Influence of test protocol in determining the blood response to model polymers

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A multi-parametric, multi-center evaluation of three polymers was performed measuring their response to blood contact. The purpose of this study was to pinpoint differences in tests performed for assessing ''basic'' hemocompatibility on identical materials at different centers and attempt to rationalize. Assays for platelet adhesion, activation, aggregability and activation of the coagulation system in addition to an *ex vivo* patency assay were performed at four centers across Europe, using protocols favored by each center for determining the blood-contacting performance of a biomaterial. Three polymers were chosen for their expected blood response spanning the range of undesirable to desirable: ethylenevinylacetate (EVA), polyvinylchloride (PVC) and PVC modified with polyethylene oxide (PEO). The assays were ranked in terms of their efficacy compared to cost and simplicity. A correlation between assays was calculated, indicating the ability of one test to correctly determine the blood response compared to another. Some assays were unable to distinguish between materials, but of the assays which could, the materials were ranked in the following order: EVA; PVC; PVC-PEO, EVA producing the most undesirable response. It is concluded that many commonly used assays for determining hemocompatibility are inappropriate, but there are simple and reliable test methods available which correlate well with the more sophisticated protocols.

1. Introduction

There is an ever-present need to characterize the hemocompatibility performance of blood-contacting biomaterials, not only for the purpose of creating better-performing medical devices, but also for regulatory purposes. The best methods for measuring the blood response, however, have been the subject of long debate for a long time [1–5], especially with regard to the need of standardized and efficient protocols.

Difficulties already arise from the definition of the bio- or hemocompatible material itself [6–10]. The manufacturer of a medical device might wish to know how the whole host responds to the device under realistic operating conditions, whereas it is important for regulatory approval to judge the safety of the component materials by measuring their reaction to individual pathways under standard conditions. For example, Dacron has been shown to be thrombogenic, but this is a requirement in large diameter vascular prostheses for the generation of a neointima. The selection of appropriate tests for regulatory approval is, however, the responsibility of the manufacturer, suggesting a paradox between the tests a manufacturer may wish to perform and those which would demonstrate a lack of safety. Furthermore, the end user of a blood contacting biomaterial often wishes to know if the material is biocompatible without regard to its application.

Many different systems appear to play a crucial role in determining the interaction of a biomaterial to blood and thus it can be argued that, if the host response is to be understood, the assessment of the hemocompatibility of a biomaterial requires the evaluation of most, if not all, of these systems [5, 11–13]. It is widely accepted that protein adsorption is the primary precursor to all subsequent events [14, 15] excepting mechanical forces, being: red blood cell lysis, platelet reactions

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(adhesion, activation, aggregation, microparticle shedding, membrane inversion, pseudopodia formation) leukocyte reactions (adhesion, activation, aggregation, cohesion with platelets), activation of the coagulation and complement systems. Many researchers determine the response of platelets (adhesion or activation) as the most indicative of blood-contacting performance [16, 17], since they are central to influencing coagulation and inflammation. All these events are, however, heavily interconnected by virtue of negative and positive feedback loops. Other issues regarding the toxicity of leachables etc., may also be an important consideration regarding material safety.

The variations in assay procedure do not stop at the parameters being tested. Many alternative material-blood contacting regimes are in use: for example human or animal models [18], *in vitro*, *ex vivo* or *in vivo* [19, 20], short or long term, static or dynamic, different contact times, different shear rates, different flow chambers and different types of flow: steady or pulsatile [21, 22], different biological media: normal blood, whole anticoagulated blood (citrate, heparin, CPD, P-PACK, hirudin), platelet rich plasma or protein solutions. In fact, no two tests are identical in an environment where each testing laboratory proposes its own type of test model with different sets of preferable variables.

There is an evident need of standardization, therefore, since national and international bodies (e.g. International Standards Organization (ISO) [23], British Standards Institute (BSI), National Institute of Health (NIH) [24]) merely recommend using an appropriate method providing a structural test selection system. However they do not provide detailed test methods or evaluation criteria but they cite various applicable references. Because of the complex nature of blood compatibility, however, deference to vertical standards for specific devices may be more appropriate than attempting to develop a single comprehensive standard [25, 26].

In the current study the authors have tested the hemocompatibility of three model polymers: ethylenevinylacetate (EVA), polyvinylchloride (PVC) and PVC modified with polyethylene oxide (PEO) in four centers around Europe. Each center tested the polymers for the basic blood response, in order to ascertain which material produces a desirable or undesirable response, using assays they would normally use for this type of assessment. The results of the test procedures have been correlated to establish the ability of each to correctly evaluate the response, and each protocol has been compared in terms of its efficacy, cost and simplicity.

This study does not aim to find, or even suggest at the present stage, a solution to the problem of standardization, but it attempts to clarify variations in different, well-established test procedures so as to provide some best practice guidelines in order to achieve a simple, low-cost but reliable method for testing the blood-compatibility of a new or existing material.

2. Materials and methods

Three materials were tested: ethylenevinylacetate, commercially available polyvinylchloride and PVC modified with polyethylene oxide (a blend of plasticized PVC and a copolymer containing PEO), all kindly provided by Solvay S.A. (Brussels, Belgium). All materials were constructed in tubular form with internal diameters (i.d.) of either 1 or 4 mm.

Three blood-contacting test arrangements were used, two being *in vitro* methods:

1. A capillary perfusion model introduced by Cazenave and Mulvihill and modified by Poot [27] and Rhodes *et al*. [28], which is described extensively in previous studies [29, 30]. Briefly, this involved cutting 30 or 50 cm sections of the 1 mm i.d. tubes, fitting 20 ml syringes to either end using tapered, polypropylene ports as connectors. 10 ml of blood was introduced into one of the syringes then perfused through the test section at shear rates varying from $100\,s^{-1}$ to $3000\,s^{-1}$ for 10 min. The direction of perfusion was switched when all the blood had been dispensed to allow continued perfusion of blood through the material. The tapered connectors were designed so as to reduce turbulence of the inflowing blood generated within the syringe. Human blood was used, anticoagulated with sodium citrate at a final concentration of 0.38% (w/v). A Harvard 22 microprocessor controlled syringe pump (South Natick, MA, USA) was used with a modification to allow 10 syringes to be perfused at once. This allowed all the polymers to be tested concurrently.

2. A closed loop system combined with the thromboelastography method introduced by Schulze *et al*. [31]. The test has been described in detail in previous studies [32]. Briefly, 18 ml of freshly drawn human blood is mixed with 2 ml citrate, 5 ml of the volume is filled into each 4 mm tube being tested and 5 ml are used as reference. The ends of each tube are connected with a small piece of a silicone tubing forming a closed loop. The loop is then positioned on the vertical circular cylinder and rotated for 30 min at a constant speed of 16–24 revs $*$ min^{-1} (approximate shear rate $750\,s^{-1}$). After blood-material contact, test and reference blood are recalcified and then tested simultaneously in four resonance thrombography (RTG) instruments and the results of the RTG plots obtained (Fig. 1) are compared according to the specified clotting parameters.

3. An *ex vivo* clotting time assay using a modified Dudley-Eloy test described in detail in previous study

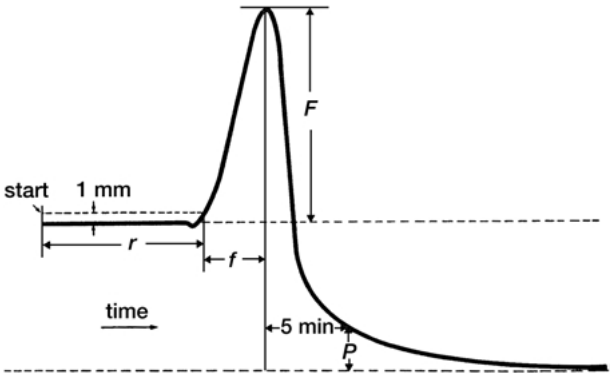


Figure 1 RTG plot and its characteristic coagulation parameters: r = clotting time (min); f = fibrin formation time (min); F = fibrin amplitude (mm); P = platelet amplitude (mm) (5 min after maximum).

[33]. The assay allows the study of the blood-material interaction under dynamic conditions. Catheters of the tested polymers 30 cm long and 1 mm i.d. are flushed with saline and closed at one end with a plug. Then they are simultaneously implanted via a short cutaneous incision and phlebotomy in the cephalic veins of anaesthetized rabbits over a length of 2 cm and secured. The plug is removed and the chronometer is started. The blood flow rate is set to 4 ml/min by adjusting the height of the distal end of the catheter and continuously monitored by ultrasound microflow velocimeter. The blood effluents are collected at given time intervals for further examination until clotting occurs within the catheter. The tested materials were tested two by two in six rabbits (4 experiments per material). The time related blood flow and the time for the blood to clot is measured. It has been demonstrated [34] that the blood flow and the flow time is strongly related to the properties of the material.

Test Parameters: The test parameters that were selected are assays used routinely to evaluate the haemocompatibility of biomaterials. They include those recommended by ISO, NIH and BSI involving the determination of platelet response (adhesion, activation, aggregability and morphology) and activation of the coagulation system.

2.1. Platelet adhesion

2.1.1. Radiolabeling

Cr^{51} was used to label platelets using a method similar to that of Sakariassen *et al*. [35]. Citrated whole blood (0.38% (w/v) final concentration of sodium tri-citrate) was split into two portions. One half was centrifuged at 150 g for 10 min to generate platelet rich plasma (PRP). A washed platelet suspension was prepared from this by adding an equal volume of Krebs-Ringer solution (4 mM KCl, 107 mM NaCl, 20 mM Na_2SO_4, 19 mM trisodium citrate, 0.5% (w/v) glucose, pH 6.1), successive centrifugation at 500 g for 10 min and resuspension in fresh Krebs-Ringer solution. Radiolabeling was achieved by incubation of the platelet suspension with 10 μCi/ml sodium-Cr^{51}-chromate (Amersham International, Amersham, UK) at room temperature for 20 min. Excess sodium chromate was removed by three successive washings with Krebs-Ringer solution. The radiolabeled platelets were resuspended in native platelet free plasma, obtained from another portion of blood from the same donor, and the original red blood cells were added to give a platelet concentration of 200×10^3/ml and a hematocrit of 44%. The number of platelets adhered on the biomaterial surface was determined directly by means of a gamma counter, quantifying the recorded counts by comparing those generated by 1 ml Cr^{51}-labeled blood.

2.1.2. Platelet retention number

Platelet adhesion was estimated by extrapolation from the number of platelets within blood after blood-biomaterial contact using a Sysmex K1000 Whole Blood Counter (Toa Medical Electronics Co. Ltd, Japan). The number of platelets in the influent and effluent blood were counted before and after contact of citrated human whole blood (0.38% (w/v) final concentration) with the test polymers in the capillary perfusion model test arrangement. The percentage of retained platelets for each polymer was calculated from this difference.

2.1.3. Relative platelet amplitude

The platelet amplitude value (P) is defined as being the value reached 5 min after the RTG plot curve has reached its maximum and depends inversely on the number of active thrombocytes (platelets) present in the sample blood (see Fig. 1). Relative platelet amplitude is defined as the ratio of platelet amplitude of the test blood to the one of the reference blood.

2.2. Platelet activation

2.2.1. Beta thromboglobulin (β-TG) release

The degree of α-granule release was evaluated by measuring the concentration of β-TG, a protein found only in platelet α-granules, released extracellularly [36]. Blood collected after contact with the test polymers in the perfusion arrangement was spun at 13 000 g (high speed) in a micro centrifuge (MSE MicroCentaur, Sanyo, Thistle Scientific, UK) for 30 s to generate platelet rich plasma (PRP). The concentration of β-TG within the plasma was quantified using an enzyme-linked immunosorbent assay (ELISA) from Stago Diagnostica (France) and compared with non-contacted blood as the negative control. This technique measured the release of β-TG from both adhered and fluid-phase platelets.

2.2.2. P-selectin expression

The degree of platelet activation was also estimated by observing changes in the expression of P-selectin, a 140 kD α-granule membrane protein also referred to as GMP140 or CD62P, which appears on the platelet surface after α-granule secretion [37]. Blood contacted in the perfusion test arrangement was collected and stained with CD62P conjugated with FITC (fluorescein isothiocyante) and CD41 conjugated with PE (phycoerythrin). Staining was performed at 4 °C for 20 min. The degree of P-selectin expression was assessed by flow cytometry (Becton Dickinson FACSort, San Jose, CA, USA), using the platelet marker CD41 to assess only platelets in whole blood, and rejecting the measurement of aggregates. This technique allowed the assessment of platelets in the fluid phase only.

2.3. Platelet morphology

Scanning electron microscopy (SEM) was used to visualize the degree of activation that adhered platelets had undergone. After blood-material contact in the capillary perfusion model test arrangement, all traces of blood were removed by rinsing carefully in PBS (phosphate buffered saline). Platelets were fixed using 2.5% gluteraldehyde, species were critical point dried and then sputter coated with gold. Platelets were

visualized on the surfaces of the polymers at 25 kV, in a JEOL ISM 5200 scanning electron microscope.

2.4. Platelet function

The functional integrity of platelets after contact with test materials was assessed by observing their response to three aggregating agonists: ADP, collagen and epinephrine (at clinically used final concentrations 2×10^{-5} M, 19 mg/ml and 10^{-3} M respectively). Lack of aggregating response, a lag before aggregation occurred, or a reduction in the degree of aggregation were all taken as evidence of a lack of platelet compatibility [38]. Test materials were contacted with blood in the perfusion test arrangement, then effluent blood spun to give PRP. This was then tested in a PAP4 platelet aggregometer (Biodata Corporation, USA) using the three agonists, non-contacted platelet rich plasma acting as a negative control.

2.5. Activation of the coagulation system

2.5.1. Fibrinopeptide A (FPA) generation

The degree of coagulation system activation was measured in two ways. Firstly, the degree of thrombin activity was measured by the generation of fibrinopeptides, molecules not consumed at the end of the reaction. Blood was collected after contact with the test polymers in the perfusion arrangement and platelet poor plasma (PPP) was generated by spinning control and test citrated human whole blood at 1200 g for 15 min at room temperature. The generation of FPA was determined in control and test samples using a competitive enzyme-linked Immunoassay (CELIA) technique from Stago Diagnostica (France). The basis of the assay assumes that higher levels of coagulation system activation will result in larger final concentrations of FPA [39].

2.5.2. Non-activated partial thromboplastin time (naPTT)

A second assay of coagulation activation measured the rate of fibrin generation. Citration of plasma reduces extracellular calcium concentration, which prevents coagulation. Addition of Ca^{2+} and platelet membrane lipid substitute allows the later reactions of coagulation to proceed, larger concentrations of factors XIIa, XIa and IXa generated during plasma-material contact producing commensurately faster rates of clotting [40]. Blood was collected after contact with the test polymers in the perfusion arrangement and platelet poor plasma (PPP) was generated by spinning control and test citrated human whole blood at 1200 g for 15 min at room temperature. Then the turbidity of the solution was measured after the addition of 25 mM $CaCl_2$ and platelet cephalin substitute (Diagnostica Stago, CK Prest kit, France) in a coagulometer (ST4, Diagnostica Stago, France) at 37 °C. Clotting time was taken as the start of fibrin generation. Non-contacted plasma was used as a negative control.

2.5.3. Resonance clotting (coagulation) time

Coagulation (or clotting) time is defined as the time between the start of the pin movement and the beginning of the formation of the resonance curve in the RTG plot (see Fig. 1). Relative coagulation time is defined as the ratio of coagulation time of the test blood to that of the reference blood.

2.5.4. Relative fibrin amplitude

Fibrin amplitude is defined as the maximum of the pin movement described by the RTG plot (see Fig. 1) obtained when the natural frequency of the system becomes equal to the excitation frequency. It is inversely dependent on the concentration of active fibrinogen in the test blood. Relative fibrin amplitude is defined as the ratio of fibrin amplitude of the test blood to that of the reference blood.

3. Results and discussion

3.1. Platelet adhesion

Platelet adhesion data determined using ^{51}Cr platelet radiolabeling are presented in Fig. 2 for three different shear rates (100, 1000 and 3000 s^{-1}).

Relative platelet amplitude values (multiplied by 10 for presentation purposes) after blood-biomaterial contact are presented in Fig. 3, using the mean wall shear rate within the closed loop system of 750 s^{-1}. Platelet retention numbers after blood–biomaterial contact using a Whole Blood Counter are also presented in Fig. 3. The shear rate was 1000 s^{-1}.

Comparison of the results of the three tests demonstrates that the first test method (using ^{51}Cr radiolabeled

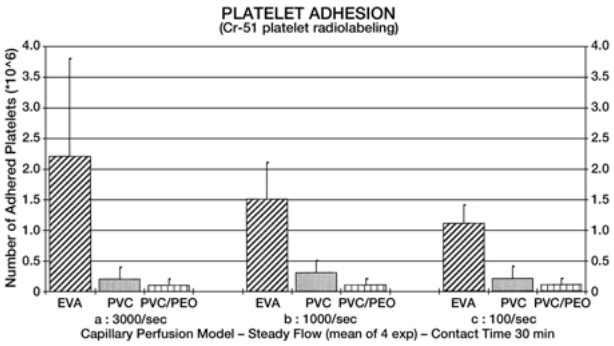


Figure 2 Platelet adhesion determined using ^{51}Cr radiolabeling for three shear rates: (a) 3000 s^{-1}, (b) 1000 s^{-1}, (c) 100 s^{-1}, (capillary perfusion model).

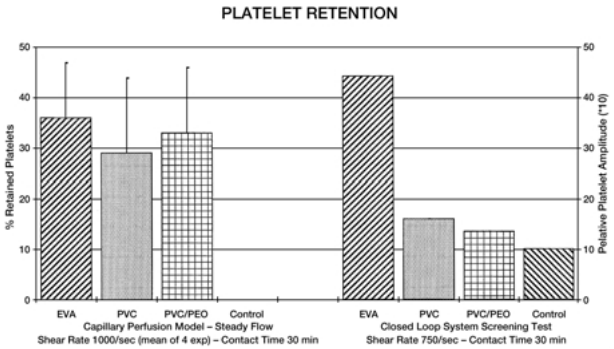


Figure 3 Platelet adhesion: relative platelet amplitude (closed loop system) and platelet retention numbers (capillary perfusion model).

TABLE I Overall test correlation

	CD62	BTG	pltadh100	pltadh1000	pltadh3000	pltret1000	relpltampl	*ex vivo*	naPTT	FPA	relcoagt	relfibramp
CD62	1.00											
BTG	0.98	1.00										
pltadh100	0.98	0.91	1.00									
pltadh1000	0.99	0.92	1.00	1.00								
pltadh3000	0.97	0.90	1.00	1.00	1.00							
pltret1000	0.68	0.50	0.81	0.79	0.82	1.00						
relpltampl	0.97	0.90	1.00	1.00	1.00	0.83	1.00					
ex vivo	− 0.76	− 0.88	− 0.60	− 0.63	− 0.59	− 0.03	− 0.58	1.00				
naPTT	− 0.92	− 0.98	− 0.82	− 0.84	− 0.81	− 0.33	− 0.81	0.95	1.00			
FPA	0.95	0.87	1.00	0.99	1.00	0.87	1.00	− 0.52	− 0.76	1.00		
relcoagt	− 0.92	− 0.98	− 0.82	− 0.84	− 0.80	− 0.33	− 0.80	0.95	1.00	− 0.76	1.00	
relfibramp	0.79	0.91	0.65	0.68	0.64	0.09	0.63	− 1.00	− 0.97	0.57	− 0.97	1.00

platelets) is better able at differentiation of the performance of the materials in respect to platelet adhesion (see Table II). Furthermore, differentiation is more evident at medium and high shear rate flow fields. This confirms the results of Turitto and Baumgartner [41, 42], as well as previous results of the authors [30] that better discrimination of biomaterials requires the use of high shear rate flow fields ($p = 0.05$ comparing PVC to PEO coated PVC), where the adherence of platelets is reaction rate controlled, rather than static or low shear rate flow fields where adherence is diffusion rate controlled ($p = 0.19$ respectively).

Quite good differentiation was also produced by the more global second test, whereas the high standard deviations obtained in the last test did not permit a statistically significant differentiation of the results. To improve the output of this last test method one could propose known strategies (e.g. EDTA/EDTA-formalin method [43]) could be included in the test protocol in order to discriminate between platelet adhesion and platelet aggregation so that more reliable results were obtained. In any case the accuracy of the results could never be better than the poor accuracy and linearity of the measuring system – namely the whole blood counter itself.

3.2. Platelet activation

Platelet activation (platelet beta thromboglobulin release and P-selectin expression – values multiplied by 100) after blood-biomaterial contact are presented in Fig. 4.

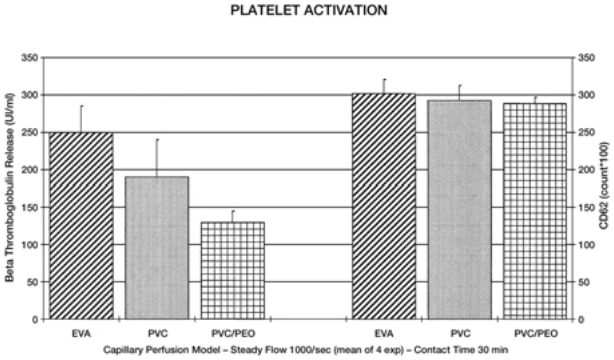


Figure 4 Platelet activation: beta thromboglobulin release and P-selectin expression (capillary perfusion model).

P-selectin results did not demonstrate statistically significant differences in the ranking of the performance of the three tested materials. On the other hand beta thromboglobulin results produced a good differentiation of the performance of the three biomaterials.

The absence of clear differentiation between the tested materials and the high statistical deviations in P-selectin measurement using flow cytometry do not suggest that this method is appropriate for ranking blood-contacting polymers. The methodology is appropriate for examining the fluid-phase insult of a biomaterial to the blood environment, but the possibility of fluid-phase, activated platelets adhering to other parts of the circuit reduce the efficacy of the protocol for determining the reactivity of a surface to platelets. Long term exposure of blood in this

TABLE II Statistical evaluation of the test methods

	pltadh100	pltadh1000	pltadh3000	pltret1000
PVC/PVC-S	0.19	0.09	0.05	0.37
PVC-S/EVA	0.01	0.01	0.04	0.35
EVA/PVC	0.01	0.02	0.05	0.25
	CD62	BTG	pltaggradp	pltaggrcollagen
PVC/PVC-S	0.38	0.04	0.33	0.43
PVC-S/EVA	0.17	0.03	0.16	0.23
EVA/PVC	0.30	0.15	0.37	0.30
	naPTT	FPA	*ex vivo*	pltaggrepin
PVC/PVC-S	0.03	0.50	0.02	0.26
PVC-S/EVA	0.01	0.01	0.01	0.48
EVA/PVC	0.14	0.01	0.90	0.25

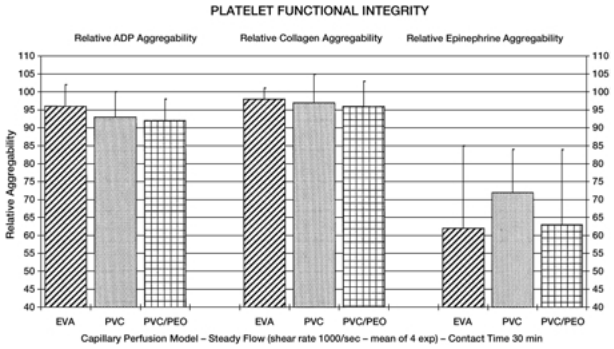


Figure 5 Platelet aggregability in response to ADP, collagen and epinephrine (capillary perfusion model).

system will additionally lead to observations of P-selectin shedding.

3.3. Platelet functional integrity

Platelet aggregability results in response to ADP, collagen and epinephrine are presented in Fig. 5. No statistically significant differentiation was observed among the tested materials (see Table II), partly due to high standard deviation values in these types of experiments. Another possible explanation for this poor differentiation could also be that the reagent concentrations used ''mask'' the biomaterial influence on the result. Platelet responses to lower concentrations could possibly better differentiate the performance of the biomaterials but this supposition has yet to be determined.

3.4. Platelet morphology

Typical images of SEM surface characterization results are presented in Fig. 6(a)–(c) for the three tested materials (PEO modified PVC, PVC and EVA respectively). Qualitatively the results confirm the same ranking between the three materials as do the platelet adhesion results.

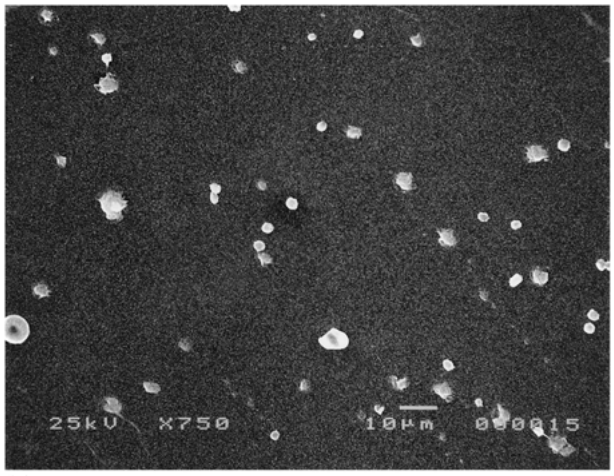
3.5. Activation of the coagulation system

Activation of the coagulation system results produced by measurement of relative fibrin amplitude and measurement of relative coagulation time after blood-biomaterial contact in the closed loop system using the resonance thrombography screening method are presented in Fig. 7.

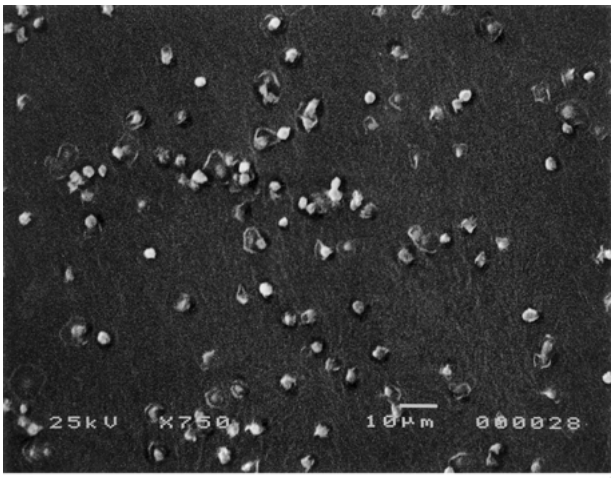
FPA generation (values multiplied by 10), non-activated partial thromboplastin time values (multiplied by 5) provided by blood-material interaction using the capillary perfusion model and clotting times provided by the *ex vivo* modified Duddley-Eloy test are presented in Fig. 8.

The relative coagulation time results did not produce a good differentiation between the three tested materials whereas measurement of relative fibrin amplitude gave better results.

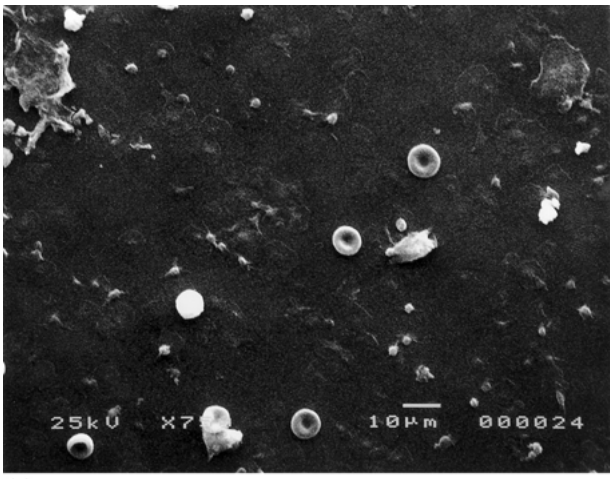
Measurement of FPA produced a good discrimination between the EVA material compared to the two types of PVC but could not differentiate the performance of the last two materials. Use of more prolonged blood-material



(a)



(b)



(c)

Figure 6 Platelet adhesion morphology images by SEM (capillary perfusion model). (a) PEO modified PVC: only a few adhered platelets with minimal activation and aggregation. (b) PVC: a lot of adhered platelets and quite a lot of spread (activated) platelets. (c) EVA: a lot of adhered platelets most of which are completely spread (activated) and some have formed aggregates.

contact times could help this differentiation (especially if we note that the generated FPA values are just higher than the ones of a normal donor (< 3 ng/ml)). However, we have to note here that FPA generation is a dynamic procedure and selection of a series of data in different blood-biomaterial contact times could be expected to

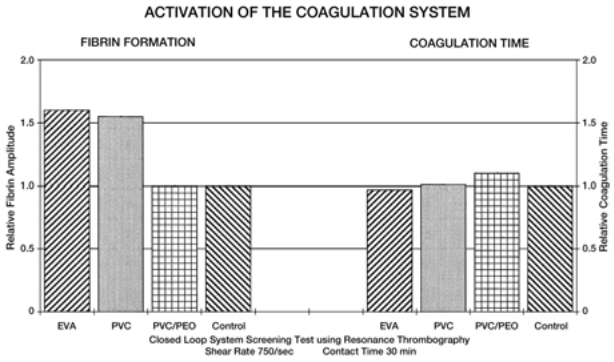


Figure 7 Activation of the coagulation system: relative fibrin amplitude and coagulation time (closed loop system).

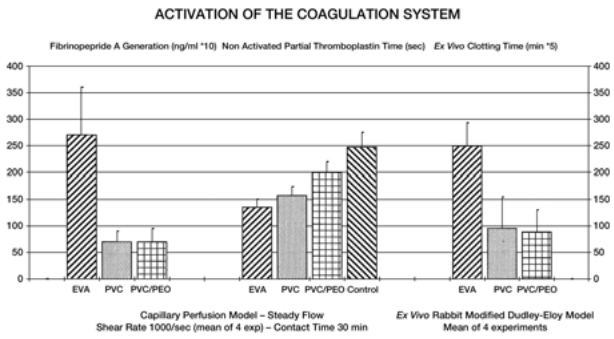


Figure 8 Activation of the coagulation system: FPA generation, non-activated partial thromboplastin time values (capillary perfusion model) and clotting time (*ex vivo* modified Dudley-Eloy test).

produce a better discrimination between the three materials tested.

Measurement of the naPTT values did produce statistically significant results (see Table II) and a good differentiation between the materials tested. This confirms previous results of the authors that this is a suitable method to differentiate materials with respect to their ability to activate the coagulation cascade [40]. We should note here once more the importance of using naPTT values (see also NIH, ISO instructions), since the use of activators included in the standard PTT assays (kaolin, ellagic acid, etc.) masks the acceleration of the coagulation system caused by contact of plasma with biomaterials and devices. In this case the materials to be tested serve as the activators.

Finally, the determination of clotting times in the *ex vivo* animal model used produced a clear differentiation of the performance of PEO modified PVC compared to EVA or PVC, but not a good discrimination between EVA and PVC. The ranking of the materials was the same one as in the naPTT and relative fibrin amplitude results.

Briefly commenting on the materials evaluation we may say that PEO modified PVC produced the best response followed by uncoated PVC and EVA. The results confirm the suggestion that the use of PEO to modify polymeric surfaces improves their blood compatibility, which is also reported in previous studies. [44–46].

Statistical evaluation of the results was performed in the following way: Firstly, correlation values (Pearson correlation) of the output results of the test methods were determined. Table I presents the overall correlation values for all the test methods evaluated.

Table II presents p-values between the materials for the tested parameters evaluated by single factor ANOVA. Finally the ability of the various test methods to discriminate the performance of the tested materials was estimated according to the previous table.

Table III presents a characterization of the test methods evaluated as excellent $(p < 0.05)$, good $(0.05 < p < 0.15)$, moderate $(0.15 < p < 0.25)$ and bad $(p > 0.25)$. Results of the closed loop system were not evaluated because of the lack of standard deviation values (mean of two experiments). The test methods were also characterized in terms of their cost (per measurement) and their simplicity (necessity of qualified personnel, time needed etc.).

4. Concluding remarks

In conclusion, we may say that there is an urgent need to establish well defined test parameters and well described test methods and procedures for the evaluation of the hemocompatibility performance of blood contacting materials and devices. From the experience obtained through this study we could make the following remarks:

1. The test parameters and test methods to be selected should try to give an output concentrating in the blood-tested material interaction and excluding any other

TABLE III Evaluation of the test methods in terms of hemocompatibility characterization effectiveness, cost and simplicity

	pltadh100	pltadh1000	pltadh3000	pltret1000	CD62	BTG
Haem. char. effect	Very Good	Excellent	Excellent	Very Bad	Bad	Very Good
Cost	High	High	High	Low	High	High
Simplicity	Complex	Complex	Complex	Simple	Complex	Complex
	pltaggradp	pltaggrcollagen	pltaggrepin	naPTT	FPA	*ex vivo*
Haem. char. effect	Bad	Very Bad	Very Bad	Very Good	Good	Good
Cost	Medium	Medium	Medium	Medium	High	High
Simplicity	Medium	Medium	Medium	Medium	Complex	Complex
	rlpramp	rlcotime	refibamp			
Haem. char. effect	—	—	—			
Cost	Low	Low	Low			
Simplicity	Simple	Simple	Simple			

external influence which very often masks the materials performance leading to confusing or even false results. For example, in the capillary perfusion model determination of platelet adhesion using ^{51}Cr labeled platelets is a rather specific method to measure this parameter. On the other hand the indirect determination of the same parameter using a whole blood counter gives results which, in the case of materials with comparable performance, are very difficult to interpret with respect to determining the influence of the whole system compared with the material alone (especially bearing in mind the accuracy and measuring method of a whole blood counter). Another relevant example would be the use of inappropriate concentrations of agonists when measuring platelet functional integrity by means of an aggregometer or the previously mentioned false use of activators (kaolin etc.) when measuring partial thromboplastin time values for the evaluation of the coagulation system after blood-material interactions.

2. When a test parameter and a test method are selected special care should be paid to the selection of the secondary parameters of the test (contact time, flow conditions etc.). These secondary parameters should be carefully designed and determined so that the test methods produce the best differentiation possible between the tested materials. For example, in most cases where determination of dynamic parameters (FPA generation, platelet adhesion and activation etc.) is involved, the appropriate contact time should be carefully chosen, or a plot of the parameter as a function of time should be reported, cost permitting. Flow conditions also play a crucial role in blood-material interactions and this implies that the test procedure selected for the hemocompatibility evaluation of a material or a device should necessarily take into account the final application of the product.

Commenting on the test methods and procedures we examined in this study we created a table characterizing simplicity, cost and hemocompatibility characterization effectiveness for the various test procedures and parameters. What we could additionally note is that we can distinguish in principle two major categories of test methods:

1. Preliminary or general screening methods, such as, for example, the closed loop *in vitro* test method evaluated here, which is very useful as a first hemocompatibility characterization step for the materials tested. The difficulty with these methods is that they examine the blood-biomaterial interface as a multiparametric system, making it difficult to determine the influence of each one of the sub-systems (platelet response, activation of the coagulation system, complement activation, thrombosis etc.) from the global parameters determined. Additionally, these methods generally appear incapable of differentiating between biomaterials giving similar performance, possibly due to inherent variation in biological systems.

2. More sophisticated and analytical test methods specially aiming to the determination of a specific parameter at a time and its role in a multiparametric system must be very carefully designed and applied in order to obtain a better understanding of blood–biomaterial interactions, and finally develop relatively simple and reliable tests to evaluate the hemocompatibility of new and existing materials. An invaluable tool towards the last stated goal is the modeling of the whole interaction process. There is a wealth of data but there is a lack of appropriate modeling which could certainly correlate and possibly group together the large number of parameters involved in the blood-material interactions.

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