An *in vitro* study of the adhesion of blood platelets onto vascular catheters. Part I.

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The adhesion of human blood platelets onto vascular catheters was studied using a specially designed perfusion chamber. Polyurethane catheters were exposed to citrated human blood for different periods (up to 20 min) and at different wall shear rates (190, 260, 330 sec^{-1}). The rate of platelet adhesion was determined using ^{111}In-labeled platelets, while the morphology of adhering platelets was investigated using scanning electron microscopy. A linear increase in platelet adhesion was found within the first 10 min of perfusion, after which a plateau value was reached. The number of adhering platelets did not vary significantly with the shear rates applied, which may indicate that within the range of shear rates studied, the adhesion of platelets onto the catheter surface is mainly determined by the rate of the reaction between the platelets and the material surface. Catheters coated with a conjugate of heparin and albumin showed a four- to five-fold reduction in platelet adhesion as compared to uncoated catheters. This reduction in platelet adhesion was not only due to the presence of albumin moieties at the surface but also to the presence of heparin residues in the adsorbed albumin–heparin conjugate.

INTRODUCTION

Currently, intravascular catheters are widely applied in clinical practice, but thromboembolic complications,[1–3] infections, and inflammatory responses[4] have been frequently reported. When indwelling catheters are used, the percentage of complications is increased significantly.[5–6]

Although the number of complications is certainly related to the experience of the clinician introducing and positioning the catheters,[7] both physicochemical and mechanical properties of the catheters (surface roughness, chemical composition, surface charge, etc.)[8–10] are of major importance.

The blood compatibility of intravascular catheters can be improved by immobilization of the anticoagulant heparin at the external surfaces.[11–20] A promising approach for improving blood compatibility was reported by Hennink, et al.[21] who used a covalently coupled albumin–heparin conjugate (alb-hep) combining the antithrombogenic properties of albumin[22] and heparin. Physical adsorption of the conjugate onto different polymeric surfaces resulted in a substantial improvement of the blood compatibility of these materials *in vitro*.

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The use of flow devices for the investigation of blood–material interaction phenomena *in vitro* with a variety of both newly developed and commercially available polymeric materials has been reviewed by Olyslager.[23]

Several investigators have emphasized the critical role of flow conditions for the *in vitro* examination of biomedical materials.[24–26] An increase in both thrombus formation and platelet adhesion onto a number of artificial surfaces with increasing wall shear rate (γ_w) was observed. Baumgartner and Haudenschild[27] devised an annular perfusion chamber to study the blood platelet interaction with subendothelium under flow conditions corresponding to those found in human veins and large arteries (vessel wall shear rates less than 800 sec^{-1}). The same flow cell was used by Sakariassen[28] to elucidate the role of factor VIII-von Willebrand factor, in platelet adhesion onto human subendothelium *in vitro*.

In our study, a modified Baumgartner cell was used to measure platelet adhesion onto polyurethane catheters as a function of both wall shear rate and perfusion time. In addition, the influence of albumin and alb-hep treatments of these catheters on platelet adhesion and morphology was investigated.

MATERIALS AND METHODS

Perfusion system

The perfusion system used was similar to that described by Sakariassen et al.[28] The perfusate was circulated through an annular catheter cell using a nonocclusive peristaltic roller pump (VRE 200, Verder, Düsseldorf, F.R.G.), while steady flow was obtained by means of gravity. A schematic drawing of the system is presented in Figure 1.

The catheter cell, depicted in Figure 2, is a modification of the original Baumgartner cell. The most important changes concern the central rod of the chamber. Due to the reduced external diameter of this rod (o.d. 1.35 mm) it was necessary to replace the original rod material (PMMA) by stainless steel. Furthermore, an insertion block was introduced to ensure fixation of the catheters under flow conditions. The internal diameter of the PMMA-based flow chamber is 6.35 mm.

Catheters

All experiments were carried out with angiographic Ducor catheters (Cordis Europe, Roden, The Netherlands), which are polyesterurethane based (Estane, B. F. Goodrich Company, Akron, Ohio, U.S.A.). Thin-walled F7 catheters (i.d. 1.35 mm, O.D. 2.30 mm) were cut into pieces with a final length of 6.5 cm, tip closed and cleaned with Freon. Sterilization was performed with ethylene oxide. It should be emphasized that the results obtained with untreated Ducor catheters in this investigation cannot be used as a measure of the antithrombogenic properties of commercially available

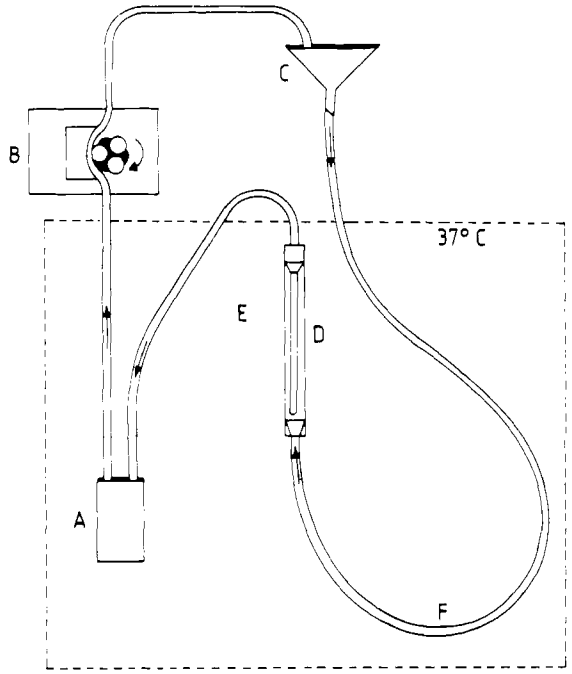

Figure 1. Schematic drawing of the perfusion system according to Saka riassen[28]: A = container with perfusate; B = roller pump; C = funnel; D = perfusion chamber; E = catheter; F = Silastic tubings.

Cordis Ducor catheters because the commercial catheters are provided with an antithrombogenic coating, which was not used in this study.

Albumin heparin conjugate treatment

Heparin from porcine mucosa (160 U/mg, Diosynth, Oss, The Netherlands) was covalently bound to albumin (Sigma A 954, St. Louis, MO) using a water-soluble carbodiimide as a coupling agent.[29] The heparin was used as obtained without further fractionation with regard to molecular weight and/or AT III affinity. The treatment of each individual catheter with the albumin-heparin conjugate was achieved by incubation of the sterilized catheter segments for 2 hr with a solution of alb-hep (5 mg/mL) in phosphate buffered saline (PBS), pH = 7.35. Subsequently a three-step rinsing procedure was carried out with PBS (each step consisting of a 10-min incubation with fresh PBS under static conditions) to remove the excess of alb-hep not firmly attached to the surface. Control experiments were carried out with albumin treated Ducor catheters, in which the catheters were treated with a 5 mg/mL solution of albumin in PBS, while further preparation of the catheters was similar to that used for the alb-hep–treated catheters. After the rinsing procedure, all catheters were kept in PBS until they were used in the perfusion experiments.

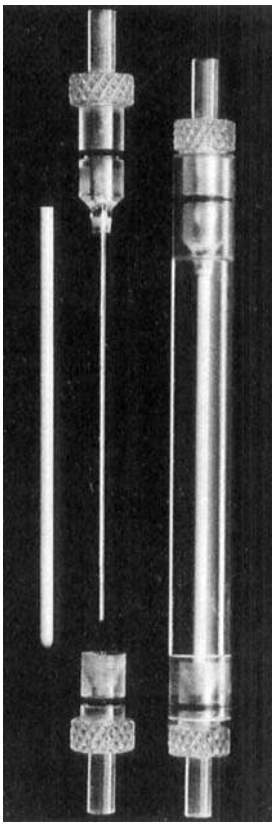

Figure 2. Perfusion chamber (left). On the right the open chamber with next to it a catheter.

Perfusates

Freshly drawn blood from healthy human donors who did not use drugs containing acetyl salicylic acid and/or oral contraceptives was anticoagulated with sodium citrate (1/10 vol, 3.8%). Then platelet labeling, washing, and resuspension of the different blood fractions was carried out according to Sakariassen[28] with the exception of the aspirin treatment. A final concentration of ^{111}In-labeled platelets of 1.2×10^{11}/L was used, while 40% (*v*/*v*) of the perfusate consisted of red blood cells (RBC). The latter is in close agreement with average RBC concentrations found in whole human blood (haematocrit: 0.35–0.50). The contents of a single blood bag (= 500 mL citrated human blood) provided 15–25 perfusates of 15 mL each.

^{111}Indium-labeled platelets and RBC-suspensions were mixed just before testing and incubated for 5 min at 37°C.

Perfusions

Perfusions with untreated catheters were carried out at three wall shear rates (190, 260, and 330 sec^{-1}) and six perfusion times (1, 3, 5, 7, 10, and

20 min). Shear rate variations were accomplished by adjusting the flow rate with the roller pump and the position of the funnel. A comparative study concerning platelet deposition onto untreated, albumin and alb-hep treated catheters was performed at one shear rate (γ_w = 330 sec^{-1}), using the same perfusion times as mentioned above.

All perfusion experiments were carried out at 37°C. Immediately after perfusion, the flow chamber was rinsed with 25 mL 10 mM HEPES-buffered saline, pH = 7.35, to remove all nonadhered blood constituents. The catheter was then removed from the flow chamber and placed in a 2.5% glutaraldehyde solution in PBS, which was used as a fixative for the surface-bound platelets.

Determination of wall shear rate

The wall shear rate at the catheter surface under controlled flow conditions in the catheter cell was calculated from the fluid dynamic equation derived by Bird et al.[31]:

$$\gamma_w = \frac{2Q}{\Pi R_0^3} \cdot \frac{[2k/(1 - k^2)] + [(1/k \ln k)]}{1 + k^2 + (1 - k^2)/\ln k}$$

where γ_w = the catheter wall shear rate (sec^{-1}), Q = flow rate (cm^3 sec^{-1}), R_0 = internal radius of the flow cell (cm), R_c = (outer) radius of the catheter (cm), and $k = R_c/R_0$. This relationship is valid for a fully developed (laminar) flow of a Newtonian fluid in the anulus.

Since blood behaves Newtonian at shear rates >100 sec^{-1},[32] it is acceptable to use this equation for shear rate calculations in the catheter flow cell. Furthermore, Aarts et al.[33] showed this assumption to be correct by Laser-Doppler velocimetry experiments using a flow chamber with a similar geometry.

As a consequence of both the restricted flow rate capacity of the roller pump in the perfusion system described (max ca 150 cm^3/min) and the dimensions of the catheter cell (internal diameter = 0.64 cm), a maximum wall shear rate at the catheter surface of 330 sec^{-1} could be obtained (k = 0.36).

Determination of surface-bound platelets

After perfusion, catheters were cut in five sections of 1.05-cm length each (surface area per segment: 0.726 cm^2) with a specially designed cutting knife, starting at the closed end of the catheters. The residual 1.25 cm was not used for further examination. Surface-bound radioactivity (expressed in cpm/cm^2) was determined for all sections by means of a gamma counter (Trigamma 600, Baird Atomic Inc., Bedford, MA).

Specific platelet activity (cpm/platelet) was calculated from platelet counts (Thrombocounter, Coulter Electronics Ltd., Harpender, U.K.) and total radioactivity (cpm) of the final platelet resuspensions. The number of adher-

ing platelets per cm^2 catheter surface was obtained by dividing surface bound radioactivity by the radioactivity per platelet.

Morphological evaluation

Glutaraldehyde fixation (one week) of the catheter segments was followed by a dehydration procedure using a series of ethanol–water mixtures (ethanol contents: 25, 50, 75, and 100% (*v/v*), respectively, 5 min incubation with each solution), after which the samples were dried under flowing N_2, glued to alumina discs and finally gold sputtered to achieve optimal resolution in the scanning electron microscopical analysis. The electron microscope used was an ISI SS 40 (International Scientific Instruments Inc., Santa Clara, CA).

Hemolysis and aggregation

Hemolysis of red blood cells as a function of perfusion time was determined by measuring the free hemoglobulin concentration spectrophotometrically at 567 nm (Ref. 34) after centrifugation (10 min at 2000*g*) of the perfusates.

The influence of the washing procedure on platelet size distribution and ADP induced aggregation (final ADP concentration = 10 μM) was investigated with a blood cell analyzer (Baker Diagnostic Hematology Series 810, Baker Instruments, Bethlehem, PA) to ascertain that perfusions were carried out with blood samples containing functional platelets.

RESULTS

Influence of wall shear rate

Platelet deposition onto untreated Ducor catheters (n = 100) at three different wall shear rates (190, 260, and 330 sec^{-1}) was measured as a function of perfusion time. The results are summarized in Figure 3. From the figure it appears that the adhesion of blood platelets onto Ducor catheters increases linearly with time during the first 10 min of perfusion, while plateau values (ca 15×10^5 plt/cm^2) are found after 10 min of blood contact. With the three different wall shear rates used in the present study, no significant difference in platelet adhesion values were observed.

Alb-hep treatment

Preliminary adsorption studies using ^{125}-Iodine labeled alb-hep (5 mg/mL in PBS) showed a surface concentration on Ducor catheters of 0.8 ± 0.4 μg/cm^2 (± s.d., n = 8) after 2 h incubation under static conditions

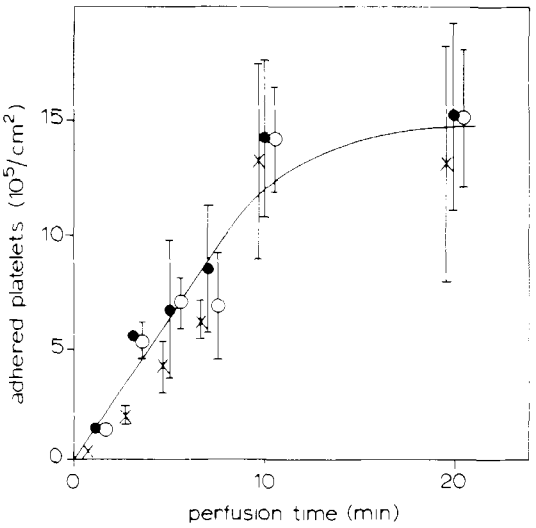

Figure 3. Platelet deposition on Ducor catheter surfaces as a function of perfusion time and shear rate: ● = 190 sec^{-1}, × = 260 sec^{-1}, ○ = 330 sec^{-1}.

(t = 20°C). This value is in close agreement with the results found for alb-hep adsorption onto a number of other polymeric materials.[35]

From desorption experiments with perfusate under flow conditions (γ_w = 330 sec^{-1}), it appeared that after 20 min of perfusion, 25% of the amount of alb-hep initially present on the catheter was desorbed, probably by exchange with other plasma proteins.

A comparative study with respect to platelet adhesion as a function of blood contact time was carried out for the untreated (n = 50), albumin (n = 27), and alb-hep conjugate treated catheters (n = 48) at a constant catheter wall shear rate (γ_w = 330 sec^{-1}). The results, presented in Figure 4, show a strong reduction of the number of adhering platelets for the alb-hep–coated catheters as compared to the untreated catheters over the whole range of perfusion times examined. The platelet adhesion data for albumin-treated catheters are in between those found for the uncoated and alb-hep–coated catheters. Comparing albumin with alb-hep–coated surfaces this difference was significant for t = 20 min (Student's t-test; $0.02 > p > 0.01$). No significant difference was found when the results obtained with the albumin-coated catheters were compared with those of untreated catheters.

Distribution of platelets

The number of adhering platelets for catheter sections (1.05 cm) at various axial distances from the catheter tip were determined as a function of time. Average platelet adhesion numbers on a particular catheter segment as a function of time were calculated from data obtained in a series of experiments carried out at the same conditions.

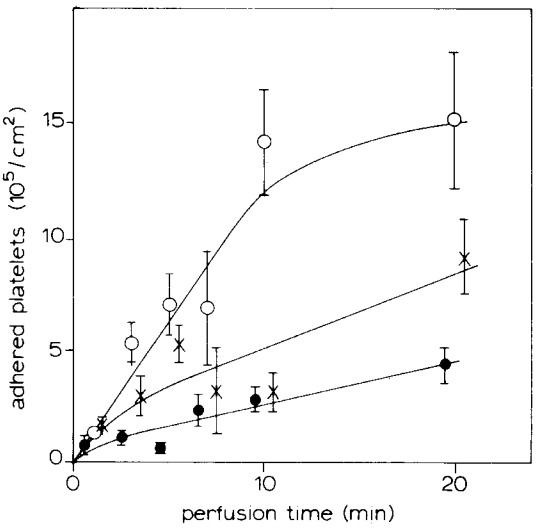

Figure 4. Platelet deposition at constant shear rate (γ_w = 330 sec^{-1}) on Ducor catheter surfaces as a function of perfusion time and surface treatment: ○ = untreated; × = albumin treated; ● = alb-hep treated.

In Figure 5 the results for alb-hep–coated and untreated catheters (n values 4–16) are plotted for the six perfusion times used. A tendency for increasing platelet adhesion onto surfaces positioned at increasing distance from the tip (along the flow direction) was only observed at longer perfusion times.

Similar distribution patterns were observed either when experiments were carried out with untreated as well as treated catheters at γ_w = 190 sec^{-1} and 260 sec^{-1}, nor when the catheters were coated with albumin. (Results not shown.)

Morphological evaluation

Microscopical analysis (SEM) of the platelets attached to both untreated and alb-hep–treated catheters did not show significant differences in morphology after 20 min perfusion. Most platelets were spread on the surface (spread conformation) while pseudopods were fully developed (see Fig. 6b and 6c). In addition, moderate amounts of small clusters of platelets (2–10 plts/cluster) were found regularly distributed over the surfaces (see Fig. 6a). Large aggregates could not be detected.

Hemolysis and aggregation

Both contact of the perfusate with foreign surfaces and air and the use of a peristaltic pump may induce damage of blood cells. Table I shows the free hemoglobin concentrations in the perfusates as a function of perfusion

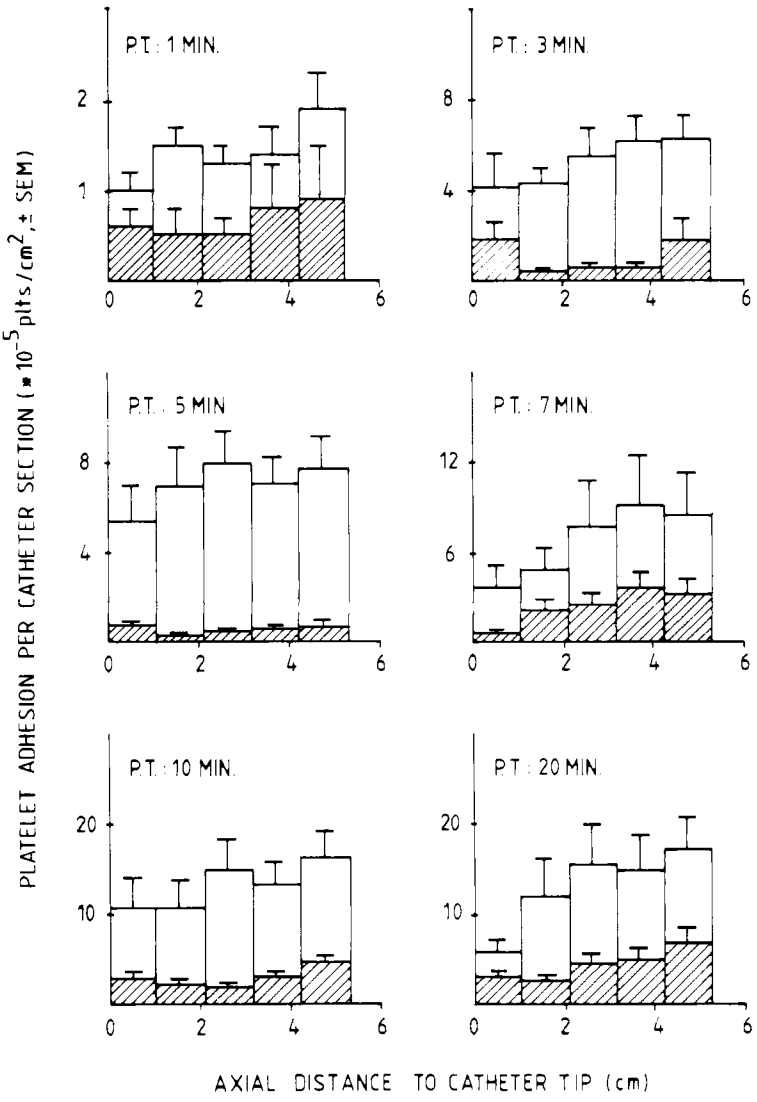

Figure 5. Number of adhering platelets as a function of catheter position for different perfusion times (P.T.): □ = untreated; ▨ = alb-hep coated.

time and shear rate. From this table it appears that a slight increase in free hemoglobin levels is observed with increasing perfusion times.

Table II shows the results of ADP-induced aggregation of platelets in perfusates as a function of both shear rate and perfusion time. This table shows that ADP induced aggregation of platelets is substantially decreased as a function of perfusion time.

We also studied the effect of washing and labeling procedures on platelet distribution and ADP-induced aggregation of platelets. Figure 7 and Table III present the results. It appears that both the platelet distribution and the

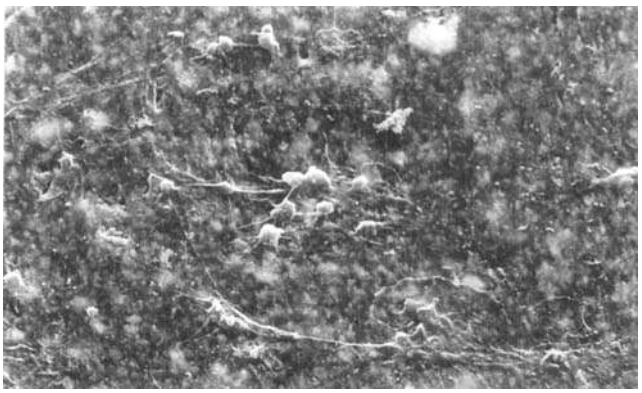

Figure 6a. Representative SEM photograph of platelets adhered on uncoated catheters. Perfusion time: 20 min; $\gamma_w = 330\ sec^{-1}$.

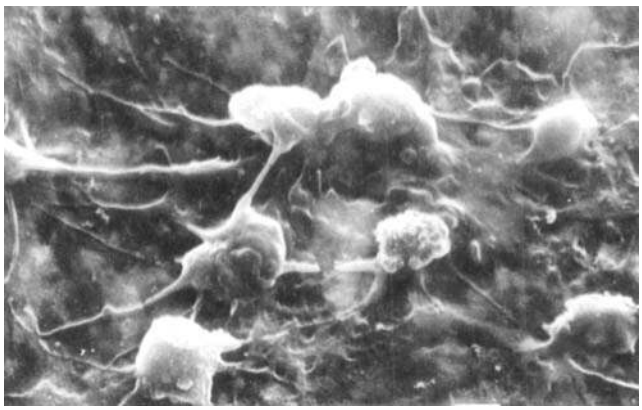

Figure 6b. Representative SEM photograph of platelets adhered on uncoated catheters. Perfusion time: 20 min; $\gamma_w = 330\ sec^{-1}$.

ADP-induced aggregation were not significantly altered by the washing or labeling procedures.

DISCUSSION

Figure 4 shows that the deposition of platelets onto catheters pretreated with alb-hep is substantially reduced (four- to five-fold) as compared to that onto uncoated catheters, emphasizing the promising characteristics of this compound for improving the blood compatibility of biomaterials.

For the adhesion of blood platelets onto foreign surfaces, two extreme cases can be distinguished. First, the adhesion of platelets is determined by

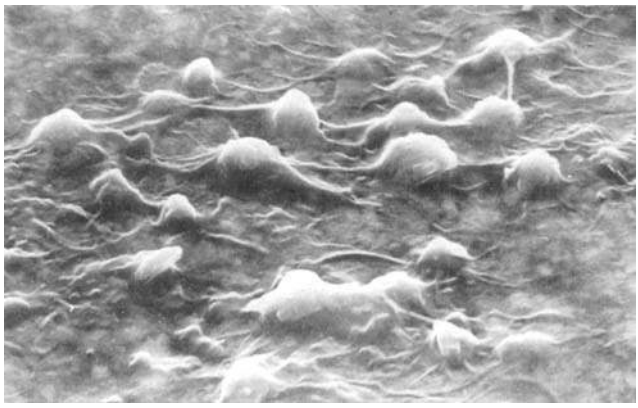

Figure 6c. Representative SEM photograph of platelets adhered on alb-hep–coated catheters. Perfusion time: 20 min; γ_w = 330 sec^{-1}.

TABLE I
Hemoglobin Concentrations (% ± SEM, n = 3–6) in Perfusate as a Function of Perfusion Time and Shear Rate after Perfusion. The Concentration after the Washing and Labeling Procedure (75 μg/mL ± 12 μg/mL (n = 3)) was Taken as 100%

	Hemoglobin concentration (% ± SEM)		
Perfusion time (min)	γ_w = 190 sec^{-1}	γ_w = 260 sec^{-1}	γ_w = 330 sec^{-1}
1	151 ± 64	68 ± 5	144 ± 83
3	60 ± 33	125 ± 28	179 ± 61
5	141 ± 87	127 ± 36	161 ± 45
7	77 ± 20	167 ± 55	167 ± 31
10	124 ± 51	180 ± 9	248 ± 17
20	121 ± 51	237 ± 57	264 ± 32

TABLE II
ADP-Induced Aggregation of Platelets after Perfusion as a Function of Perfusion Time and Shear Rate Indicated as the Height of the Aggregation Curve 5 min after ADP Administration (% ± SEM, n = 3–4 unless indicated otherwise). ADP Aggregation of Platelets after the Washing and Labeling Procedure was Taken as 100%

	Aggregation height (% ± S.E.M.)		
Perfusion time (min)	γ_w = 190 sec^{-1}	γ_w = 260 sec^{-1}	γ_w = 330 sec^{-1}
1	115 (n = 1)	96 ± 9	60 (n = 1)
3	72 ± 27	74 ± 13	77 ± 13
5	38 (n = 1)	63 ± 15	90 (n = 1)
7	51 ± 18	62 ± 12	51 ± 22
10	30 ± 4	35 ± 12	37 ± 4
20	24 ± 1	40 ± 16	27 ± 1

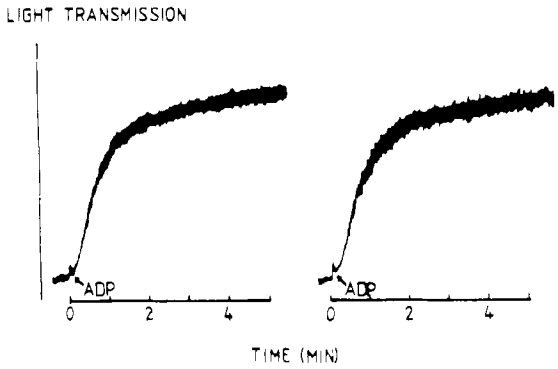

Figure 7. ADP induced aggregation of platelets before (left) and after (right) the washing and labeling procedure.

the reaction rate of the platelets and the material surface; and second, this process is governed by the diffusion of the platelets from the bulk to the material surface.[36] To be able to compare different materials with regard to platelet adhesion, this process should not be diffusion controlled. It is likely that our test system fulfils this requirement because an increased mass transfer of platelets (higher shear rate: higher diffusion coefficient of platelets[36]), does not yield a higher platelet deposition rate.

Another indication that platelet adhesion on our surfaces is not a diffusion controlled process is shown in Figure 5. The numbers of adhering platelets on different catheter segments are similar (or even increasing along the flow direction). For a diffusion-controlled process, a decrease in platelet adhesion numbers along the flow direction would be expected, due to the depletion of platelets in the boundary layer.

These observations suggest that the adhesion of blood platelets on Ducor catheters under the test conditions applied is determined by the rate of reaction of platelets with the material surface.

However, the range of the shear rate values applied is somewhat narrow and additional studies are necessary to establish at which shear rate diffusion limited platelet adhesion will occur.

TABLE III
Platelet Distribution in Platelet-Rich Plasma before and after Washing and Labeling Procedure

	Platelet distribution			
	MPV (μm^3)	PDW	Mode (μm^3)	Medn (μm^3)
Before	6.5	1.55	5.5	6.2
After	6.5	1.56	5.0	5.9

MVP = Mean platelet volume; PDW = Platelet distribution width; Mode = Platelet volume of the largest fraction; Medn = Platelet volume of the median fraction.

In order to perform reliable perfusion experiments it is necessary that the platelets used are equally reactive as platelets normally circulating in the bloodstream. Our results show that after the washing and labeling procedures, neither spontaneous platelet aggregation nor changes in reactivity of the platelets toward ADP (Fig. 7) were observed.

These results suggest that the function of the platelets used was the same as that of nontreated platelets. Since the perfusate is recirculated, it can also be expected that some damage to blood elements will occur. Table I shows that a maximum concentration of free hemoglobin of 198 μg/mL was measured after 20 min perfusion, corresponding with a hemolysis of only 0.2%. On the other hand, Table II shows that a substantial decrease in the function of platelets occurred, as indicated by the ADP-induced aggregation. Therefore perfusion times were limited to 20 min.

From the observation that albumin-coated catheters showed higher platelet adhesion numbers than alb-hep–coated ones (see Fig. 4), it can be concluded that the reduction in numbers of adhering platelets onto alb-hep–coated catheters cannot only be ascribed to the presence of albumin moieties of the conjugate at the surface. This was also concluded from a previous study in which another type of test system was applied and dog blood instead of human blood was used as a source of platelets.[23]

Scanning electron microscope photographs revealed that after 20 min perfusion, clusters of blood platelets with fully developed pseudopods are present on uncoated catheters (Fig. 6a). This suggests that the platelet release reaction has taken place[37] in which platelet aggregating constituents have been released (e.g., ADP).[38]

Figure 6c shows that platelets adhering onto alb-hep conjugate coated catheters have a similar shape as those adhered onto noncoated catheters (see Fig. 6b). In addition, platelet aggregates, as present on uncoated catheters, were also observed on alb-hep–coated catheters.

In conclusion, although platelet adhesion is only one of the parameters needed to assess the blood compatibility of surfaces, the test system described in this paper provides a useful tool for the estimation of the thrombogenicity of catheter surfaces *in vitro* and hopefully can be useful for predicting the performance of the catheters *in vivo*. From experiments using alb-hep–coated catheters, it was found that this coating substantially reduced the adhesion of blood platelets as compared to uncoated catheters.

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