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# Immobilization of Theophylline on Medical-Grade Polyurethane Inhibits Surface-Induced Activation of Blood Platelets

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**Abstract:** Conjugate molecule **2**, in which theophylline and a 4-azidobenzoyl group are separated by a short spacer chain, was adsorbed onto polyurethane sheets and subsequently irradiated with ultraviolet light. The resulting photoreaction at the polymer surface led to immobilization of theophylline. The modified polymer surface thus obtained was subjected to different physico-chemical experiments, i.e., X-ray photoelectron spectroscopy, attenuated total reflection infrared spectroscopy, and water contact-angle measurements. These experiments clearly pointed out that theophylline is linked to and exposed at the surface of the modified polymer. In addition, the modified surface was subjected to various *in vitro* biochemical tests, i.e. (i) a thrombogenicity assay in which the surface was contacted with either platelet-rich blood plasma (PRP) or platelet-free blood plasma (PFP), and formation of thrombin was monitored as a function of time and (ii) studies focussed on adhesion of blood platelets to the surface, both in a static system, and under flow conditions. The latter experiments were conducted with a parallel-plate flow chamber. The biochemical tests revealed that the modification leads to a marked increase of the lag-time for surface-induced thrombin formation provided that PRP was used in the test (experimental lag-times for the modified surface and the untreated control: ca. 1270 and 576 s, respectively). Using PFP, the lag-times of the modified and untreated surfaces do not differ substantially (698 and 549 s, respectively). Studying adhesion of blood platelets in a static set-up, the modified surface showed adhesion of only few platelets with nearly unchanged morphology, whereas extensive adsorption of activated platelets (evident from spreading and formation of pseudopods) was found for the untreated control surface. These observations were made with scanning electron microscopy. The experiments with the parallel-plate flow chamber showed adhesion of approximately 30.000 platelets per square cm of the modified surface, for shear rates in the range 12.5−300 $s^{-1}$. The density of adhered platelets for the untreated surface was found to be higher by one order of magnitude.

## Introduction

The search for artificial surfaces with perfect or near-perfect bloodcompatibility continues to be one of the most important items in biomaterials science.[1,2] Truly bloodcompatible polymeric materials would be extremely useful in extracoporeal medical devices (e.g., hemodialysis)[3] and in the creation of small-diameter blood vessel prostheses.[4] So-called "heparin-

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(1) See, for instance: (a) Peppas, N. A.; Langer, R. *Science* **1994**, *263*, 1715. (b) Test Procedures for the Blood Compatibility of Biomaterials; Davids, S., Ed.; Kluwer Academic Publishers: Dordrecht-Boston-London, 1993; and references cited therein. (c) Hakim, R. M. *Cardiovasc. Pathol.* **1993**, *2*, 187S.

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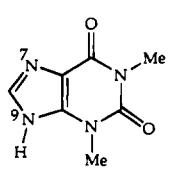
ized" surfaces are probably the best artificial surfaces (in terms of bloodcompatibility) known to date. Fragments of heparin are attached to a polymer surface,[5] either via a covalent bond[5a-c] or via attractive electrostatic forces.[5d] Heparin fragments appear to retain a marked anticoagulant activity after surface immobilization.

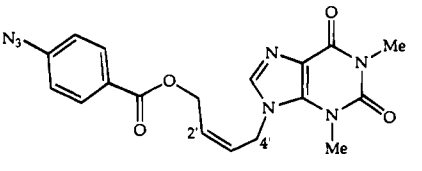
Herein we wish to report on a new type of surface modification, which essentially rests upon attachment of theophylline to a polyurethane surface. Theophylline (**1**) is a xanthine-based heterocycle, which potentiates the inhibition of platelet aggregation by prostaglandins. Our choice for theophylline was primarily based on earlier work by Bamford et al.,[6] who have incorporated the drug into a new type of water-soluble polymer. These workers observed that the resulting materials exhibit a marked inhibitory effect with respect to the activation of blood platelets.[6a]

Two important introductory remarks can be made at this point. First: we attached theophylline to the surface of a medical grade polyetherurethane sheet (Pellethane 2363 55-D).[7] The material did not show any change with respect to the mechanical properties as a result of the surface modification. Secondly: theophylline was coupled to the polymer surface by means of a *photoreaction*. The surface modification was carried out with a newly synthesized molecule, **2**, a conjugate system in which theophylline is linked via a short spacer to a 4-azidobenzoyl moiety. The latter system is well-known for its photoreactivity. Adsorption of **2** to the polymer surface and subsequent irradiation with UV light induced a photoreaction of the 4-azidobenzoyl group, which presumably involves a nucleophilic site at the polymer surface. Then, the result is that theophylline is covalently linked to the surface.

We describe here (i) the chemical synthesis of **2**; (ii) our method of photochemical surface modification; (iii) physicochemical analyses on the modified surface; and (iv) *in vitro* biochemical tests of the modified surface, e.g., thrombogenicity for platelet-rich blood plasma and platelet-poor blood plasma, morphology of adhered blood platelets, and adhesion of blood platelets, both in a static system and under flow conditions.



**1**



**2**

## Experimental Section

**a. Synthesis. General Method.** Unless otherwise noted, all starting materials were obtained from commercial suppliers and used without further purification. Pyridine and dichloromethane were distilled from $CaH_2$ and stored over 4Å molecular sieves. Dimethyl sulfoxide (DMSO) was distilled at reduced pressure. A relatively large prerun (ca. 20%) was discarded; the main fraction was collected and stored over 4Å molecular sieves. Nitromethane was predried with $CaCl_2$, distilled, and stored over 4Å molecular sieves. Methanol was predried with iodine and magnesium turnings, distilled, and stored over 3Å molecular sieves. Triethylamine was distilled from $CaH_2$ and stored over KOH. Reactions and chromatographic separations were monitored by thin-layer chromatography (TLC) using precoated glass plates of 250-$\mu$m thickness silica gel with a fluorescent indicator. Preparative column chromatography was carried out by using Merck Silica Gel 60 (particle size 0.063−0.200 mm). Infrared (IR) spectra were recorded on a Mattson-Polaris IR-100-410B spectrophotometer. Bands are in units of $cm^{-1}$. Ultraviolet (UV) spectra were recorded on a Beckmann DU 7500 spectrophotometer. $^1$H NMR spectra were recorded at 399.9 MHz on a Varian Unity-Plus spectrometer, as solutions in DMSO-$d_6$ or $CDCl_3$. Chemical shifts are expressed in parts per million (ppm, $\delta$ units), relative to tetramethylsilane (TMS, 0.0 $\delta$). $^{13}$C NMR spectra were recorded at 100.6 MHz on the same instrument, on the same samples, and the chemical shifts are also reported in ppm relative to TMS (0.0 $\delta$). Low-resolution mass spectra (EI) were measured on a Kratos MS80 mass spectrometer. Exact masses were obtained by peak matching.

**1-(Triphenylmethoxy)-4-hydroxy-*cis*-2-butene**. Triphenyl methyl chloride (10.00 g, 35.9 mmol) was added in 10 equal portions over a period of 3 h to a stirred solution of *cis*-2-butene-1,4-diol (60.00 g, 681.8 mmol) in dry pyridine (100 mL). Stirring was continued for 24 h. Then, all volatiles were removed on a rotatory evaporator. Last traces of pyridine were removed by coevaporation with toluene. The residue was dissolved in diethyl ether and washed with water (2×), saturated sodium bicarbonate solution (2×), and water (4×). The excess of diol was removed in these washing steps. The organic layer was dried ($MgSO_4$), filtered, and concentrated. The residue was chromatographed on a silica gel column using a gradient of petroleum ether 40−60/ethyl acetate (4:1 v/v to 1:1 v/v). This yielded 9.95 g (30.1 mmol, 84%) of pure 1-(triphenylmethoxy)-4-hydroxy-*cis*-2-butene as a slightly colored solid. $^1$H-NMR ($CDCl_3$) $\delta$ 1.90 (1H, bs, OH), 3.69 (2H, d, $CH_2$-O-Tr), 3.98 (2H, d, $CH_2$-OH), 5.63−5.80 (2H, m, olefinic Hs), 7.19−7.50 (15H, m, aromatic Hs); $^{13}$C-NMR ($CDCl_3$) $\delta$ 58.74, 60.15, 87.11, 127.10, 127.83, 128.56, 128.79, 131.05, 134.92. Anal. Calcd for $C_{23}H_{22}O_2$: C, 83.6; H, 6.7. Found: C, 84.5; H, 7.2.

**1-(Triphenylmethoxy)-4-chloro-*cis*-2-butene (3)**. 1-(Triphenylmethoxy)-4-hydroxy-*cis*-2-butene (10.00 g, 30.3 mmol) was dissolved in 150 mL of dry dichloromethane. The solution was magnetically stirred, and 4-(*N*,*N*-dimethylamino)pyridine (3.70 g, 30.3 mmol), *p*-toluenesulfonyl chloride (11.53 g, 60.5 mmol), and triethylamine (6.11 g, 60.4 mmol) were added in sequence.[8] After 3 h, TLC showed complete conversion. Then the reaction mixture was poured into 100 mL of water. The organic layer was separated, concentrated, redissolved in 150 mL of *n*-hexane, and washed successively with water (2×), saturated bicarbonate (2×), and water (2×). The organic phase was dried ($MgSO_4$), filtered, and concentrated, affording a yellowish oil. Further purification using column chromatography (eluent petro-

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leum ether 40−60/ethyl acetate (9:1 v/v) gave pure **3** as a white solid ($R_f$ = 0.59): yield 7.94 g (22.8 mmol, 75%); mp = 31.7 °C; $^1$H-NMR ($CDCl_3$) $\delta$ 3.71 (2H, d, $CH_2$-O-Tr), 3.95 (2H, d, $CH_2$-Cl), 5.66−5.73 (1H, m, olefinic H close to Tr), 5.78−5.85 (1H, m, olefinic H close to Cl), 7.19−7.50 (15H, m, aromatic Hs); $^{13}$C-NMR ($CDCl_3$) $\delta$ 39.48, 59.79, 87.20, 127.26, 127.26, 127.73, 128.59, 130.96, 143.84; mass spectrum, *m/e* 348.128 ($M^+$ calcd for $C_{23}H_{21}OCl$ 348.125). Anal. Calcd for $C_{23}H_{21}OCl$: C, 79.2; H, 6.1. Found: C, 80.0; H, 6.5.

**9-*N*-(1′-Hydroxy-*cis*-2′-buten-4′-yl)theophylline (5).** Theophylline (**1**; 5.42 g, 30.1 mmol) was added to a magnetically stirred solution of **3** (7.00 g, 20.1 mmol) in 100 mL of dry DMSO. Dry potassium carbonate (7.00 g, 50.7 mmol) was added.[8] After 5 h, the reaction mixture was poured into 200 mL of water. Ethyl acetate was added until clear phase separation resulted. The organic layer was separated and repeatedly washed with water. Washing was stopped when unreacted **1** could no longer be detected in the organic phase (TLC). These washing steps simultaneously removed (most of the) DMSO from the organic phase. The organic phase was separated, dried ($MgSO_4$), filtered, and concentrated to afford crude **4** as a white solid. For analytical purposes, a sample of this material was further purified by column chromatography using ethyl acetate as eluent: $^1$H-NMR ($CDCl_3$) $\delta$ 3.40 (3H, s, Me), 3.60 (3H, s, Me), 3.82 (2H, d, H1′), 4.82 (2H, d, H4′), 5.70-5.80 (1H, m, H2′), 5.92−6.00 (1H, m, H3′), 7.20−7.50 (15H, m, aromatic Hs) , 7.39 (1H, s, H8); $^{13}$C-NMR ($CDCl_3$): $\delta$ 27.97, 29.71, 43.79, 59.99, 69.88, 87.27, 125.03, 127.18, 127.93, 128.55, 132.12, 140.42, 143.69; mass spectrum *m/e* 492.215 ($M^+$ calcd for $C_{30}H_{28}N_4O_3$ 492.216). Anal. Calcd for $C_{30}H_{28}N_4O_3$: C, 73.2; H, 5.7; N, 11.4. Found: C, 75.0; H, 6.0; N, 11.2.

Crude **4** (5.00 g, 10.2 mmol) was dissolved in a mixture of dry nitromethane (80 mL) and dry methanol (20 mL). Zinc bromide (6.85 g, 30.5 mmol) was added, and the solution was stirred for 24 h.[9] Then water (1 mL) was added, and the mixture was concentrated *in vacuo*. The residue, a colored syrup, was dissolved in 20 mL of ethanol. Column chromatography using ethanol as the eluent afforded pure **5** (2.50 g, 10.0 mmol) as a white solid ($R_f$ 0.76); $^1$H-NMR ($CDCl_3$) $\delta$ 3.40 (3H, s, Me), 3.60 (3H, s, Me), 4,35 (2H, t, H1′), 5.07 (2H, d, H4′), 5.51-5.60 (1H, m, H2′), 5.83−5.95 (1H, m, H3′), 7.65 (1H, s, H8); $^{13}$C-NMR ($CDCl_3$) $\delta$ 27.58, 29.43, 43.20, 56.98, 105.88, 124.39, 134.61, 142.01, 148.31, 150.98, 154.36; mass spectrum *m/e* 250.106 ($M^+$ calcd for $C_{11}H_{14}N_4O_3$ 250.107). Anal. Calcd for $C_{11}H_{14}N_4O_3$: C, 52.8; H, 5.6; N, 22.4. Found: C 52.0; H, 5.6; N, 22.2.

**9-*N*-[1′-(4-Azidobenzoyl)-*cis*-2′-buten-4′-yl]theophylline (2).** 4-Azidobenzoic acid[10] (0.90 g, 5.5 mmol), 4-(*N*,*N*-dimethylamino)pyridine (0.19 g, 1.6 mmol), and *N*,*N*′-dicyclohexylcarbodiimide (2.04 g, 10,0 mmol) were added to a magnetically stirred solution of **5** (1.24 g, 5.0 mmol) in 50 mL of dry dichloromethane.[11] The reaction was quenched after 24 h through addition of water (10 mL). Subsequently, all volatile matters were removed *in vacuo*. The residue was taken up in ethyl acetate (50 mL) and washed with water (2×), 5% aqueous acetic acid (3×), and water (2×). These washing steps partly removed the *N*,*N*′-dicyclohexyl urea. The organic phase was dried ($MgSO_4$), filtered, and concentrated. The residue was taken up in dry ethyl acetate (25 mL) and filtered to remove traces of the urea. Concentration−dilution−filtration was repeated several times until no urea resided on the filter. The residue was chromatographed on a silica gel column using ethyl acetate/ethanol (19:1, v/v) as the eluent. This afforded pure **2** (1.96 g, 65%) as a slightly yellowish solid: $^1$H-NMR ($CDCl_3$) $\delta$ 3.40 (3H, s, Me), 3.60 (3H, s, Me), 5.02 (2H, d, H1′), 5.18 (2H, d, H4′), 5.85-5.98 (2H, m, H2′,3′), 7.08 (2H, d, aromatic Hs), 7.72 (1H, s, H8), 8.03 (2H, d, aromatic Hs); $^{13}$C-NMR ($CDCl_3$) $\delta$ 27.90, 29.70, 43.57, 59.87, 106.75, 118.81, 126.10, 128.18, 128.36, 131.39, 140.88, 145.00, 148.75, 151.56, 155.15, 165.45; mass spectrum *m/e* 395.134 ($M^+$ calcd for $C_{18}H_{17}N_7O_3$ 395.134). Anal. Calcd for $C_{18}H_{17}N_7O_3$: C, 54.7; H, 4.3; N, 24.8. Found: C, 55.5 ; H, 4.8; N, 24.3. UV spectroscopy (isopropyl alcohol) $\lambda_m$= 272 nm, log $\epsilon$ = 4.35; $\lambda_m$= 202 nm, log $\epsilon$ = 4.48.

**b. Surface Modification/Immobilization.** Surface modification was carried out with a medical-grade polyetherurethane (Pellethane 2363 55-D from Dow Chemical, thickness 0.13 mm). Pieces of 30 × 40 mm were cut and cleaned by repeated washing with isopropyl alcohol. Then, the foils were dried *in vacuo*. Each dry and clean foil was pressed between two horizontal glass plates (60 × 30 × 4 mm), held together by two metallic clamps. The upper glass plate contained a circular hole with a diameter of 20 mm. A solution of **2** in isopropyl alcohol (5 mM, 200 $\mu$L) was transferred into the hole. The device was placed in a vacuum oven which was thermostated at 40 °C. Horizontal positioning of the device was carefully maintained. First, the solvent was allowed to evaporate at atmospheric pressure. Secondly, the pressure was lowered to remove last traces of solvent. The device was removed from the vacuum oven and exposed for 15 min to UV light, using a medium-pressure metal halide lamp. The lamp is made of ozone-free quartz and constructed such that optimum UV-A is emitted. Technical details on the lamp: nominal watt power: 930 W; lamp voltage: 133 ± 15 V; radiation output ($\mu$W/cm$^2$, measured at 1.00 m distance): UV-A: 2500; UV-B: 900; UV-C: 230. After irradiation, the foil was removed from the glass device and washed extensively, first with isopropyl alcohol and then with water. The foils were dried in air and stored in dry form.

**c. Physicochemical Characterization.** The modified surface was characterized using X-ray photoelectron spectroscopy (XPS), attenuated total reflection infrared (ATR FT-IR) spectroscopy, and water contact-angle measurements. The modified surface was compared with a control surface, designated surface A. Preparation of surface A was performed exactly according the procedure as described above; the only difference was that the circular hole in the glass device was filled with pure isopropyl alcohol, instead of the 5 mM solution of **2** in isopropyl alcohol.

For the XPS experiments, polymer foils were mounted on a standard sample holder and inserted via a separately pumped load lock into a Leybold MAX200 XPS instrument. A sample area of 7 × 4 mm$^2$ was analyzed. Mg K$\alpha$ (1253.6 eV) radiation of a Mg/Al double anode X-ray tube (13 kV, 20 mA) was used. The spectrometer was calibrated using Ag, Cu, and Au, according to ref 12. At the used pass-energy of 48 eV, the FWHM for Ag 3d5/2 was 1.1 eV. The base pressure in the analysis chamber was well below 1 × 10$^{-9}$ mbar. The instrument was controlled by a HP A400 computer and a Leybold DS100 data system was used for data aquisition and analysis. For the quantitative analysis the spectra were corrected for the analyzer transmission function according to ref 13, and a linear background was subtracted. Sensitivity factors were calculated, using the approach of Nöller et al.[14] and the Scofield cross-sections.[15] For energy referencing a C 1s binding energy of 285.0 eV for aliphatic carbon was used.[16] ATR FT-IR spectra of the modified surface and surface A were recorded on a Bio-Rad FTS-60 spectrophotometer. Water contact angles were measured with the laser-beam method, according to ref 17.

**d. Biochemical Characterization.** The modified surface was subjected to the *in vitro* thrombogenicity assay as described in our previous work.[18] As reference materials, we used surface A, polyethylene (PE), and polyvinyl chloride (PVC).[19] Note that the blood plasma (either PRP or PFP) was anticoagulated with citrate prior to the *in vitro* thrombogenicity assay; the addition of free $Ca^{2+}$ (in excess to citrate) marks the actual start of the experiment. Anticoagulation with heparin

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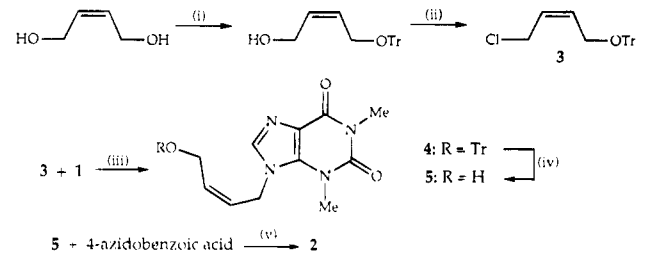
**Scheme 1**[a]




[a] (i) TrCl in pyridine; (ii) *p*-toluenesulfonyl chloride, 4-(*N*,*N*-dimethylamino)pyridine, $NEt_3$ in $CH_2Cl_2$, see ref 8; (iii) KOH in DMSO, see ref 8; (iv) $ZnBr_2$ in $MeNO_2$/MeOH; (v) DCC, 4-(*N*,*N*-dimethylamino)pyridine in $CH_2Cl_2$.

is impractical, as the effect of heparin cannot be eliminated as instantaneously and effectively, as for the system citrate−$Ca^{2+}$. Scanning electron microscopy (SEM) was used to study morphology of blood platelets, adhered to the four different surfaces after incubation with platelet-rich blood plasma (PRP).[18d] Furthermore, a flat parallel plate perfusion chamber was used to study platelet adhesion under PRP-flow conditions. The device used was a modification of the perfusion chamber described by Sakariassen et al.[20] In a polymethylmethacrylate block (dimensions 75 × 26 × 7 mm), a central hole with a depth of 0.2 mm, a width of 4.5 mm, and a length of 28 mm was made. The chamber volume is 30 μL. The two inlets of the chamber (1 mm i.d.) gradually taper off over 7 mm to the rectangular cross section of the central hole at an angle of 20°. A rectangular piece of the test material (dimensions: 60 × 10 × 0.2 mm) served as the roof of the flow chamber; approximately 1.5 $cm^2$ of the test surface was in contact with flowing PRP. The wall shear rate was calculated from the formula: $\gamma = 6Q/bd^2$, where $Q$ is the volumetric flow rate ($cm^3/s$), $b$ (cm), and $d$(cm) are the width and depth of the slit, respectively. The flow was controlled by a syringe pump (Harvard Apparatus Co, South Natrick, MA, U.S.A.). In each experiment, the chamber was perfused with PRP for 10 min. Volumetric flow rates of 0.1 or 1.0 mL/min (corresponding to wall shear rates of 50 and 500 $s^{-1}$, respectively) were used. After perfusion, the chamber was rinsed with PBS buffer for 10 min at a wall shear rate of 50 $s^{-1}$. Platelet deposition was determined as described.[18]

## Results and Discussion

**Preparation and Immobilization of Conjugate 2.** Synthesis of **2** is outlined in Scheme 1. First, *cis*-2-butene-1,4-diol was converted into its monotritylated congener by reaction with tritylchloride in pyridine (isolated yield: 84%). This product was reacted with 4-toluenesulfonic acid in the presence of 4-(*N*,*N*-dimethylamino)pyridine and triethylamine, affording **3** in 75% isolated yield. Compound **3** was coupled with theophylline after Zemlicka *et al.*[8] This reaction proceeds in DMSO, in the presence of $K_2CO_3$. *A priori*, formation of two reaction products is expected, one through coupling at N7, and one through coupling at N9. In line with the results of Zemlicka et al., who performed analogous couplings with adenine,[8] we observed reaction at N9 with >99% regioselectivity. Subsequently, the trityl group was cleaved by reaction with $ZnBr_2$ in nitromethane/methanol. Isolated yield after coupling and detritylation: 65%. The resulting alcohol was reacted with 4-azidobenzoic acid,[10] thus yielding conjugate **2** in 65% isolated yield.

Compound **2**, adsorbed on the polyurethane foil, was irradiated with UV light. This induces a photoreaction of the aryl azide, presumably via a 1-azacyclohepta-1,2,4,6-tetraene intermediate.[21] This highly reactive intermediate can react with nucleophilic sites at the polymer surface (e.g., N−H of the urethane amide groups). In this way, a covalent link between theophylline and the polymer surface is generated.

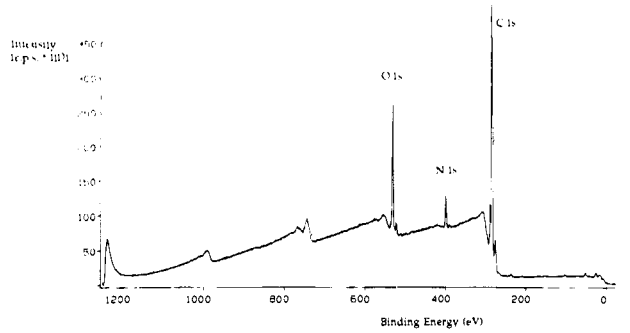



**Figure 1.** XPS scan of the modified surface.

**Table 1.** Elemental Atomic Percentages and Elemental Ratios Measured by XPS for the Modified Surface and Surface A

| | percentage | |
|---|---|---|
| atom | modified surface | surface A |
| C | 79 | 81 |
| O | 16 | 16 |
| N | 4.8 | 2.9 |
| | **elemental ratio** | |
| atom | modified surface | surface A |
| C/O | 4.9 | 5.1 |
| C/N | 16.5 | 27.9 |

**Surface Characterization.** Both the modified surface and surface A were studied with XPS. As expected, oxygen, nitrogen, and carbon are detected. No contaminations were found. Figure 1 shows the XPS surface scan of the modified surface. The O 1s, N 1s, and C 1s narrow-scan spectra were used to get more detailed chemical information. The spectra were analyzed by applying curve deconvolution, using a Gaussian line shape. The observed binding energies for the oxygen, nitrogen, and carbon species are in good agreement with those of Beamson and Briggs.[16] Table 1 gives the atomic % C, N, and O, and the elemental ratios C/O and C/N for the modified surface and surface A, as calculated from the XPS spectra.[22] These numbers provide an indication of how the elemental concentrations of O, N, and C vary from the modified surface to surface A. The decrease of the elemental ratio C/N from 27.9 for surface A to 16.5 for the modified surface indicates an increase of the nitrogen concentration in the top layer, which has to arise from the immobilized conjugate molecule. A comparison of the narrow-scan XPS spectra of surface A and the modified surface shows small but significant differences. Firstly, as already observed in the surveys, the nitrogen concentration increases from 2.9 to 4.8 atom %. Secondly, there are changes in the surface chemistry, which can be derived from changes in the peak-shape of the narrow-scan spectra. Figure 2 shows expansions of the O 1s, N 1s, and C 1s narrow-scan spectra, together with the difference spectra.

(19) The polymers PE (batch MöS 2790) and PVC (polyvinylchloride with tri(ethylhexyl)trimellitat as plasticizer, batch 29 859) were a kind gift of Dr. W. Lemm, Eurobiomat, Berlin, Germany. The polymers were 50 μm films.

(20) Sakariassen, K. S.; Aarts, P. A. M. M.; de Groot, P. G.; Houdijk, W. P. M.; Sixma, J. J. *J. Lab. Clin. Med.* **1980**, *102*, 522.

(21) (a) For a recent review on the photochemistry of aryl azides, see: Schuster, G. B.; Platz, M. S. *Adv. Photochem.* **1992**, *17*, 69. (b) Nielsen, P. E.; Buchardt, O. *Photochem. Photobiol.* **1982**, *35*, 317. (c) *Azides and Nitrenes;* Scriver, E. F. V., Ed.; Academic Press, Inc.: Orlando, FL, 1984.

(22) The relative intensities (the concentrations) of the different chemical groups present in the topmost surface layers of surface A can be used to calculate the hard segment to soft segment ratio. From the ether/carboxyl oxygen ratio (= 4.5), the ether/carboxyl carbon ratio (= 8.1), and from the observed nitrogen concentration, the hard segment to soft segment ratio of the polyurethane can be estimated to approximately 1:9.

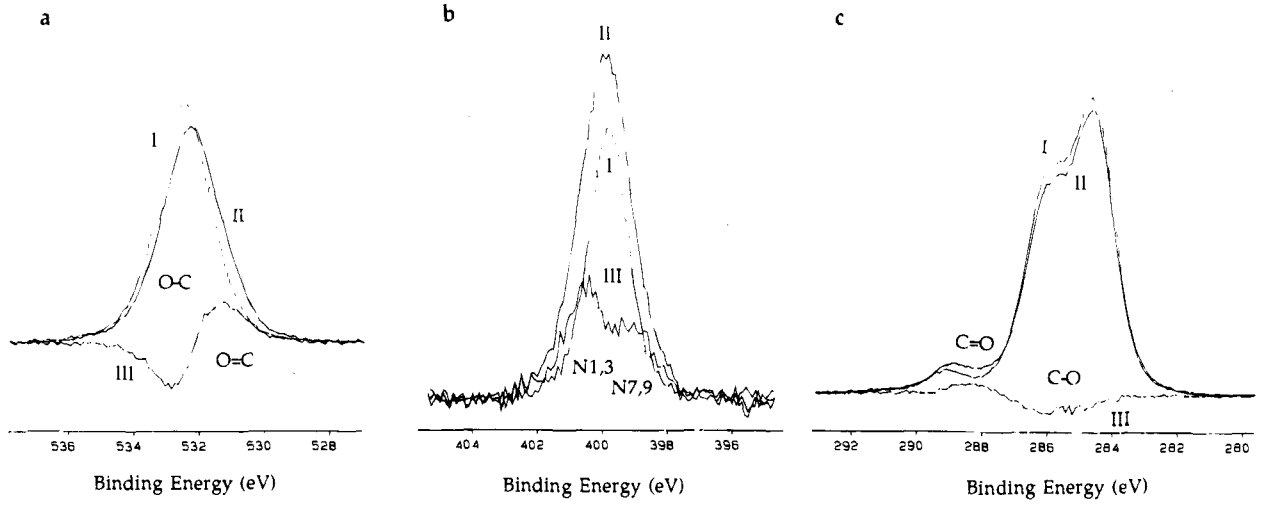



**Figure 2.** Narrow-scan XPS spectra. I: surface A; II: modified surface; III: difference spectra. (a) O 1s spectra showing that C−O decreases and C=O increases after surface modification. (b) N 1s spectra showing that the nitrogen content in the topmost layer increases upon surface modification. Also, the difference spectrum indicates that the surface modification is associated with introduction of another type of nitrogen. (c) C 1s spectra, also showing that the surface modification leads to decreased C−O and increased C=O content at the surface.

More detailed expansions are provided in the supporting information. The O 1s spectra (Figure 2a) indicate an increase of the C=O and a decrease of the C−O concentration. The N 1s line of the modified surface is more intense (Figure 2b). This intensity increase and broadening suggest the presence of two other nitrogen functionalities. The C 1s spectrum (Figure 2c) shows a higher carboxyl concentration and a decrease of the C−O functionality. All this information indicates that immobilized theophylline is indeed present on top of the modified surface. Although it is difficult to calculate the actual concentration, the XPS data indicate that the concentration is relatively high.

ATR FT-IR measurements, also performed for the modified surface and surface A, produced highly similar spectra. Only one subtle difference could be detected: a small extra peak at 1660 $cm^{-1}$ for the modified surface. It is important to note that the IR spectrum of **2** shows four strong lines, i.e., at 2120, 1670, 1650, and 1260 $cm^{-1}$. The bands at 2120 and 1260 $cm^{-1}$ are due to the azide group, while the other bands can be ascribed to the C=O groups of the ester and the heterocycle. The FT-IR spectrum of the modified surface is therefore consistent with the presence of surface-immobilized theophylline. The bands at 2120 and 1260 $cm^{-1}$ should disappear as the azide group reacts in the photoreaction, and the bands at 1670 and 1650 $cm^{-1}$ are expected to stay. The IR spectrum of **2** as well as the FT-IR spectra of the modified surface and surface A, are provided as supporting information.

The modified surface and surface A were also compared with respect to the water contact angles. These were measured according to the laser beam method.[17] Six specimens of the modified surface and five specimens of surface A were used. On each specimen, a small water drop was placed, and reflections were measured on both the left and right edges. The data are compiled in Table 2. It is obvious from these results that the surface has become more hydrophilic upon the photoreaction. This is as expected, since theophylline is capable of forming hydrogen bonds with surrounding water molecules. It should be realized that the contact angle data do not provide exclusive proof for the presence of theophylline on the surface, nor can they be taken as a measure for the amount of theophylline per surface unit.

**Biochemical Characterization.** *In vitro* thrombogenicity tests were performed according to the procedure as described previously.[18,23] The assay produces a thrombin generation curve for each surface tested. The most important parameter abstracted from a thrombin generation curve is the lag time, i.e., the time elapsing between the moment of recalcification of the plasma and the moment on which the free-thrombin concentration starts to increase. The test was performed with the modified surface, surface A, and the EUROBIOMAT controls polyethylene (PE), and polyvinyl chloride (PVC).[19] Two test runs were made, one with PRP, and one with PFP. When PRP is used, the combined effect of contact activation and surface-induced activation of platelets is measured. Using PFP, only the effect of contact activation is monitored. Figure 3 shows four thrombin-generation curves, three refer to independent experiments with the modified surface in contact with PRP, and one corresponds with surface A in contact with PRP. Table 3 gives a full compilation of the lag times measured for the different surfaces. Another important parameter, the maximum concentration of free thrombin, reached during each experiment, is also listed in Table 3. It should be noted that all thrombin generation curves were corrected for the residual amidolytic activity of the thrombin−$\alpha_2$-macroglobulin complex, according to the method of Hemker et al.[24] The results in Table 3 show that the thrombogenicity of the different surfaces *in contact with PRP* decreases in the following order: PE > surface A > PVC > modified surface. Noteworthy, the surface modification leads to a pronounced reduction of the thrombogenicity of the polyurethane; the lag time is seen to increase from 549 s (surface A) to approximately 1270 s. The results obtained with PFP do *not* show large differences between the different surfaces (Table

**Table 2.** Contact Angles Measured by the Laser-Beam Method for the Modified Surface and Surface A[a]

| modified surface | | surface A | |
|---|---|---|---|
| left | right | left | right |
| 50 | 52 | 70 | 72 |
| 48 | 50 | 75 | 77 |
| 62 | 55 | 70 | 70 |
| 50 | 50 | 70 | 68 |
| 55 | 57 | 70 | 70 |
| 50 | 52 | | |

[a] Mean for modified surface: 53 deg, standard deviation: 4 deg; mean for surface A: 72 deg; standard deviation: 3 deg.

(23) Lindhout, Th.; Baruch, D.; Schoen, P.; Franssen, J.; Hemker, H. C. *Biochemistry* **1986**, *25*, 5962.

(24) Hemker, H. C.; Willems, G. M.; Béguin, S. A. *Thromb. Haemost.* **1986**, *56*, 9.

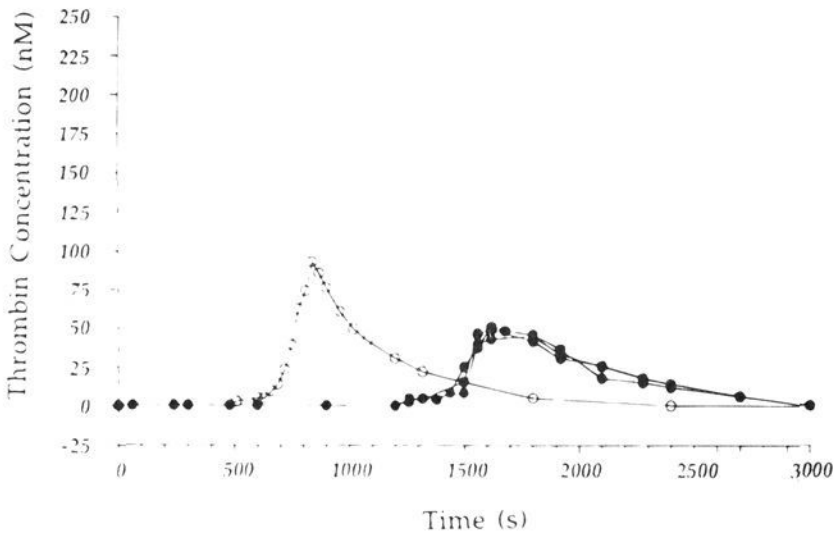



**Figure 3.** Thrombin generation curves measured for the modified surface (filled circles, measured *in triplo*) and measured for surface A (open circles). The increased lag time as a result of surface modification is clearly visible. The thrombin generation curves were corrected for the amidolytic activity of the $\alpha_2$m-thrombin complex, according to ref 24.

**Table 3.** Results of the Thrombin Generation Assay Using PRP and PFP

| | platelet-rich plasma | | platelet-free plasma | |
|---|---|---|---|---|
| surface | lag time (s) | max thrombin concn (nM) | lag time (s) | max thrombin concn (nM) |
| modified | 1257 | 51 | 698 | 163 |
| | 1283 | 49 | | |
| | 1272 | 45 | | |
| surface A | 576 | 93 | 549 | 237 |
| PE | 278 | 217 | 485 | 217 |
| PVC | 707 | 75 | 582 | 180 |

3). Also, the effect of the modification is only marginal; the lag time is found to increase from 549 s (surface A) to only 698 s. Taken together, these data point out that theophylline, immobilized on the surface of the polymer, markedly inhibits activation of blood platelets, while contact activation is largely unaffected. Noteworthy, Bamford et al. reached essentially the same conclusion in their studies focussed on theophylline covalently coupled to water-soluble polymers.[6]

As in our previous work, we also performed an *in vitro* static platelet adhesion test.[18a-c] The modified surface, surface A, PE, and PVC were incubated with citrated PRP for 15 min, washed, and prepared for scanning electron microscopy (gold sputtering). The PE surface was found to be fully covered with spread and aggregated platelets. PVC and surface A gave similar micrographs, showing extensive but not complete covering of the surfaces with activated platelets. On the other hand, the modified surface was covered to only a small extent (< 10%). The adhered platelets showed virtually no surface-induced morphology changes. Scanning electron micrographs of the four surfaces are provided in the supporting information. Figure 4A is a typical magnification of platelets adhered to surface A. Formation of pseudopods, spreading, and aggregation are clearly visible, i.e., the platelets are activated. Figure 4B is a typical magnification of platelets on the modified surface. In this case, it is seen that the adsorbed platelets hardly changed shape. Some small pseudopods can be found, but spreading and/or aggregation are not observed at all.

Furthermore, the modified surface and surface A were tested in a parallel plate flow chamber. This type of experiments provides insight into platelet adhesion under flow conditions.[20] Citrated PRP was led through the flow chamber at four different wall shear rates: 12.5, 25, 50, and 300 $s^{-1}$ (corresponding to the flow-rates 25, 50, 100 and 600 $\mu L \cdot min^{-1}$). Perfusions with 12.5 and 50 $s^{-1}$ shear rate lasted 30 min, but those with 25 and

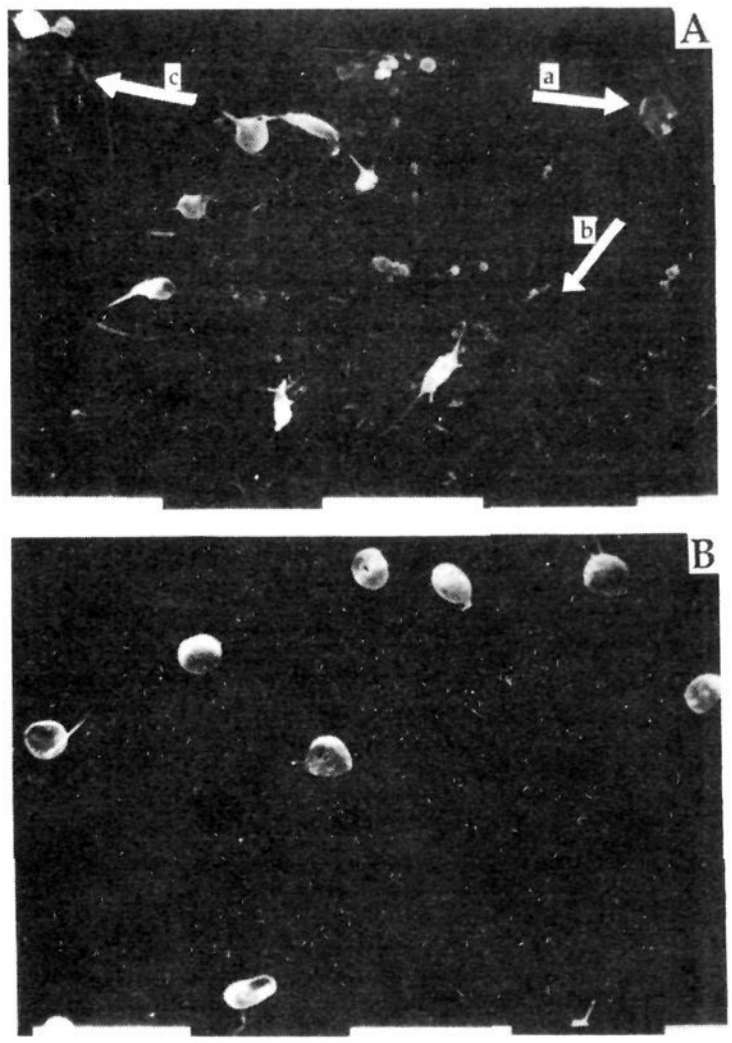



**Figure 4.** (A) Typical detailed scanning electron micrograph of platelets adhered to surface A. Activation (spreading and formation of pseudopods) is clearly visible. (B) Typical detailed scanning electron micrograph of platelets adhered to the modified surface. Note that these platelets retained their discoid shape. Length of the bar: 10 $\mu$m.

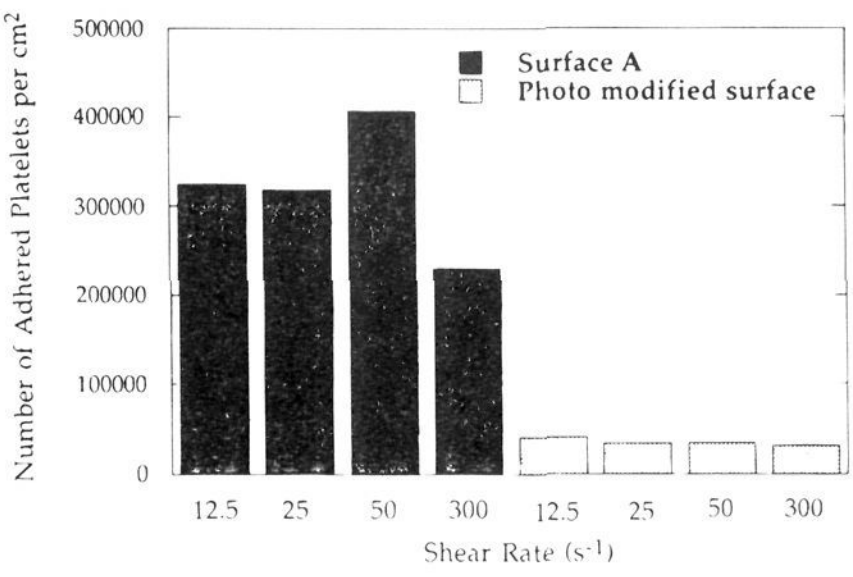



**Figure 5.** Histogram showing the density of adhered platelets for surface A (black) on the modified surface (dashed), measured in the parallel-plate flow chamber for different shear stresses.

300 $s^{-1}$ shear were stopped after 10 min. After each perfusion, the chamber was flushed with PBS buffer (see: Experimental Section). Then, the specimens were removed from the chamber and prepared for scanning electron microscopy as in the static platelet adhesion test (vide supra). For each specimen, the surface-density of adhered platelets was determined. The results are depicted in Figure 5. The data reveal that platelet adhesion decreases substantially as a result of the surface modification. The density of adhered platelets is reduced by approximately one order of magnitude. Apparently, this effect is not impaired by increasing the shear rate up to 300 $s^{-1}$, and/or by increasing duration of the perfusion up to 30 min.

It is important to note that, *a priori*, most of the observations made in this work could be due to theophylline leaching off the modified surface. In order to investigate this possibility, we have executed a series of experiments, comprising 24

identical specimens of the modified surface. Six specimens were incubated with citrated human PRP, for 1, 2, 4, 8, 16, and 48 h. Six other specimens were incubated with citrated human PFP (same time intervals); six were sonicated in demineralized water at 37 °C (same time intervals), and six were immersed in hot water (80 °C, same time intervals). In all cases, the surface-to-volume ratio was 0.75 mL/0.5 $cm^2$. The liquid phase was centrifuged (in the case of PRP or PFP) and filtered (50 $\mu$m). The filtrates were subjected to HPLC analysis, using a C18 reverse-phase column (i.d. 4 mm, length 200 mm), $H_2O-CH_3CN$ (80−20 v/v) as eluent, and UV extinction ($\lambda$ 270 nm) for detection. The system was set up such that theophylline had a retention time of approximately 12′30″; the detection limit was as low as 0.05 $\mu$M. In none of the 24 cases, a theophylline peak could be detected, i.e., if there is any leaching of theophylline, the concentration in the supernatant is lower than 0.05 $\mu$M. Subsequently, the thrombin generation test (*vide supra*) was run with untreated polyurethane surface and a set of PRP batches to which free theophylline was added in different concentrations (0, 0.05, 0.10, 0.20, and 1.0 $\mu$M). The resulting lag times are 540/550 s (duplo, no theophylline added), 550/562 s (duplo, 0.05 $\mu$M theophylline), 556/540 s (duplo, 0.10 $\mu$M theophylline), 560 s (single experiment, 0.10 $\mu$M theophylline), and 570 s (duplo, 0.20 $\mu$M theophylline), and 620/630 s (duplo, 1.00 $\mu$M theophylline). Thus, a clear effect of free theophylline is seen only if the concentration is > approximately 1 $\mu$M. Taken together, these results show that the substantial reduction of the thrombogenicity of the photomodified surface (vide supra) must be due to the presence of immobilized theophylline and *not* to leaching. In fact, we have no positive evidence of any leaching of theophylline after photoimmobilization and subsequent washing steps.

## Concluding Remarks

The present results show that UV-irradiation of conjugate **2**, adsorbed onto the surface of a polyurethane sheet (Pellethane 2363 55-D), leads to a marked decrease of the surface thrombogenicity. The results of the physicochemical and biochemical experiments are clearly consistent with the idea that theophylline is linked to and exposed at the surface of the modified polymer. Our biochemical analyses reveal that the modified surface exerts a strong inhibitory effect with respect to activation of blood platelets. This finding is in agreement with studies reported by Bamford et al., who covalently attached theophylline to a series of water-soluble polymers.[6]

There is an analogy between our method of surface modification, on one hand, and other well-known processes based on photoreactivity of aryl azides, on the other hand (e.g., photoaffinity labeling, photodying of textiles and paper).[25] Therefore, our tentative explanation of the present results is that irradiation of **2** first transforms the 4-azidobenzoyl group into a highly reactive species (presumably 1-azacyclohepta-1,2,4,6-tetraene, *vide supra*),[21] which is trapped by a nucleophilic group at the polymer surface. It should be noted, however, that our XPS measurements do not provide conclusive evidence for this type of covalent attachment of theophylline. Our future work will be concentrated on defining the scope of this photochemical surface modification with respect to the development of new bloodcompatible artificial surfaces.

**Supporting Information Available:** XPS scan and narrow-scan XPS spectra and ATR-FTIR spectra of surface A and the modified surface, IR spectrum of **2**, and scanning electron micrographs of platelets (15 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(25) See, for instance: (a) Griffiths, J.; McDarmaid, I. *J. Soc. Dyes and Color* **1977**, *93*, 455. (b) Griffiths, J.; Fagbule M.; McDarmaid, I. *Textilveredlung* **1972**, *12*, 807. (c) Griffiths, J.; McDarmaid, I. *J. Soc. Dyes and Color* **1978**, *94*, 65.