHODS

Polyurethane films

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 by slowly adding a 5% (w/v) solution of PU in dimethylformamide to a tenfold excess of water whilst stirring at high speed. The precipitate was dried *in vacuo* at 60°C. To prepare films, a 10% (w/v) solution of the purified PU in tetrahydrofuran was poured 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 with the glass surface of the dishes, was used for leucocyte adhesion studies.

Unless specified otherwise, the films were washed with analytical grade organic solvents to remove contaminating surface active agents. This washing procedure involved two 15 min periods of sonication in cyclohexane, followed by two further periods of sonication for 15 min in ethanol. The composition of the extract was analysed by Fourier transformed infrared spectroscopy (FTIR) using a FTS-60 spectrometer (Bio Rad Digilab Division) and by gel permeation chromatography (GPC) using a system consisting of a HPLC system (Millipore Waters, type 510), μSteragel columns (exclusion limits 10 000, 1000 and 100 nm successively) and a differential refractometer (Millipore Waters, type 411). The washed films were dried *in vacuo* and stored in a dessicator in the dark at room temperature until used for adhesion studies.

Surface modification with poly(ethyleneimine)

PU films (2.5 × 7.5 cm) were coated with PEI (mol wt 40 000–60 000; Sigma, St. Louis, MO, USA) by immersion of the films for 1 h in a solution of 1% (w/v) PEI in water. Weakly adsorbed PEI was removed by rinsing with distilled water, followed by triple sonication in a phosphate buffered sodium chloride solution (PBS, pH 7.4) and triple sonication in water successively (15 min each). The films, encoded as PU-NHx, were finally dried *in vacuo*, and stored in a dessicator in the dark at room temperature.

Surface modification with poly(acrylic acid)

Attempts to immobilize poly(acrylic acid) (PAA, mol wt approximately 5000; Aldrich, Brussels, Belgium) on to PU films by the method used for PEI immobilization were unsuccessful. Consequently a two step gas plasma technique was used to graft acrylic acid (AA) on to PU films. The gas plasma reactor (*Figure 1*) consisted of a glass reactor tube (volume approximately 3 l) connected to a vacuum system (Balzers) and three externally capacitively coupled electrodes to generate plasmas by a 13.56 MHz radiofrequent generator (ENI Power Systems). AA (Merck, Darmstadt, Germany) was first degassed by means of a freeze/thawing procedure, and fed into the reactor using the system depicted in *Figure 1*.

In the first step, four PU films (2.5 × 7.5 cm each) were exposed for 10 min to AA vapour in the reactor at reduced pressure (2.0 mbar). During this process the pressure in the reactor dropped to about 1.5 mbar, due to absorption of AA into the PU films. The excess AA vapour was removed by evacuating the reactor for 1 min, and subsequent flushing with argon gas (Ar > 99.999%, Hoekloos, Schiedam, The Netherlands) for 1 min (0.2 mbar, 10 ml/min).

In the second step AA was covalently coupled to the PU films using a radiofrequent argon plasma (15 Watt, 0.2 mbar, 10 ml/min) for 1 min. After the coupling step, the films were exposed to an argon gas flow for 2 min (0.2 mbar, 10 ml/min). The treated films were washed afterwards to remove non-bound fractions like monomer and homopolymer by triple sonication in PBS, followed by triple sonication in distilled water for 15 min each. The films, encoded as PU-COOH, were finally dried *in vacuo*, and stored in a dessicator in the dark at room temperature.

Surface characterization

Wettability

Water contact angles were determined at room temperature by means of three different methods. To characterize both sides of the films separately, contact angles were determined using the captive bubble method[30] and the sessile drop method[31]. Drops of ultrapure water and air

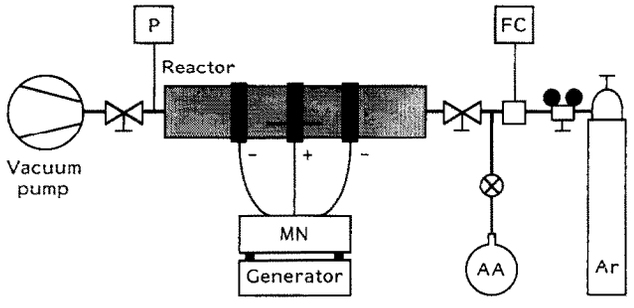

Figure 1 Schematic representation of the gas plasma modification system. AA = acrylic acid supply; Ar = argon gas supply; MN = matching network; P = pressure sensor; FC = mass flow controller. The PU films are placed in the centre of the reactor.

bubbles in ultrapure water, both with an approximate diameter of 2–3 mm, were photographed within 30 s after contact with the film surface. The contact angles were obtained directly from the photographs using a goniometer. Contact angles were also measured dynamically, using the Wilhelmy plate method[32]. Samples, both sides of which were treated identically, were immersed in ultrapure water at a speed 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 (θ_a and θ_r) were calculated[32]. To detect ionizable groups at modified surfaces, θ_a was also determined as a function of pH, essentially as described by Holmes-Farley *et al.*[33]. In our case, unbuffered solutions were used. The pH was adjusted with 1 M HCl or 1 M NaOH, and did not change during the experiment.

Surface charge

Zeta (ζ) potentials of the films were calculated from streaming potentials, measured with a parallel plate system[34]. For this purpose, very thin PU films (thickness < 100 μm) were glued on to glass plates and placed at a distance of 100 μm. The streaming potential (ΔE), which develops by passing an electrolyte solution (0.01 M KCl and 1 mM phosphate buffer, pH 7.4, 8.0 or 8.5) along the plates, was measured as a function of the driving pressure (ΔP). The ζ potential was calculated from $\Delta E/\Delta P$, assuming non-conductivity of the polymer film surface[34].

Surface composition

X-ray photo electron spectroscopy (XPS) was used to analyse the elemental composition of the film surfaces[35]. XPS measurements were performed using a Kratos XSAM 800 (Kratos analytical, Manchester, UK), using X-rays from a Mg-K_α source (15 kV/15 mA) at a take off angle of 90° between the film surface and the analyser. Under these conditions, the detection depth was about 7 nm. For quantitative analysis, detail scans were made of C1s, O1s, N1s and Si2p peaks (diameter of the spot size 3 mm). Empirically derived sensitivity factors were used to convert the peak areas in surface elemental composition.

Staining methods

Ionic dyes were used to qualitatively detect charged groups on the surface of the PU films. To stain either positively charged or negative charged surfaces[36], the films were immersed for 10 min in solutions of 0.01% (w/v) crystal violet (Merck, Darmstadt, Germany) or 0.05% (w/v) acid blue 80 (Aldrich, Brussels, Belgium), respectively, either in water or PBS. The films were subsequently rinsed with water to remove excess of dye. Carboxylic acid groups on to PU films were detected in a similar way by staining them with a solution of 0.02% (w/v) Azure-A[37] (Fluka AG, Buchs, Switzerland) in 0.01 M phosphate buffer (pH 7.6).

Leucocytes

Leucocytes were isolated from freshly collected citrated human blood, as described by Roos and de Boer[38]. One unit (500 ml) of blood was centrifuged at 400**g** for 5 min, followed by separation of the leucocyte-rich interface (buffy coat) from the plasma layer and the packed red cells. The buffy coat was diluted with PBS, containing 13 mM trisodium citrate and 0.5% (w/v) human albumin (medium-G; 290–295 mOsm, pH 7.2–7.4) to a final volume of 200 ml, and layered on a Percoll® (Pharmacia, Uppsala, Sweden) suspension with a specific gravity of 1.077 g/ml. Leucocytes were fractionated by centrifugation for 20 min at 1000**g** (room temperature).

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

To prepare a granulocyte suspension, cells from the pellet formed below the Percoll® layer were collected. In order to lyse erythrocytes, cells were resuspended 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). To complete the lysis, the suspension was gently shaken for 20 min while kept on ice. To remove red cell stroma and haemoglobin, the suspension was centrifuged at 4°C for 5 min at 400**g** and the granulocyte containing pellet was resuspended in medium-G. This procedure was repeated three times.

All cell suspensions were adjusted to 6.4×10^5 cells/ml in medium-G. Leucocyte counts were determined using a Bürker counting chamber, after staining the cells with Türk's solution. The purity of the cell suspensions was determined microscopically. For this purpose the cells were stained with May-Grünwald/Giemsa staining. Lymphocyte suspensions had a purity of at least 93%. Monocytes were the main contaminants. Granulocyte suspensions had a purity of 95–98%. The viability of the cell suspensions was always more than 98%, determined by trypan blue exclusion[39]. Before being used in adhesion experiments, leucocyte suspensions were stored at room temperature for not more than 1 h.

Leucocyte adhesion

Leucocyte adhesion experiments were performed with PU- and surface-modified PU films. Films were mounted in Teflon® well plates (four wells) with test surfaces of 1.2 cm^2/well, essentially as previously described[40]. Each well was first rinsed with 1.0 ml of PBS, and subsequently filled with 1.0 ml of leucocyte suspension (6.4×10^5 cells/ml). Cell suspensions were incubated with the surfaces for 5, 15, 30 or 60 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 at the end of 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 10–20 min, the glutaraldehyde solution was removed. The films were detached from the well plates and dried in air.

Adherent leucocytes were stained by immersion of the films in a haematoxilin solution (Delafield; Merck, Darmstadt, Germany) for 10–15 min, followed by drying in air. Using a light microscope, two areas of 0.1–0.4 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 adhesion experiment was repeated at least four times.

Scanning electron microscopy (SEM)

Immediately after fixation with glutaraldehyde, small samples of the films were prepared for scanning electron microscopy (SEM). Samples were first dehydrated by 10 min immersion in successively 25, 50, 75% ethanol, 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 scanning electron microscope (Japan Electron Optics Laboratory) using a 15 kV accelerating voltage.

Statistical analysis

A two-way analysis of variance was carried out to evaluate the significance of differences between leucocyte adhesion to the treated and control surfaces as a function of time. To make direct comparisons between two sets of data, a two-tailed *t* test for paired data sets was used. P values <0.05 were accepted to consider data as significantly different.

RESULTS

Surface modification

The XPS data (*Table 1*) clearly demonstrate that the elemental composition of the PU film surfaces changes as a result of the different modification and washing procedures. It appears from these results that silicon-containing surface active agents were almost completely removed from the untreated films by washing them with cyclohexane and ethanol. Analysis of the extracts revealed that some polyether-rich low molecular weight oligomer was also removed by the washing procedure. The incorporation of amine groups by modification of PU films with PEI (PU-NHx) was confirmed by the increased surface concentration of nitrogen (3.8 at.%) as compared to untreated PU films (2.4 at.%). Although PEI does not contain oxygen, modified surfaces contained relatively high amounts of oxygen. This may be caused by a rearrangement of PU molecules, as a result of the interaction with adsorbing PEI. Similar effects have been reported by Ratner and Yoon[41]. The elemental composition of washed PAA-treated surfaces (PU-PAA) was not different from untreated PU. This demonstrates that it is not possible to incorporate carboxylic acid groups into the film surface by adsorption of PAA. A successive treatment of the PU films with AA vapour and Ar gas plasma (PU-COOH), however, led to a considerable increase of the surface concentration of oxygen, suggesting that carboxylic acid groups had been introduced. Control experiments, in which PU films were either treated with AA vapour (PU-AA) or Ar gas plasma (PU-Ar) alone, did not lead to this result. When the Ar gas plasma treatment step was applied without the AA-pre-adsorption step (PU-Ar sample), a significant amount of nitrogen was incorporated into the PU film surface (3.7 at.%). This is probably caused by the post-plasma reaction of long-living radicals at the film surface with atmospheric nitrogen.

Carboxylic acid groups can be detected qualitatively by the Azure-A staining method[37]. Only PU films which were successively treated with AA vapour and Ar plasma, were intensively stained using this method. After thorough washing, the surface concentration of oxygen of these films decreased slightly, demonstrating that only a small fraction of the introduced carboxylic acid groups, probably PAA homopolymer, had been removed.

The surface modification of the films was also demonstrated by contact angle measurements (*Table 2*). Contact angles measured with the sessile drop and

Table 1 Chemical analysis (XPS) of modified and untreated PU film surfaces

Sample	Side	Elemental composition (at.%)			
		C	N	O	Si
PU untreated	Top	77.4 ± 0.3	2.9 ± 0.3	18.6 ± 0.1	1.1 ± 0.2
PU untreated	Bottom	77.9 ± 0.4	2.2 ± 0.2	18.3 ± 0.6	1.6 ± 0.1
PU washed	Top	80.1 ± 0.4	2.6 ± 0.1	17.3 ± 0.5	0.1 ± 0.1
PU washed	Bottom	79.6 ± 0.4	2.4 ± 0.2	18.1 ± 0.5	0.1 ± 0.1
PU-NHx washed	Bottom	72.8 ± 0.4	3.8 ± 0.2	22.7 ± 0.2	0.7 ± 0.1
PU-PAA	Bottom	78.6	2.4	19.1	Not tested
PU-PAA washed	Bottom	79.2	2.6	18.2	Not tested
PU-COOH	Bottom	70.8	2.3	26.8	Not tested
PU-COOH washed	Bottom	74.8 ± 0.1	2.0 ± 0.2	23.1 ± 0.4	0.2 ± 0.1
PU-AA washed	Bottom	76.9	2.6	20.3	Not tested
PU-Ar washed	Bottom	74.6	3.7	21.6	Not tested

Concentrations are the means of two measurements + standard deviation. Surfaces which were not used for leucocyte adhesion experiments were examined once.

Sample codes; PU = polyurethane film, PU-NHx = PU film with amine groups incorporated by means of immersion in poly(ethyleneimine) solution, PU-PAA = PU film immersed in poly(acrylic acid) solution, PU-COOH = PU film with carboxylic acid groups incorporated by means of a two-step gas plasma treatment, PU-AA = PU film exposed to acrylic acid vapour, and PU-Ar = PU film treated with an argon plasma. For washing procedures see section on Materials and Methods.

Table 2 Water contact angles of modified and untreated PU film surfaces

Sample	Wilhelmy plate		Sessile drop		Captive bubble	
	θ_a	θ_r	Top	Bottom	Top	Bottom
PU untreated	100 ± 1	51 ± 4	71 ± 2	69 ± 1	66 ± 2	67 ± 8
PU washed	97 ± 2	43 ± 2	75 ± 3	76 ± 3	67 ± 6	70 ± 5
PU-NHx washed	89 ± 3	33 ± 3	71 ± 3	77 ± 5	59 ± 3	63 ± 7
PU-PAA	90 ± 1	24 ± 1	-	-	-	-
PU-PAA washed	92 ± 1	48 ± 2	-	-	-	-
PU-COOH	55 ± 1	11 ± 8	-	-	-	-
PU-COOH washed	70 ± 3	15 ± 8	53 ± 5	44 ± 6	47 ± 3	39 ± 5
PU-AA washed	93 ± 1	42 ± 1	-	-	-	-
PU-Ar washed	56 ± 1	5 ± 8	-	-	-	-

Contact angles (degrees) are expressed as the mean + standard deviation; $n = 3$ for Wilhelmy plate method and $n = 5$ for sessile drop and captive bubble method. For sample codes see *Table 1*.

captive bubble method were similar on both sides of each film. The Wilhelmy plate method was therefore used to measure contact angles dynamically. Using this method, it was shown that the removal of silicon containing compounds from untreated PU films led to a slightly increased wettability of these films. Modification of the washed PU films, with either amine or carboxylic acid groups, resulted in a further increase of the wettability. The advancing contact angle of films modified with carboxylic acid groups was nearly constant in the pH range of 2–10, whereas at a higher pH, a significant drop in the contact angle was observed (*Figure 2*). This effect was not observed for washed films and films modified with amine groups.

The results of ζ potential measurements (*Table 3*) show that the surface charge of the films was not significantly changed after modification. An increase of the pH from 7.4 to 8.5 had no effect on the ζ potential of the different surfaces. Staining methods, to detect the charge at the surface of biomaterials[36] also failed to demonstrate changes of surface charge after modification.

Leucocyte adhesion

The results of the adhesion experiments were evaluated by a two-way analysis of variance. *Figures 3* and *4* demonstrate that the number of both granulocytes and lymphocytes which adhered to the PU films largely depends on the type of modified surface used. For each surface, the number of adherent leucocytes increased significantly in time. No plateau was reached after 60 min of exposure. It also appears from *Figures 3* and *4* that for each surface at a particular exposure time the number of adherent granulocytes was significantly higher than the number of adherent lymphocytes. The numbers of leucocytes adherent to surfaces modified with amine groups were significantly higher than the numbers of leucocytes adherent to untreated surfaces. The numbers of adherent leucocytes on PU modified with carboxylic acid groups were only significantly higher than control surfaces after an exposure time of 60 min.

Table 3 Zeta potentials of modified and untreated PU film surfaces

Sample	Zeta potential (mV)		
	pH 7.4	pH 8.0	pH 8.5
PU untreated	−31.7 ± 2.3	−31.5 ± 0.8	−31.3 ± 1.3
PU washed	−35.5 ± 1.9	-	-
PU-NHx washed	−32.2 ± 3.0	-	-
PU-COOH washed	−32.0 ± 2.9	−31.8 ± 1.5	−36.0 ± 5.0

Zeta potentials are expressed as the mean + standard deviation from at least three measurements. For sample codes see *Table 1*.

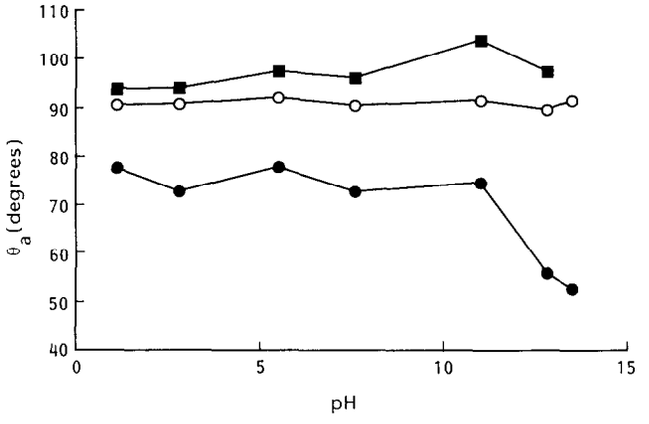

Figure 2 Dependence of the advancing contact angle (θ_a) on the pH of the wetting solution, for test and control polyurethane surfaces. Untreated PU, —■—; PU-NHx, —○—; PU-COOH, —●—. Each data point represents the mean of at least two measurements (Wilhelmy plate method).

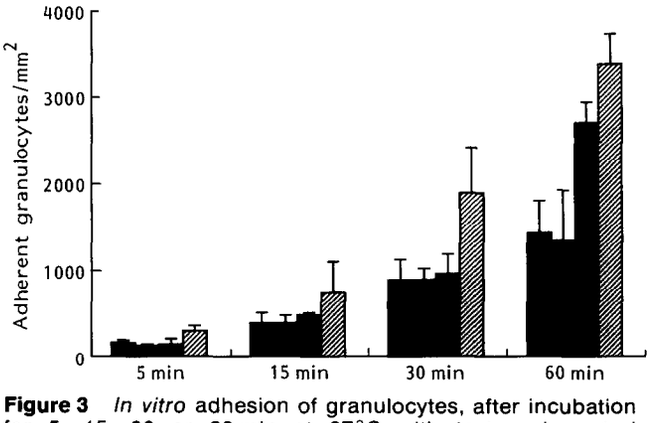

Figure 3 *In vitro* adhesion of granulocytes, after incubation for 5, 15, 30 or 60 min at 37°C with test and control polyurethane surfaces. Untreated PU, ■; washed PU, ▨; PU-COOH, ▩; PU-NHx, ▨. The error bars shown are standard deviations, $n = 5$ for 30 min incubation and $n = 4$ for all other experiments.

Figures 5 and *6* show typical micrographs of granulocytes adherent to a washed PU film surface and a PEI-modified PU film surface after 30 min of incubation. In general, adherent cells maintained their original spherical shape. No cell spreading was observed. Also no differences were observed between the morphology of lymphocytes adherent on the various surfaces at different exposure times. Lymphocytes occasionally formed large pseudopods (*Figure 7*).

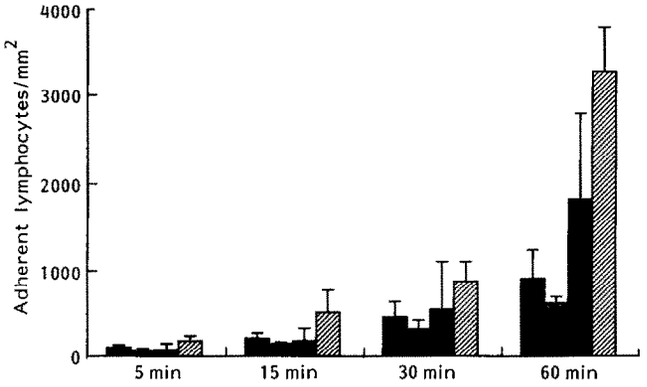

Figure 4 *In vitro* adhesion of lymphocytes, after incubation for 5, 15, 30 or 60 min at 37°C with test and control polyurethane surfaces. Untreated PU, ■; washed PU, ▨; PU-COOH, ▩; PU-NHx, ▨. The error bars shown are standard deviations, $n = 6$ for 30 min incubation and $n = 5$ for all other experiments.

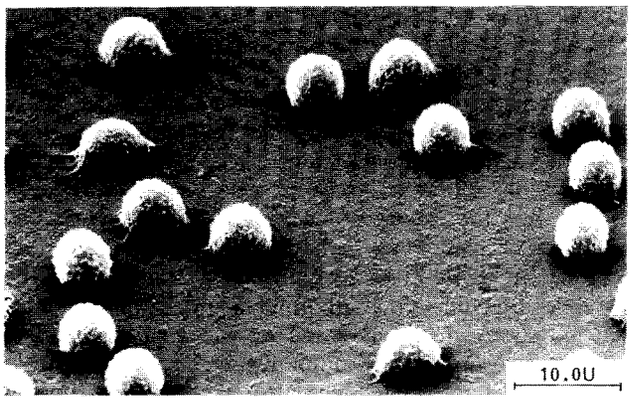

Figure 5 SEM micrograph of granulocytes adherent to a washed polyurethane film surface, after 30 min of incubation at 37°C. Stage tilt 60°, original magnification ×2000.

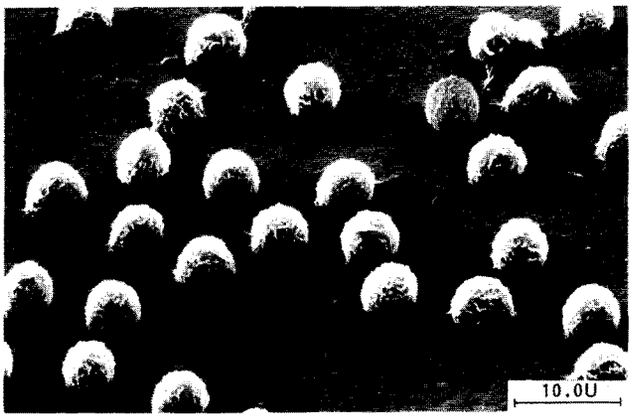

Figure 6 SEM micrograph of granulocytes adherent to a PEI-modified polyurethane film surface, after 30 min of incubation at 37°C. Stage tilt 60°, original magnification ×2000.

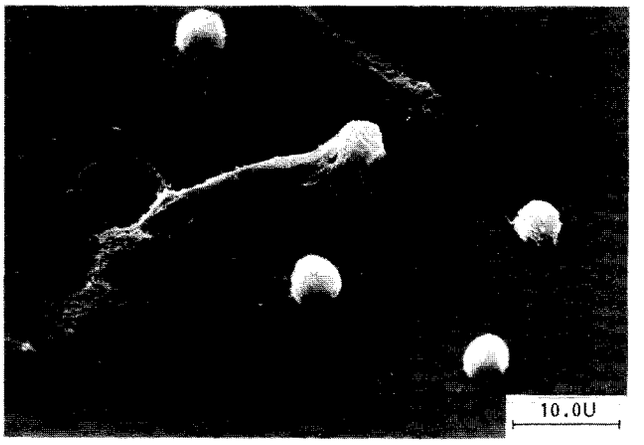

Figure 7 SEM micrograph of lymphocytes adherent to a washed polyurethane film surface, after 30 min of incubation at 37°C. Stage tilt 60°, original magnification ×2000.

DISCUSSION

When leucocyte adhesion to polymer surfaces is studied in the context of leucocyte filtration, it is important to distinguish between the adhesion behaviour of different subpopulations of leucocytes. Since the concentration of monocytes in human blood is relatively low, and their adhesion to artificial surfaces[42, 43] is efficacious from the viewpoint of leucocyte filtration[44, 45], the adhesion behaviour of these cells was not included in this study. Granulocytes form the largest population of leucocytes in human whole blood and generally adhere well to artificial surfaces[10, 46]. Lymphocytes form the second largest group, and are generally less reactive towards polymer surfaces[10, 47], which probably accounts for their relatively high concentration in filtered blood[9, 44, 45]. A substantial difference in adhesiveness between granulocytes and lymphocytes was also found in the present study. When the adhesion of granulocytes to the surfaces studied was compared with the adhesion of lymphocytes, a similar trend in adhesion behaviour was observed, although the absolute adhesion values for the two types of cells were different. *Figures 3* and *4* show that the adhesion of leucocytes to PU films modified with either amine or carboxylic acid groups is increased over their adhesion on untreated films. The increase of cell adhesion due to carboxylic acid groups was unexpected, since negatively charged groups at solid surfaces usually diminish cell adhesion[12, 13, 24]. However, some authors reported an increase of cell adhesion due to the incorporation of carboxylic acid groups into the surface[48].

It is questionable whether carboxylic acid groups incorporated into PU surfaces are indeed negatively charged. Although the surface modification obviously resulted in the presence of carboxylic acid groups at the PU film surface, as concluded from the combined results of XPS and Azure-A staining experiments, the ζ potentials of the untreated and modified films were practically the same in the range of pH 7.4-8.5 (*Table 3*). This may be further explained by the results of pH-dependent contact angle measurements which suggest that carboxylic acid groups incorporated into the PU films only became negatively charged at above pH 11 (*Figure 2*). A similar phenomenon has been reported for polyethylene surfaces

modified with carboxylic acid groups[49]. Remarkably, surfaces modified with amine groups do not show a change of the contact angle in the range of pH 1–13. It should be noted that a pH-dependent change of the contact angle can only be observed when ionization of the amine groups leads to a significant change of the surface free energy. Apparently, this is not the case for the present surfaces, probably due to the high extent of hydrogen bonding at the solid–water interface, at both protonated and unprotonated surfaces. This different behaviour of surfaces modified with amine groups as compared to surfaces modified with carboxylic acid groups has also been reported by other authors[33].

The ζ potential of the films modified with amine groups was not significantly different compared with the ζ potential of the untreated films. It is remarkable that the films modified with amine groups had a negative ζ potential, since the surface charge of pure PEI has been reported[50] to be +33.0 mV. At least 10–30% of PEI will be protonated in aqueous solutions at pH 7.4, depending on its molecular weight, degree of branching, concentration and ionic strength of the solvent used[51, 52]. It cannot be excluded that the ionization of immobilized PEI is also restricted. The fact that all surfaces studied had a negative ζ potential may also be explained by the adsorption of negatively charged ions to the surface. A time-dependent change of ζ potential, due to ion diffusion into the boundary layer, has been observed by several authors[53–55]. Van Damme even reported that the ζ potential of a polymer film containing quaternary amine groups, in contact with an electrolyte-containing buffer solution, was initially positive but became negative over time[56].

Since the ζ potential of the PU films had not significantly changed by incorporation of ionizable groups into the film surface, long-range electrostatic interactions between the cells and the solid surface were comparable in all experiments. Therefore, the increased number of adherent leucocytes to modified surfaces as compared to untreated surfaces cannot be explained in terms of differences between electrostatic interactions[57]. In the literature, enhanced leucocyte adhesion to solid surfaces is often related to a higher surface free energy[9] or to an improved wettability[10]. Such a relation would explain the enhanced adhesion of leucocytes to surfaces which are modified with carboxylic acid groups, since the wettability of the modified films was increased compared with untreated films (*Table 2*). However, the concept of surface free energy to explain differences in cell adhesion is only valid when leucocyte adhesion is studied at a state of thermodynamic equilibrium. This was not the case in our experiments, because no plateau values for cell adhesion were reached within 60 min (*Figure 3*). Moreover, the substantial increase of leucocyte adhesion to surfaces modified with amine groups cannot be ascribed to a change of surface free energy, since the wettability of these films was virtually unaffected by the modification procedure. We therefore assume that the enhanced cell adhesion to the modified surfaces is related to the chemical nature of the functional groups incorporated into the film surfaces. Leucocytes have a heterogeneous membrane structure which may even rearrange during the adhesion process. Ionizable groups are locally present at the cell surface or appear during adhesion. Such groups will not affect the net cell charge, but may attribute to short-range interactions with chemical groups at the modified PU surface. In the case of surfaces modified with amine groups, negatively charged groups on the leucocyte membrane, such as sialic acid groups[22, 27], may interact with amine groups at the modified surface. It has been reported that high ionic concentrations or extreme pH conditions fail to remove yeast cells from PEI-treated cloth[29]. We suggest that in addition to specific ionic interactions of cells with modified PU surfaces, other interactions are involved. For example, hydrogen bonding between groups on the leucocyte membrane and electronegative atoms on the modified-film surface may favour cells to adhere. The development of pseudopods by the lymphocytes (*Figure 7*) suggests an active attachment process[58]. However, the original spherical shape of adherent granulocytes (*Figures 5* and *6*) indicates that these cells were not activated during adhesion.

In contrast to the effect of chemical surface modification, washing of the PU films with cyclohexane and ethanol led to a reduction of the amount of adhering leucocytes. This was most pronounced in the case of lymphocytes. However, this phenomenon was only significant after short incubation times (5 min). Suppression of cell adhesion to washed PU surfaces occurred despite an improved film wettability, which generally favours cell adhesion[10]. Marchant *et al.* have attributed a reduced leucocyte adhesion on to washed PU surfaces to the removal of cytotoxic moieties by the washing procedure[59]. Organic silicon compounds such as polysiloxanes are known to contaminate polymer surfaces[35]. Our results (*Table 1*) demonstrate that washing of the PU films led to the removal of silicon containing compounds. Similar results have been reported by other authors[59, 60]. Analysis of the extract with FTIR and gel permeation chromatography (GPC) analysis revealed that some polyether-rich low molecular weight oligomer was also removed. It is most likely that such PU oligomers migrate from the polymer bulk to the surface, and may thus be extracted[61]. After washing, the chemical composition of the film surface was practically the same on both sides of the PU film (~80% C, ~18% O, ~2% N). This composition of the surface is in agreement with results published by others[60, 62]. The washing procedure used is, therefore, appropriate to obtain reproducible and well-defined PU film surfaces.

Up to 60 min, cell adhesion had not reached plateau values, indicating that there was not yet a thermodynamic equilibrium. In adhesion studies using other surfaces and granulocytes, Absolom *et al.*[9] found that a state of saturation was reached after 20–30 min. Leucocyte adhesion after 30 min exposure has been studied by others[12, 14]. It is likely that the level of leucocyte adhesion in our experiments was not restricted by the rate of sedimentation, because it can be predicted on a theoretical basis[63] that all cells will reach the substrate surface within an exposure time of 60 min. Therefore this study does not allow us to conclude whether the adhesion of leucocytes to the test materials is governed by kinetic or thermodynamic effects[64]. When short-range interactions between the cells and surfaces studied are of major importance, as was suggested, the strength of the bond between cell and substrate rather than the activation

energy for adhesion will determine the extent of adhesion after prolonged exposure times.

The substantial increase in leucocyte adhesion to the surfaces modified with amine groups, which was most pronounced for lymphocytes, and the simplicity of the coating procedure used, offer opportunities for the development of improved leucocyte filters. In this respect, it should be noted that the influence of plasma protein adsorption, haematocrit, platelets, divalent ions or flow conditions have to be further evaluated.

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