

# Platelet reactions to modified surfaces under dynamic conditions

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The influence of surfaces on the reactions of platelets in whole blood under laminar flow was investigated in a cone and plate viscometer. Citrated whole blood was exposed to steel, PMMA and PMMA modified with PEO at low ( $500 \text{ s}^{-1}$ ) and high ( $4000 \text{ s}^{-1}$ ) wall shear rates at room temperature for a period of 100 s. Treated blood samples were fixed with paraformaldehyde, stained with a monoclonal antibody for CD41 (platelet GPIIb/IIIa) conjugated with phycoerythrin and analyzed by flow cytometry. The reactions of platelets (microparticle generation and formation of platelet–platelet, platelet–red blood cell and red blood cell–microparticle aggregates) to these environments were quantified. Additionally, the size of platelet–platelet aggregates was assessed. The percentage platelet aggregation and numbers of microparticles generated were independent of surface type at any shear rate. The composition of the aggregates formed was influenced by the surface: at low and high shear rates PMMA caused the generation of platelet–platelet aggregates of the greatest size. The numbers of red blood cell–platelet and red blood cell–microparticle aggregates also varied depending on the surface. Fewer red blood cell–platelet aggregates were formed at higher shear rates, whereas the reverse was true for red blood cell–microparticle aggregates. It is concluded that these variations may help to explain the differential effects of surfaces to the induction of distant thrombotic events: microparticles may be protected from loss from the blood stream by their association with red blood cells at high shear rates.

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## 1. Introduction

Platelets are versatile cells capable of displaying a variety of reactions, including adhesion to surfaces, secretion of internal granules, expression of surface markers, shedding of microparticle (MP) vesicles and formation of aggregates (with other platelets and red blood cells, RBCs). The major physiological role of platelets lies in the control of thrombosis and haemostasis with an additional involvement in inflammation. The observation that platelets interact with foreign materials placed within blood is well known, but a general theory relating the chemical features of the surface and haemodynamic conditions to platelet reactions has not been developed. Indeed, relatively little is known about how different surfaces influence the reactions of platelets in different flow conditions, especially those observed in the fluid-phase, other than the usual observations of adhesion and activation.

The presence of MPs was observed many years ago as platelet dust, but more recently they have been quantified in relation to laminar wall shear rate [1]. The exact physiological significance of MPs has not been established, but they are thought to contribute to the modulation of atherosclerotic plaques [1], the

growth of fibrin deposits [2] and the activation of leukocytes in inflammation [3]. However, we have previously observed that MPs have the ability to form aggregates with RBCs [4], although whether or not the association was strong has not been confirmed. We have also previously demonstrated that P-selectin (CD62P) can be expressed on the surface of MPs [4] suggesting possible interaction with leukocytes and the potential for influencing an inflammatory response. MPs are membrane vesicles, shed from the surface of platelets, which express active phospholipid species capable of potentiating coagulation on their surface [5]. MPs can be formed in response to a number of soluble activating compounds [6–9] and surfaces [10], but are also formed in response to shear forces [1] without chemical agonist involvement. The fact that MPs can be associated with RBCs suggests that a subpopulation of these very small bioactive components may have different transport properties to isolated particles. This study investigates the hypothesis that different surfaces within sheared environments affect MP generation and their association with RBCs.

The literature has many views on the effect of surfaces on platelet aggregation, with little firm evidence.

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Because the presence of large clumps of platelets in the blood has an adverse effect on patient health we wished to investigate the extent of material and shear rate involvement on the platelet aggregation response, and to test the hypothesis that platelet aggregate formation is influenced by the nature of the surface.

## 2. Materials and methods

### 2.1. Collection of blood

Blood was taken from healthy, male volunteers who had been free from medication for at least 14 d and anticoagulated with tri-sodium citrate (3.8% wt/vol) at a ratio of 1 part citrate to 9 parts blood, by venepuncture of a median cubital vein with a 19-gauge needle. Before any experiments were performed, the platelets were allowed to recover from the shock of phlebotomy by incubating the blood for 30 min at 37°C.

### 2.2. Preparation of surfaces

Stainless steel was machined from a solid block, 35 mm diameter and shaped such that it formed the plate component of the viscometer. Polymethylmethacrylate (PMMA) was purchased from RS Components (Corby, Northamptonshire, UK) as a 25 mm diameter rod, cut into 5 mm thick discs and polished using 1200 grit abrasive carbon paper, followed by four decreasing grades of diamond slurry: 9, 6, 3 and 1  $\mu\text{m}$ . Some discs were surface-modified with polyethylene oxide (PEO) using a method based on that of Desai and Hubbell [11]. Briefly, the PMMA surfaces to be modified were cleaned by incubating in Lipsol (L.I.P., Shipley, West Yorkshire, UK) for 30 min at room temperature whilst stirring, then rinsed in water and degassed under vacuum whilst sitting in water, overnight at room temperature. The surfaces were then modified by incubating them for 30 min at room temperature whilst agitating with a PEO solution, prepared by dissolving 32 g PEO-bisphenol A (18 500 molecular weight, Polysciences, Warrington, PA, USA) in 68 ml water then adding to 300 ml 80% (vol/vol) solution of acetone (99.99%, HPLC grade, Aldrich Chemicals, Poole, Dorset, UK). The effect of PEO treatment on the wettability of the surfaces was assessed by measuring the air–water contact angle using the captive bubble technique for virgin and modified PMMA. A bubble of air trapped underneath the material surface immersed in water was photographed and the incident angle measured. Ten examples of each material were measured (Table I). The PEO modification rendered the PMMA surfaces significantly more wettable ( $p = 0.0031$ ). The modified surfaces were retained in water until used.

### 2.3. Exposure to blood samples to surfaces

A series of experiments was performed in which blood was exposed to shear rates of varying magnitude (500 and 4000  $\text{s}^{-1}$ ), representing a moderate venous rate and a high arterial rate, in contact with one of three

TABLE I Air–water contact angles of virgin and modified PMMA surfaces. The difference is significant,  $n = 10$ ,  $p = 0.0031$

	Air–water contact angle (deg)	
	PMMA	PMMA–PEO
Mean	26.4	19.8
S.D.	6.2	1.7

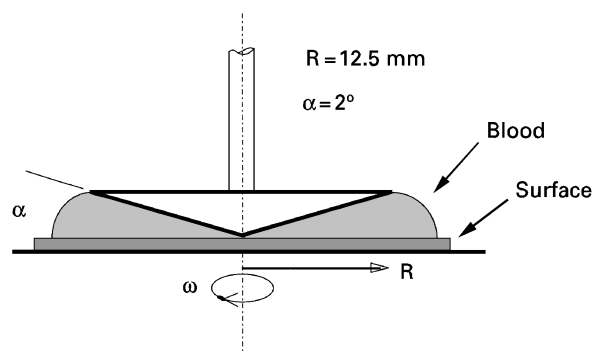


Figure 1 Schematic diagram of cone and plate viscometer.

materials: steel, PMMA and PMMA modified with PEO (PMMA–PEO). In each experiment, whole blood was subjected to the desired shear rate in contact with one of the three surfaces at room temperature in a rotational viscometer (Contraves LS-2, Zurich, Switzerland) which was modified to operate as a cone and plate apparatus. The torque-measuring mechanism of the LS-2 was isolated and its motor replaced with a unit capable of turning the plate component of the viscometer at a steady angular velocity of appropriate magnitude. The speed of the motor was under computer control, thus allowing precise, linear acceleration and deceleration of the plate to the desired level of shear. The cone component was machined from a rod of stainless steel, 25 mm diameter with a cone angle,  $\alpha$ , of 2° (Fig. 1). The height of the cone in relation to the plate was controlled by a lever.

For the different experiments, either the plate was used on its own or a PMMA or PMMA–PEO disc was attached centrally to the steel plate. A 50  $\mu\text{l}$  volume of whole blood was placed on to the test material and the cone lowered until it contacted the surface. The cone was then raised by 25  $\mu\text{m}$  to avoid physical damage to the cone and the sample. Each aliquot of blood was sheared for 100 s plus a 15 s steady acceleration and deceleration phase at the beginning and end of each cycle. The sequence of shear rate and material type to which the blood was exposed was randomized to prevent systematic errors.

### 2.4. Detection of platelet reactions

At the end of each shearing period a 40  $\mu\text{l}$  aliquot of whole blood was removed from the viscometer and placed into an equal volume of fixative (2% wt/vol) paraformaldehyde in PBS and incubated at room temperature for 10 min. Fixed blood was then stained

with anti-CD41 directly conjugated with phycoerythrin (PE) (specific for platelet glycoprotein receptor GPIIb/IIIa, from Pharmingen, San Diego, CA, USA) and incubated for 20 min at 4 °C. The samples were analyzed using a Becton Dickinson FACSsort (San Jose, CA, USA). 30 000 platelet-positive particles were acquired for each sample using a threshold on PE fluorescence (FL2), which prevented single RBCs in the sample from being acquired.

## 2.5. Data analysis

Analysis of the different platelet reactions and interactions (MP shedding, formation of platelet–platelet aggregates, platelet–RBC aggregates and RBC–MP aggregates) has been described previously [4, 12]. To summarize, particles were quantified in terms of their forward light scatter (FSC) and FL2 intensity. For each subject, a two-dimensional dot-plot (FSC versus FL2) of the control sample was constructed. The dot-plot was separated into five polygonal regions to identify: (R1) normal platelets; (R2) platelet–platelet aggregates; (R3) platelet–RBC aggregates; (R4) RBC–MP aggregates and (R5) MPs (Fig. 2). The mean number of platelets per aggregate (MPA) was estimated by dividing the mean value of FL2 for the platelet aggregate group (R2) by the mean value of FL2 for the normal platelet group (R1). A platelet aggregation index (PAI) could then be calculated by the following formula:

$$\text{PAI} (\%) = \frac{N(\text{R2}) \cdot \text{MPA}}{N(\text{R1}) + (N(\text{R2}) \cdot \text{MPA})} \cdot 100 \quad (1)$$

where  $N(\text{R1})$  and  $N(\text{R2})$  are the number of particles within regions R1 and R2.

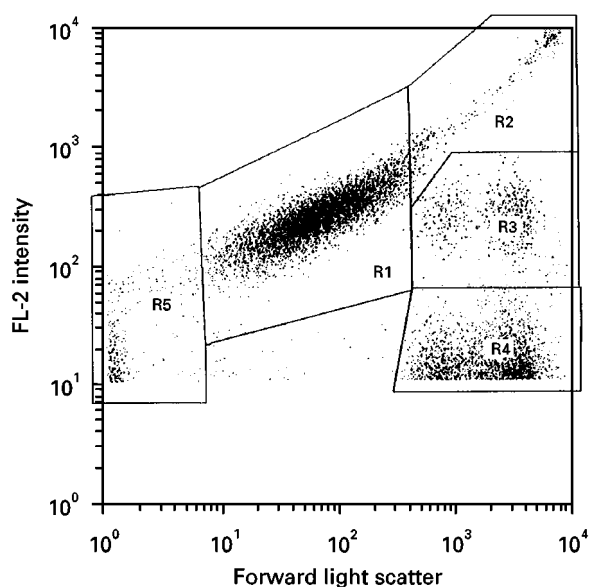


Figure 2 Dot-plot of fluorescence intensity against forward light scatter for 30 000 platelet-positive particles in whole blood exposed to  $4000 \text{ s}^{-1}$  at room temperature for 100 s. Regions consist of (R1) normal platelets; (R2) platelet–platelet aggregates; (R3) platelet–red blood cell aggregates; (R4) microparticle–red blood cell aggregates; (R5) microparticles.

An index of MP shedding (MPSI) was defined as the number of particles observed in polygonal region R5 divided by the total number of platelets involved in both platelet–platelet aggregates and single cells, as follows

$$\text{MPSI} (\%) = \frac{N(\text{R5})}{N(\text{R1}) + (N(\text{R1}) \cdot \text{MPA})} \cdot 100 \quad (2)$$

Statistical comparison of the data was achieved using a paired *t*-test.

## 2.6. Examination of platelet deposition on surfaces

Some of the surfaces were examined for the presence of platelets and sub-platelet sized particles using scanning electron microscopy (SEM) and laser-scanning confocal microscopy. For SEM, the samples were washed carefully with phosphate buffered saline (PBS) to remove non-adherent particles then fixed for 10 min at room temperature with 2.5% (vol/vol) gluteraldehyde (Emscope, Ashford, Kent, UK). Water was removed by successive incubation in 70% (vol/vol), 90% (vol/vol) and 100% (vol/vol) methanol (HPLC grade, Merck, Poole, Dorset, UK) for 20 min in each concentration at room temperature. The samples were gold sputter coated prior to examination under SEM. For confocal microscopy, the samples were washed carefully with PBS then fixed with 1% (wt/vol) paraformaldehyde in PBS for 10 min at room temperature. A drop of anti-CD41 conjugated with PE (Pharmingen, San Diego, CA, USA) large enough to cover the area of interest was placed on the surface and incubated at 4 °C for 20 min. A second layer antibody (rabbit anti-mouse conjugated with rhodamine, Cappel, Thame, Oxfordshire, UK) was applied in the same fashion and again incubated for 20 min at 4 °C. The surface was mounted with Vectashield (Vector, Peterborough, UK) to stabilize the fluorochromes, excited with a 543 nm laser and the emitted fluorescence observed after optical filtering using a 575–640 nm band pass filter with a Zeiss LSM-310 confocal microscope (Welwyn Garden City, Hertfordshire, UK).

## 3. Results

### 3.1. Platelet aggregates

The percentage platelet aggregation (as defined in this study) was independent of surface type at either shear rate (Table II), i.e. there was no difference in the ratio of platelets trapped within platelet–platelet aggregates to the total number of platelets originally within the blood sample between different surface types. However, the mean number of platelets per aggregate was variable, depending on material. Our results demonstrate that this parameter is both surface and shear rate dependent (Fig. 3). PMMA produced the largest aggregates, steel the smallest ( $p = 0.043$  for PMMA versus PMMA–PEO,  $p = 0.0094$  for PMMA versus steel). The size of the aggregates was smallest at low shear rate, the ranking of the materials remaining the same with increasing shear rate.

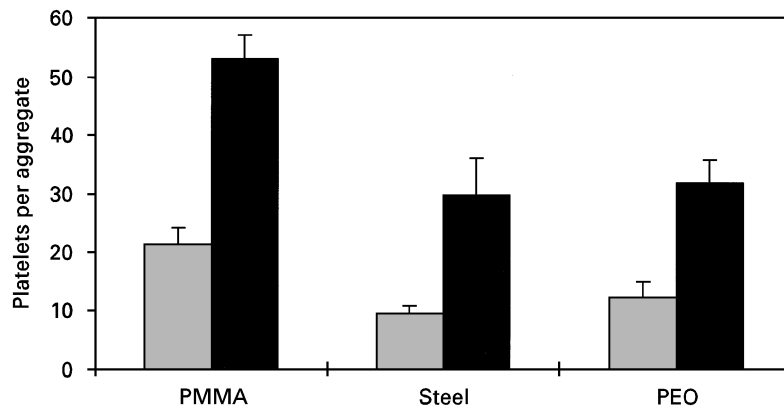


Figure 3 Numbers of platelets per aggregate within whole blood sheared at 500 and 4000 s<sup>-1</sup> for 100 s at room temperature in contact with steel, PMMA and PMMA-PEO. Mean ± SEM, *n* = 5. (□) 500, (■) 4000.

TABLE II Percentage platelet aggregation and microparticle shedding index (MPSI: see Section 2.5) for contact of whole blood with steel, PMMA and PMMA-PEO; mean ± S.D., *n* = 5

	Platelet aggregation (%)		MPSI (%)	
	500 s <sup>-1</sup>	4000 s <sup>-1</sup>	500 s <sup>-1</sup>	4000 s <sup>-1</sup>
Steel	60.0 ± 6.6	42.6 ± 17.7	4.5 ± 1.9	6.6 ± 5.3
PMMA	72.6 ± 13.5	73.7 ± 11.2	2.3 ± 2.3	4.0 ± 3.3
PMMA-PEO	60.0 ± 19.0	71.3 ± 9.1	2.9 ± 2.3	5.4 ± 3.9

### 3.2. Microparticle shedding

The number of MPs observed within the fluid phase was dependent upon shear rate, but independent of surface (Table II). However, the PMMA surfaces which were examined by SEM had a large number of sub-platelet sized particles present, postulated as being MPs, which were not evident on PMMA-PEO samples. This observation was in agreement with surfaces examined by confocal microscopy, which had numerous bright spots on the surfaces of PMMA, but not PMMA-PEO.

### 3.3. Red blood cell aggregates

RBCs have been shown to form associations with platelets and MPs in the past [4]. In this study we have shown that the degree of association is surface dependent (Fig. 4). The number of platelet-RBC aggregates was greatest at low shear rate for all materials. The reverse was true for RBC-MP aggregates. Steel induced large numbers of both types of aggregate (*p* = 0.0097 for steel versus PMMA for platelet-RBC aggregates at 500 s<sup>-1</sup>, 0.0126 for steel versus PMMA-PEO for RBC-MP aggregates at 4000 s<sup>-1</sup>).

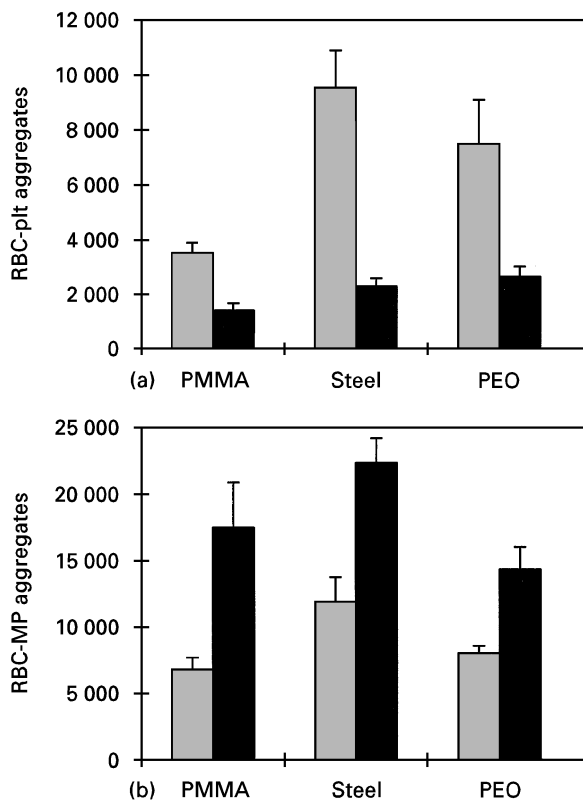


Figure 4 Numbers of (a) platelet-red blood cell aggregates and (b) red blood cell-microparticle aggregates in whole blood sheared at 500 and 4000 s<sup>-1</sup> for 100 s at room temperature in contact with steel, PMMA and PMMA-PEO. Mean ± SEM, *n* = 5. (□) 500, (■) 4000.

## 4. Discussion

Platelet reactions to biomaterials have been studied extensively in the past. Many of these studies have investigated platelet adhesion and activation in static and dynamic conditions, a few have considered platelet aggregation in dynamic situations but none has examined the composition of the different aggregates with which platelets are involved after contact with different surfaces. In this study we have presented data which compare the interaction of platelets and platelet-derived MPs with RBCs in addition to the size of platelet aggregates after dynamic contact of blood with three different surfaces.

It is generally known that platelets aggregate differentially in response to different magnitudes of shear rate. It is generally assumed that different materials can induce different degrees of platelet aggregation. Our findings, however, show that the situation is

somewhat more complicated. In fact, we have demonstrated that three very different materials caused very similar numbers of platelets to aggregate after dynamic contact. The different surfaces caused the platelets to form aggregates of different sizes, an observation not previously reported. This finding could be particularly relevant to the manufacturers of extracorporeal circuits (e.g. cardiopulmonary bypass), where large shear forces are experienced for relatively long transit times. Neurological injury is a significant problem in this situation [13–15], almost certainly caused, to a large extent, by platelet emboli, in addition to existing risk factors (age, atherosclerosis, etc.). A reduction in the size of aggregates may substantially reduce the levels of such injury.

The observation that the numbers of platelets per aggregate in platelet–platelet aggregates is variable is reasonable, especially when considered as a function of shear rate. As shear rate is increased, the probability of collisions between single platelets and hence between single platelets and growing aggregates will increase because blood is a complex fluid. Because cohesion between platelets can occur in flowing blood when platelet receptors GPIb or GPIIb/IIIa having the correct orientation interact due to the stimulation of GPIb/IX by unravelled von Willebrand factor (vWf) [16], the higher shear rates merely present a greater opportunity for platelets to collide and therefore aggregation to occur, with larger numbers of cells becoming entrapped within aggregates by chance. One might not expect that these collisions, and therefore the forming aggregates, to be influenced by the nature of the contacting surface.

Our results indicate that the surfaces *do*, however, influence the formation of aggregates: not the total number of platelets that are aggregated, but the number of platelets within each aggregate, and therefore the number of aggregates. It is known that many platelets have intimate contact with the wall without becoming adhered [17]. It would be expected that the platelets thus stimulated might preferentially form aggregates, but only if the stimulation involved the induction of receptor GPIIb/IIIa to bind fibrinogen. However, in this scenario the total number of platelets forming aggregates would be expected to increase, and therefore the percentage aggregation. It would be anticipated that the pre-stimulated platelets would form aggregates at a faster rate, increasing the probability of aggregates conglomerating into larger units. However, the total number of platelets aggregating would not increase if a proportion of the platelet population was unable to, or prevented from forming aggregates. It is known that platelets are heterogeneous in terms of their size and reactivity [18]. The data suggest that a major proportion (in the region of 30%) may be either protected or very unreactive.

It is clearly of interest, therefore, that platelets can form associations with RBCs. Because RBCs will tend to occupy the high velocity areas away from the surface, a platelet–RBC association will reduce the ability of the platelet to come into contact with either the surface or other platelets. This factor may help to

explain why so many platelets do not form platelet–platelet aggregates, even in the presence of stimulating surfaces. In the case of steel, at  $500\text{ s}^{-1}$  almost 30% of all platelets have formed associations with RBCs, but less than 10% for PMMA, where more than 20% of fluid-phase platelets are in the form of single platelets. Clearly, protection by RBCs is not providing the full explanation. Indeed, the data suggest that RBC–platelet association is surface-dependent. This would imply that these platelets are receiving some form of stimulation, probably not a mechanism involving GPIIb/IIIa which would induce aggregation.

Possibly more interesting is the observation that MPs form associations with RBCs. We have not yet investigated the strength of the association, but there remains the possibility that MPs can be deposited some distance from the site of generation. This suggests that MPs able to potentiate coagulation and having the ability to induce an inflammatory response may perform their function other than at the site of activation. Because the results show that the number RBC–MP aggregates, and the number of MPs shed into the fluid phase, are surface-dependent, the materials used in devices where high flow rates are encountered may be responsible for a range of pathological manifestations. It is necessary, therefore, to investigate both the nature of the RBC association with MPs, and that of RBCs and platelets, to understand fully how surfaces affect the platelet response in dynamic flow conditions. Of interest is examination of the contribution that individual surface features make to the overall response, and to what degree the flow conditions in their own right generate the different aggregates. For that study, we envisage investigating a series of much less activating surfaces and to elucidate the non-GPIIb/IIIa mechanism of platelet stimulation, if indeed this is a genuine phenomenon.

## 5. Conclusion

In dynamic *in vitro* experiments contacting whole blood with three different surfaces, we have demonstrated that platelets are influenced in the fluid-phase by the presence of a surface. We did not observe differences in the total number of platelets aggregating, but there were marked differences in the sizes of the aggregates. We observed that the number of single platelets and platelet MPs forming associations with RBCs was surface and shear rate dependent. The numbers of platelet–RBC aggregates decreased with increasing shear rate, and the reverse for MP–RBC aggregates. It is concluded that MPs may be protected from removal from the blood by such an association and may help to explain the dissemination of distant thrombotic events.

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