Platelet procoagulant surface as an essential parameter for the *in vitro* evaluation of the blood compatibility of polymers

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Thrombus formation at an artificial surface in contact with blood is the result of the interplay of two tightly linked biological systems, namely blood platelets and blood coagulation. While initiation of the overall process is thought to originate from proenzyme–enzyme conversions at the artificial surface, propagation of the process is only possible when a suitable phospholipid surface is available. The outer leaflet of the plasma membrane of activated platelets is such a surface; it contains negatively charged phospholipids which are normally present in the inner leaflet of the membrane. An examination of the thrombogenicity of materials, therefore, should include a quantitative assay for procoagulant sites at an artificial surface. In the present study we have evaluated polymers, exposed to platelet-rich plasma, for their procoagulant properties by using two sets of assays. With the one set, markers of blood coagulation were assayed (recalcification time of platelet rich plasma and kallikrein-C1-Inhibitor complex formation) and with the other set the surfaces were analysed for platelet adherence and procoagulant sites utilising annexin V, which has a high affinity for negatively charged phopholipids. For the polymers, the fastest rate of contact activation, as determined from kallikrein-C1-Inhibitor generation, was found with polyethylene. In spite of that, the conventional partial thromboplastin time (PTT) could not reveal differences between the various materials. However, when clotting was performed with platelet-rich plasma, it was found that the polymers differed significantly in their clot promoting activities. The shortest clotting time (5 min) was found with polyethylene (PE), and polyvinyl chloride (PVC) gave the longest clotting time (10 min). These findings closely correlated with the amount of procoagulant sites generated at the platelet-rich plasma–polymer interface.

1. Introduction

Thrombus formation at the blood–polymer interface occurs in response to the activation of two tightly linked biological systems [1], namely blood cells (platelet reactions) and plasma proteins (blood coagulation). Whereas initiation of the blood clotting is likely to be triggered by the interaction of the plasma proteins (factor XII and prekallikrein) with the surface, propagation of the process requires the presence of a phospholipid membrane containing negatively charged phospholipids (phosphatidylserine). Such a procoagulant surface becomes available when platelets are activated by the final product of the coagulation pathway, thrombin [2]. Thus, the trace amounts of thrombin that are initially formed by contact activation of the blood coagulation pathway are able to activate platelets, which in turn provides a procoagulant surface to accelerate the generation of thrombin. Obviously, such procoagulant sites are readily formed at the polymer surface when activated platelets bind to the polymer surface. Alternatively, procoagulant sites may also be generated when the interaction of the polymer with platelets causes their activation. Once thrombin generation is started at a polymer surface it is difficult to control, because thrombin bound to the polymer surface and/or bound to fibrin retains its procoagulant activity but escapes from inactivation by its natural inhibitors [3]. As a consequence, during cardiopulmonary bypass, for instance, a high dosage of heparin is required to avoid thrombus formation at, and embolization from, the polymer surface.

We reasoned that for *in vitro* testing of the thrombogenicity of a material or device intended to be used in contact with blood, the generation of cellular procoagulant surfaces must be a parameter to be considered. Therefore, platelet-rich plasma or whole blood should be the test medium of choice. Furthermore, the methods to evaluate the interaction of polymer surfaces with platelet-rich plasma should comprise a quantitative determination of the number of platelets adhering to the surface and an estimation

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of the percentage of total platelets which are providing procoagulant sites and a quantitative assessment of the extent of activation of the blood coagulation pathway. With these test methods we examined the interaction of platelet-rich plasma with polymers that are widely used for the construction of medical devices in contact with blood.

2. Materials and methods

2.1. Materials

The polymers polyethylene (PE; batch MöS 2790), polypropylene (PP; batch 1350248002), polyetherurethane medical grade (PU; batch 910428 CENTR), polyvinyl chloride with tri-(ethyl-hexyl)-trimellitat as plasticizer (PVC-T; batch 29 859) and polyvinyl chloride with di-(ethyl-hexyl)-phthalate as plasticizer (PVC-D; batch 29 858) were a kind gift of Dr W. Lemm, Eurobiomat, Berlin. The polymers were 50 µm films (PE, PVC and PP) or a 250 µm film (PU). Microplates (96 and 24 wells) were from Costar, Cambridge, UK. Citrated platelet-free plasma (PFP) and citrated platelet-rich plasma (PRP) were prepared as described previously [4]. All other reagents were of the highest purity commercially available.

2.2. Methods

2.2.1. Outline of the test protocol

All testing was performed on polymer films folded in the well of a 24-well microplate, unless otherwise stated. Care was taken that the test medium, citrated platelet-rich plasma (PRP), was not in contact with the container. The surface to volume ratio ($3.2\ cm^2/250\ \mu l$) was kept constant for all testings. All materials were before use incubated overnight in a phosphate buffered saline solution (PBS). The folded films were then exposed to citrated PRP (250 µl) for 15 min at 37 °C while the plate was shaken at 150 rpm on an orbit shaker (Lab-line Instruments, Metrose Park, USA). Following this incubation, two sets of assays were performed. The first set comprised methods to evaluate and quantitate platelet reactions at the polymer surface. The wells containing the polymer were washed with PBS and treated with 3.5% formaldehyde. Each of the sheets was then cut in four pieces for further analysis (platelet adhesion and morphology). The second set of assays comprised blood coagulation reactions. First, a sample was taken after the 15-min incubation period and assayed for contact activation reactions (kallikrein-C1-Inhibitor complex). Then, calcium chloride was added to the citrated PRP in the well. The clotting time of the recalcified PRP was recorded as described elsewhere. On several occasions samples were taken after the addition of Ca^{++} ions and assayed for thrombin. The assay procedures are described in detail below.

2.2.2. Kallikrein-C1-Inhibitor assay

The kallikrein-C1-Inhibitor assay was performed according to Nuyens *et al.* [5]. Briefly, aliquots (45 µl) were taken from PRP exposed for 15 min to polymers and were added to 5 µl of PBS containing EDTA (100 mM), Tween (1% wt/vol), Polybrene (0.5% wt/vol), benzamidine (100 mM), soy bean trypsin inhibitor (0.1% wt/vol) and NaN_3 (0.02% wt/vol), pH 7.4 to stop the contact activation. Sepharose-bound monoclonal antibodies (MoAb Kok 12) specific for C1-Inhibitor-protease complexes were added to the sample to catch the complexes. ^{125}I labelled anti-kallikrein antibodies were then used to detect the C1-Inhibitor-kallikrein complexes. The results were expressed as percentage binding of the labelled antibody added. A standard curve was constructed from dextran sulphate activated plasma containing by definition 100 units of kallikrein-C1-Inhibitor/mL.

2.2.3. Clotting time

The microplates, containing polymer films exposed for 15 min to citrated PRP, was placed in a plate reader (SLT Lab Instruments, Salzburg, Austria, model 340 ATCC) at 37 °C. Clot formation was initiated by the addition of 4 µl of a 0.5 M $CaCl_2$ solution. The final free Ca^{++} concentration was 4 mM. For each well the optical density at 405 nm was recorded every 15 s. The clotting time was taken as the peak value of the first derivative of the tracing.

2.2.4. Thrombin generation

Samples (2 µl) were removed from the recalcified platelet-rich plasma in the wells containing the polymers and assayed for thrombin using the chromogenic substrate S2238 (Chromogenix, Mölhdal, Sweden). The thrombin generation curves thus obtained were analysed for the amount of free thrombin according to the method of Hemker *et al.* [6].

2.2.5. Platelet adhesion

Polymer surfaces exposed to citrated PRP were rinsed with PBS and fixed with formaldehyde (3.5%). The fixed surfaces were stained with May-Grünwald-Giemsa. Platelet deposition was quantified by automated microdensitometry carried out with a Quantimed image analyser fitted to a light microscope. Alternatively, the number of platelets deposited on to the polymer surfaces were quantified by measuring lactate dehydrogenase (LDH) content. To this end, the washed polymer surface was incubated with a solution of NADH (240 µM) and 1% Triton X100 for 30 s. Pyruvate (0.5 mg/ml) was added and the change in OD at 340 nm was recorded. A standard curve was constructed from known amounts of platelets. The slope was 8.4 mU $LDH/10^6$ platelets.

2.2.6. Adhesion of procoagulant platelets

Procoagulant sites at the polymer surfaces were identified by their ability to bind fluoresceine (FITC)-labelled annexin V [7]. Annexin V was prepared by cDNA recombinant techniques with plasmid pRH291, and purified as previously described [8]. The preparation was more than 99% pure. Annexin V was

dialysed against coupling buffer (50 mM sodium borate/NaOH, pH 9.0, 150 mM NaCl and 1 mM EDTA). Dialysed Annexin V (50 μM) was mixed with 50 μM FITC isomer I (Sigma St. Louis, MO) and incubated for 2 h at 37 °C. The coupling reaction was then stopped by the addition of 100 mM glycine. The mixture was first dialysed against 50 mM Tris/HCl, pH 8.0, 80 mM NaCl and 1 mM EDTA and subsequently applied to a Mono Q column (Pharmacia, Uppsala, Sweden). The bound proteins were eluted by an NaCl gradient.

2.2.7. *Morphology of polymer-bound platelets*

The surfaces exposed to PRP were examined by scanning electron microscopy (SEM). The cells attached to a polymer surface were fixed with gluteraldehyde and then dehydrated with ethanol and subjected to critical-point drying. They were subsequently mounted on aluminium stubs, sputter-coated with gold and viewed in a scanning electron microscope (SEM 505 model, Philips, Eindhoven, The Netherlands). The morphology of procoagulant platelets was examined with fluorescence microscopy as well as by phase contrast light microscopy after incubation of formaldehyde fixed platelets with fluorescein-labelled annexin V.

3. Results and discussion

3.1. Global clotting assay

The partial thromboplastin time (PTT) is frequently used to estimate the potency of a material to induce the clotting of plasma. In this test the material is incubated with citrated platelet-free plasma for a certain time interval to activate the contact factors XII and XI. Calcium ions and phospholipids are then added to start the generation of thrombin and thus fibrin formation. The addition of phospholipids is essential, because they enhance dramatically the rate of thrombin formation and thus shorten the clotting time to a value that is of practical use. A major disadvantage of using artificial phospholipid membranes is that differences in the extent of contact activation can easily be overlooked because the clotting time is then largely determined by the rate-enhancing effect of the artificial phospholipid membranes and not by the levels of activated contact factors [9]. In view of the important role of platelets as a source for a procoagulant surface and the stimulating effect of an artificial surface (polymers) on platelet activation, we reasoned that a global clotting assay should be performed with platelet-rich plasma or even whole blood.

We have evaluated the clot promoting properties of the polymers following exposure to recalcified platelet-free plasma in the presence of artificial phospholipid membranes (partial thromboplastine time, PTT) and after exposure to recalcified platelet-rich plasma (platelet mediated clotting time, PMT). In these tests, clot formation was monitored by measuring the change in optical density as described in Section 2. First of all we examined the dependency of the clotting time on the pre-incubation time of the polymer with citrated PRP. We found that for all polymers the clotting time decreased with increasing pre-incubation time and reaching a minimum value after 10 min. Therefore, clotting was initiated by calcium following a 15-min incubation time of each polymer with citrated PRP.

Fig. 1a shows the photometer tracings of platelet-rich plasma incubated with PE and with PVC-T after recalcification. The sudden onset of the change in optical density reflects the clotting of the plasma. The time points of clot formation after the addition of calcium chloride, defined as the peak of the first derivative of the curve, were 6 and 10 min for PE and PVC-T, respectively. Fig. 1b shows the corresponding thrombin generation curves. It is seen that clotting of plasma occurs as soon as the first detectable traces of thrombin are generated. Table I summarizes the

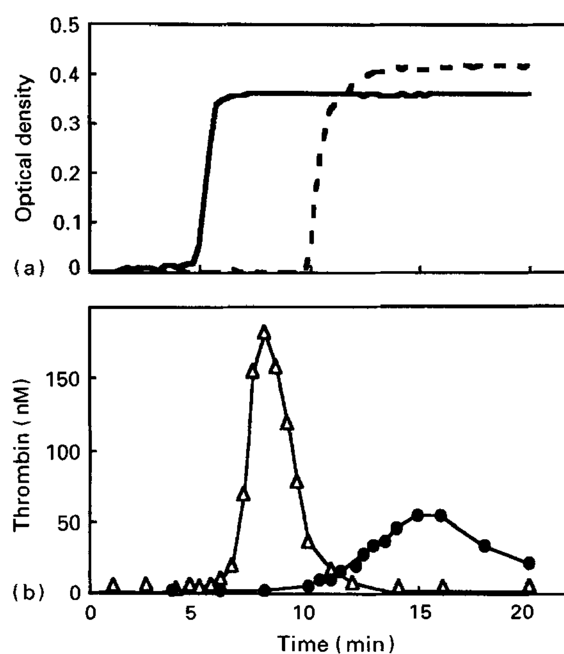

Figure 1 Thrombin formation and fibrin clot formation after recalcification of citrated platelet-rich plasma exposed to polymer surfaces. Polyethylene (—) and polyvinyl chloride (- - - -) were exposed to citrated platelet-rich plasma for 15 min. Following the addition of calcium ions clot formation was monitored as a change in optical density (a). In a separate experiment, samples were taken after the addition of calcium ions and assayed for thrombin. (b) Thrombin generation curves for (△) polyethylene and (●) polyurethane.

TABLE I Recalcified clotting time of citrated platelet-free (PFP) and platelet-rich plasma (PRP) exposed for 15 min to polymer surface

Polymer	Clotting time[a]	
	PPT (min)	PMT (min)
Polyethylene	7.4 ± 0.3	5.7 ± 0.5
Polypropylene	8.8 ± 0.3	7.3 ± 0.6
Polyurethane	8.3 ± 0.2	8.0 ± 0.4
Polyvinyl chloride T	8.2 ± 0.4	9.9 ± 0.9
Polyvinyl chloride D	8.4 ± 0.2	7.5 ± 0.7

[a] mean value of 12 determinations ± SD

clotting times obtained with the different polymers exposed to platelet-free plasma in the presence of phospholipids (PTT) and exposed to platelet-rich plasma (PMT).

Our results indicate that when the global clotting test is performed with platelet-free plasma in the presence of artificial phospholipid membranes no differences were found between the materials (Table I). However, when the same test was performed with platelet-rich plasma in the absence of artificial phospholipid, we found that PE exhibited a significant higher clot promoting activity than the other polymers.

3.2. Contact activation

Samples were taken at timed intervals from citrated platelet-rich plasma exposed to polymer surfaces and assayed for kallikrein-C1-inhibitor concentrations to assess the extent of contact activation (Fig. 2). With the positive control, glass surface, 25% activation was seen after 2 min. It took about 20 min to obtain the same degree of contact activation with PE. It is clearly seen that PVC-T is the least active surface with respect to initiation of contact activation. We have to emphasize that the kallikrein-C1-Inhibitor complex is not the only inhibitor complex that is generated during contact activation. However, it is evident that although kallikrein is also inactivated by the plasma protease inhibitor α_2-macroglobulin the amount of kallikrein-C1-inhibitor complex is a true measure for the extent of contact activation.

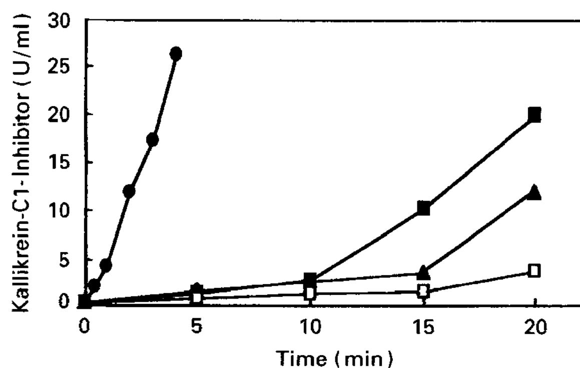

Figure 2 Activation of the contact system. Glass (●), PE (■), PU (▲) and PVC-T (□) were incubated with citrated PRP. At timed intervals aliquots were removed and assayed for Kallikrein-C1-Inhibitor complex.

3.3. Platelet adhesion

Citrated PRP was exposed for different time intervals to PE. Platelet adhesion to the surface was quantified by two methods: image analysis of stained polymer surfaces and LDH content of the surface. Fig. 3 shows that both methods are comparable. That is, with both methods we observed an increase in platelet adhesion with time, reaching a maximum after about 15–20 min. The results of platelet adhesion experiments with other polymers are depicted in Table II. Evidently, surface coverage and number of adhered platelets following a 15 min incubation of the polymers with citrated PRP is the highest for PE. Thus, PE was found the least platelet-compatible by both methods.

The free calcium concentration in citrated platelet-rich plasma is about 50 μM and probably sufficient for platelet reactions to proceed. In order to assess our assumption, we compared platelet adhesion from citrated platelet-rich plasma with that of heparinized (5 IU/ml) platelet-rich plasma in which the free calcium concentration is 1 mM. Using the LDH method, no significant differences were found in the number of platelets adhered to PE surfaces from heparinized or citrated PRP (data not shown).

3.4. Platelet morphology

When platelets interact with a polymer and become surface-activated they progress through a sequence of morphological forms: the platelets form pseudopods and these pseudopodial forms then may change

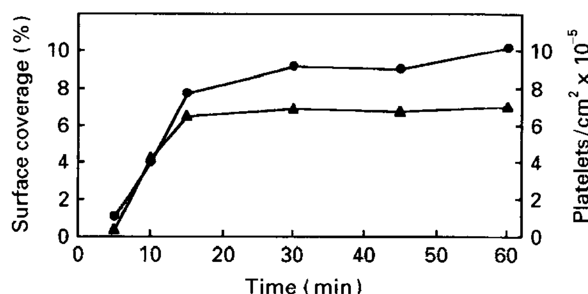

Figure 3 Platelet adhesion. Polyethylene was exposed to citrated PRP at varying time intervals. Following a mild rinsing step the percentage of surface covered with platelets (●) as well as the number of platelets as determined from LDH content (▲) were determined.

TABLE II Platelet adhesion to polymer surface exposed for 15 min to citrated platelet rich plasma. Further details are given in the text

Polymer	Platelet adhesion	
	Micro-densitometry (% surface coverage)[a]	LDH (platelets/cm^2 × 10^{-5})[b]
Polyethylene	8.7 ± 0.3	12.9 ± 0.5
Polypropylene	1.1 ± 0.4	3.4 ± 0.3
Polyurethane	2.6 ± 1.0	2.9 ± 0.2
Polyvinyl chloride T	0.3 ± 0.1	0.8 ± 0.1
Polyvinyl chloride D	0.2 ± 0.1	0.6 ± 0.1

[a] mean ± SD (n = 5)
[b] mean ± SD (n = 3)

into a fully spread, pancake-shaped, platelet [10]. Fig. 4 shows the SEM micrographs of a representative part of a PE surface after exposure for 15 min to citrated PRP. It is clearly seen that most of the platelets were fully spread (Fig. 4a) and occasionally platelets in their pseudopodial stage were seen, probably in contact with a spread platelet (Fig. 4b). Many fewer platelets adhered to the other polymers. The platelets that adhered to PU all showed shape changes, but only a very few were fully spread. Those platelets that adhered to PVC were not progressing beyond the early pseudopodial stage.

3.5. Procoagulant platelets

Although platelets adhered to PE showed dramatic shape changes, it remained to be seen whether the platelets exhibited procoagulant sites. Formaldehyde fixed surfaces were incubated with fluorescein-labelled annexin V. Fig. 5 shows the phase contrast (a) and fluorescence micrographs (b) of a sample of PE exposed to PRP. Both micrographs were taken from the same site. It is clearly seen that all platelets adhered to the surface had a fluorescent appearance. It is of interest to note that spread platelets showed a highly localized staining with the fluorescent probe. Since

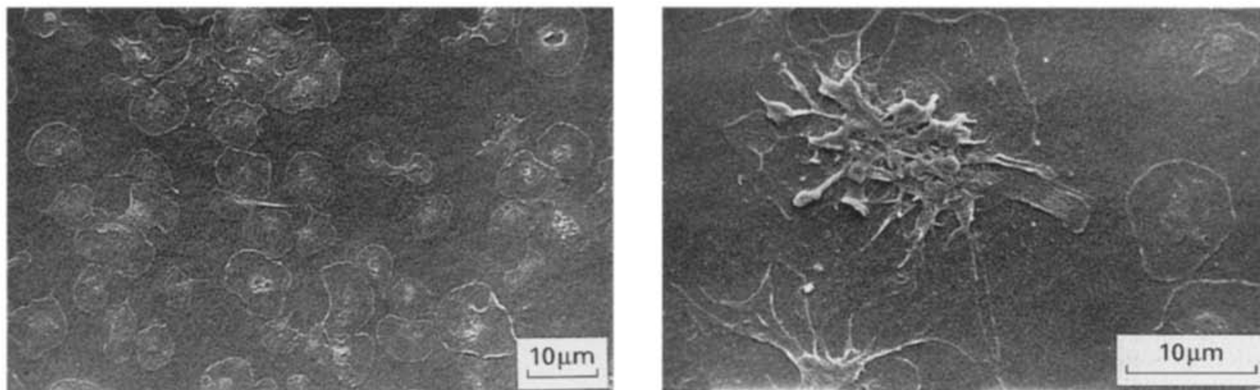

Figure 4 SEM views of platelets on polyethylene surface exposed for 15 min to citrated platelet-rich plasma.

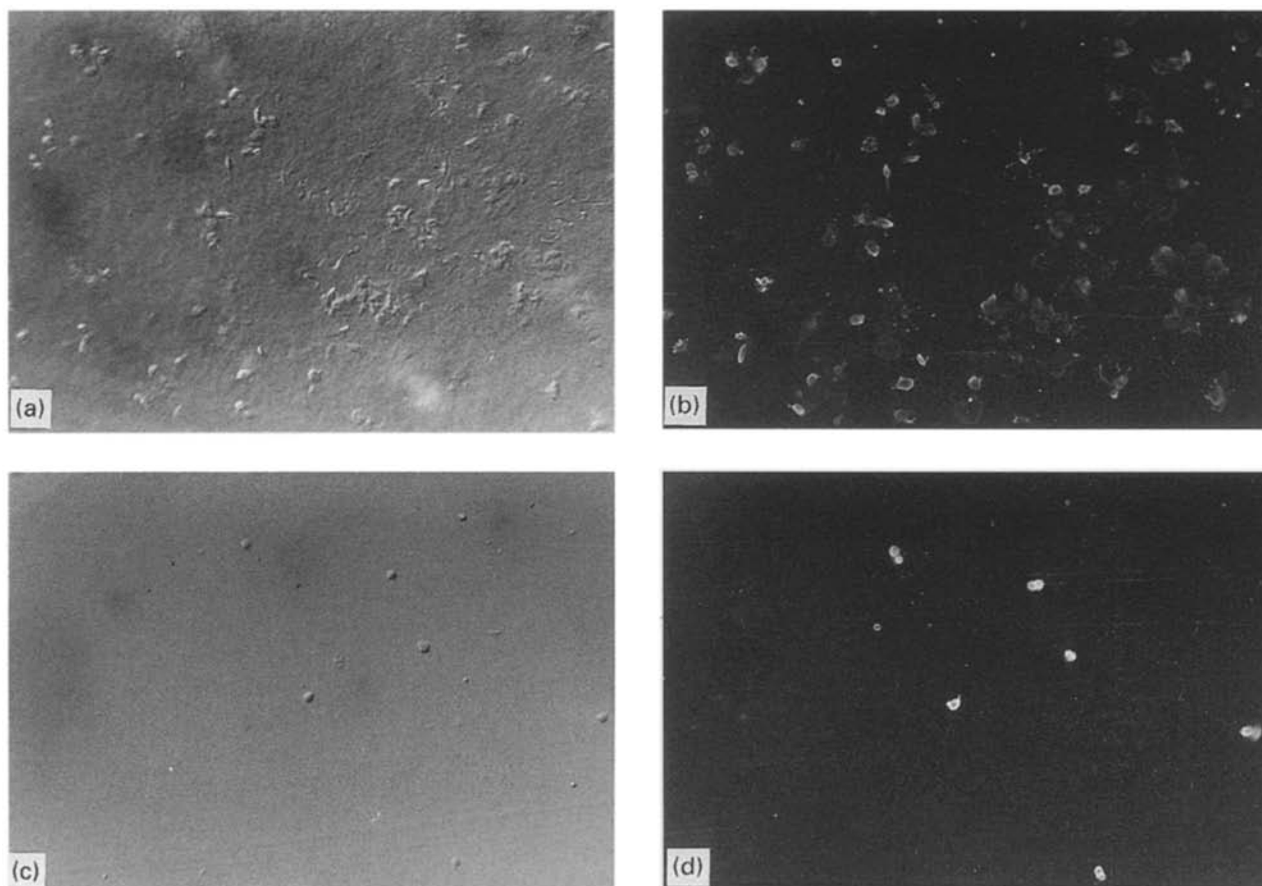

Figure 5 Platelet morphology and exposure of procoagulant sites at polymer surfaces. Phase contrast micrographs are shown for polyethylene (a) and polyvinyl chloride T (c) exposed to citrated platelet rich plasma. Fluorescence micrographs, after incubation with FITC-Annexin V, of the same PE and PVC surfaces are seen in (b) and (d), respectively. Overall magnification 1000 ×.

annexin V binds specifically to procoagulant sites, we conclude that platelets that adhere to PE become procoagulant. The phase contrast and fluorescence micrographs of the PVC-T surface (Fig. 5c and d) revealed a few round-shaped platelets. Most of them showed intense fluorescent staining, indicating the presence of procoagulant sites although pseudopod formation was not observed.

4. Conclusions

The interaction between an artificial surface (polymer) and the haemostatic system leads to the formation of thrombin and fibrin/platelet deposition. This process can be dissociated into an initiation and a propagation part. We note that the potency of a material to support the propagation of the process of thrombosis is the most relevant parameter to be tested. Since formation of procoagulant sites, as a result of surfce activation and/or thrombin-mediated activation of platelets, is essential to the propagation of the process, *in vitro* testing of the thrombogenicity of a material should include methods that examine the formation of procoagulant sites.

The test protocol as presented here does reveal significant differences between the polymers examined in this study with respect to their *in vitro* thrombogenicity. From the data of the global clotting assay it is clear that PE is the most thrombogenic surface and that PVC-T is the least thrombogenic one. This finding closely correlates with the higher amount of procoagulant sites (activated platelets) at the PE surface.

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