Plasma recalcification as a measure of contact phase activation and heparinization efficacy after contact with biomaterials

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The rate of plasma clotting was measured in order to investigate two different processes. In both cases normal, pooled platelet-poor plasma was used as a substrate for measurement of clotting. The intrinsic coagulation pathway was studied by bringing a variety of biomaterials into contact with a plasma aliquot and observing the rate of clotting diminish by virtue of factor XII activation. The efficacy of heparinization was investigated by measuring the increase in clotting time of a plasma aliquot during biomaterial contact. In both cases, clotting time was measured turbidimetrically. Marked differences in intrinsic pathway activation were observed between a variety of materials. There were clear differences between the materials and the negative and positive controls. The assay showed that heparinized materials could be distinguished from non-heparinized materials and a non-activated plasma control.

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It is widely recognized that as soon as blood or plasma contacts a foreign surface, plasma proteins are adsorbed onto that surface. This takes place during the first few milliseconds and after a degree of inorganic ion and water adsorption. It is mainly a biophysical effect of the limited solubility of proteins in plasma and reflects to some extent the basic concentrations of the individual proteins. This layer quickly starts to reorganize, however, such that proteins important in the initiation of the coagulation cascade, amongst others, are preferentially adsorbed onto the surface in concentrations greater than one would expect from a knowledge of the composition of plasma. Depending upon the nature of the surface in question, the proteins important in intrinsic coagulation (factor XII, high molecular weight kininogen, prekallikrein and factor XI) can be proteolytically cleaved and thereby initiate the cascade. The end result of the activation of these precursor molecules is a stable, cross-linked fibrin clot. The presence of materials in the vascular system which are able to initiate this cascade may give rise to thrombotic and embolic episodes. Knowledge of the ability of a group of materials to cause thrombosis via this pathway is a critical part of a multi-parametric approach to biomaterials assessment.

Attempts to limit the flux of this pathway during blood–material contact have previously been successful, especially through the use of materials with heparin attached to the surface. Heparin associates with thrombin and factor IXa, interactions which are enhanced by antithrombin III, inhibiting the functioning of the pathway. A number of the other active agents of the coagulation cascade enzymes are also inhibited in this way. However, the process by which heparin is attached to the surface can be detrimental to the efficacy of its anticoagulative properties. Initially materials were coated with heparin simply by ionic attachment or by deposition on the surface. This allows local anticoagulation, but for short-term use only. Covalent attachment is necessary for long-term anticoagulation, but some processes have been shown to restrict heparin action; early heparin conjugates were barely functional. The use of the correct spacer chains and heparin of the appropriate molecular weight has subsequently improved matters. However, it is necessary to measure the degree to which a heparinized surface will allow intrinsic coagulation in order to design the most effective blood-contacting material.

As part of a study of the in vitro methods for the assessment of blood compatibility, the activation of the intrinsic pathway by polymer surfaces has been investigated. In this paper we report on the use of plasma recalcification for this assessment and for the measurement of the deactivation of plasma by heparinized surfaces.

MATERIALS AND METHODS

Materials

All polymer materials assessed for contact phase activation were medical grade tubing (1.0 mm internal
diameter). Silicone (Shore hardness 50) was purchased from Altec (Alton, Hampshire, UK), PVC (Shore hardness 80) from Portex (Hythe, Kent, UK), LDPE from Altec (Alton, Hampshire, UK), Pellethane (Shore hardness 90) from Dow (Midland, MN, USA) and glass from Chance brothers (Malvern Links, Worcestershire, UK). W123 was an experimental material based on polyetherurethane with a Shore hardness of 98.

The materials assessed for heparinization efficacy were leads used in the construction of invasive biosensors. Two forms of experimental heparinized leads were assessed (Hep M16, Hep T16), as was a non-heparinized lead (SD1).

**Blood collection and preparation**

Fresh blood was withdrawn from an ante cubital vein of healthy, male volunteers who had refrained from any medication for at least 14 days and had fasted for at least 4 h. Collection was achieved using a 21-gauge needle placed such that vascular damage and bruising were minimized. Withdrawn blood was immediately placed in tri-sodium citrate (3.2% w/v) at a volume ratio of 1 part citrate to 9 parts blood. Platelet-free plasma (PFP) was prepared by initial centrifugation of the anticoagulated blood at 1000 g for 20 min and subsequent centrifugation of the plasma supernatant at 13 500 g for 2 min in a microcentrifuge. The PFP was pooled, 150 µl volumes aliquoted into eppendorf tubes and frozen by submersion of the tubes in liquid nitrogen. Plasma which was lipaemic was always discarded since hyperactivation tended to occur on the lipid crystals formed during freezing and thawing. All materials were assessed using plasma from the same pool and so each aliquot was effectively the same in background contact phase activity.

**Blood-material contact**

For use, plasma aliquots were thawed for 2 min at 37°C in a water bath. For the contact phase experiments, 100 µl were placed within the lumen of the capillary tubing and incubated statically at 37°C for 10 min. For the heparinized surfaces, the leads were coiled into an LP2 γ-counting tube (Luckham, Burgess Hill, UK) and incubated with 100 µl plasma for 1 min at 37°C without agitation.

**Measurement of partial thromboplastin times**

The partial thromboplastin time (PTT) was calculated by measuring the turbidity of an aliquot of plasma optically as a function of time after the addition of phospholipid and calcium ions. The PTT was taken as the start of fibrin formation, indicated by a rise in the observed turbidity.

In the case of the contact phase activation experiments, 75 µl of plasma were extracted from the material lumen and added to 150 µl of a prewarmed mixture of 25 mM CaCl₂ and platelet substitute (Diagnostic Reagents, Thame, Oxfordshire, UK). Then, 150 µl of the Ca²⁺ and phospholipid mixture were added directly to the LP2 tube in the heparinization experiments. Turbidity was measured at 37°C in a Payton 300BD (dual channel) platelet aggregometer (Toronto, Canada). Measurements were taken in relation to a negative (unactivated plasma) and, for the contact phase experiments, positive (glass-activated plasma) control.

**Treatment of clotting time data**

The clotting times relating to the contact phase activation experiments could not be directly correlated with an activating stimulus. This was attempted using different concentrations of suspended kaolin. This achieved very good correlation of clotting time against log (kaolin concentration) for clotting times under 75 s, but the sability of the suspension of the low concentrations of kaolin needed to extrapolate the curve back to clotting times in the region of that observed with materials was very poor. Instead, plasma was pooled to create a large stock, and the small variations experienced normalized against a control material.

The heparinization experiment results were correlated with an untreated plasma control.

**RESULTS**

The speed of plasma clotting induced by contact of each of the tubular, polymeric materials was determined after the incubation of plasma statically at 37°C (Table 1, Figure 1). Such static contact had an obvious effect on the activation status compared to unactivated plasma. Marked differences were obtained between all the materials, except between PVC and LDPE. The degree to which the biosensor leads deactivated plasma was determined after static incubation at 37°C (Table 2, Figure 2). It is clear that both processes of heparinization (M16 and T16) were effective in retaining the performance of the heparin moieties. The process has caused a lengthening of the plasma clotting time in relation to the negative control. The non-heparin-coated wire has, as expected, caused a shortening of the PTT.

**DISCUSSION**

The results indicate that it is possible to differentiate materials with respect to their ability to activate the coagulation cascade and clot an aliquot of plasma. There are marked differences between many of the materials. Silicone is superior to all the other materials by a comfortable margin byt Pellethane, surprisingly, has the shortest PTT of all the materials evaluated. Glass is, as expected, very much worse than any of the other materials. The results are a little inconsistent with clinical data, which tend to show medical grade polyurethanes, including Pellethane, as the best materials available for blood contacting situations. Certainly they are regarded as better than PE and PVC. The ability of a material to intensify intrinsic coagulation does not necessarily indicate that this will occur to a great degree in vivo. Indeed, the coagulation cascade is just one part of the thrombotic pathway and the interaction of platelets within this scheme of events is extremely important. However, the most promising materials are those which would perform well in both contact phase activation and platelet interactions.
Table 1  Partial thromboplastin times after plasma contact with various materials using liquid nitrogen-frozen plasma (mean ± s.d., n = 4)

<table>
<thead>
<tr>
<th>Material</th>
<th>Plasma clotting time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No activation</td>
<td>215 ± 9</td>
</tr>
<tr>
<td>Glass</td>
<td>67 ± 3</td>
</tr>
<tr>
<td>PVC</td>
<td>154 ± 11</td>
</tr>
<tr>
<td>LDPE</td>
<td>150 ± 4</td>
</tr>
<tr>
<td>Silicone</td>
<td>191 ± 4</td>
</tr>
<tr>
<td>Pellethane</td>
<td>137 ± 4</td>
</tr>
<tr>
<td>W123</td>
<td>162 ± 0.5</td>
</tr>
</tbody>
</table>

Figure 1  Partial thromboplastin times after plasma contact with various materials using liquid nitrogen-frozen plasma (mean ± s.d., n = 4).

Table 2  Partial thromboplastin times after plasma contact with heparinized biosensor leads (mean ± s.d., n = 4)

<table>
<thead>
<tr>
<th>Material</th>
<th>Plasma clotting time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No activation</td>
<td>207 ± 10</td>
</tr>
<tr>
<td>SD1</td>
<td>172 ± 15</td>
</tr>
<tr>
<td>Hep M16</td>
<td>223 ± 13</td>
</tr>
<tr>
<td>Hep T16</td>
<td>231 ± 39</td>
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The results show that this assay is also useful in providing evidence of the efficacy of the heparinization of a surface. The assay has not discriminated between the two surfaces, indicating either a similarity in the final result of heparinization or a slight vagueness in the ability of the assay to differentiate between small quantities of heparin. The results also indicate that the surfaces have in fact reduced the level of active components within the plasma compared to the negative control. Heparin is known to inactivate forming factor Xa within the coagulation cascade. This is presumably what has happened, after a small degree of initial activation within the starting plasma and the contact phase which has occurred due to the interaction of the plasma with the LP2 tubes.

CONCLUSIONS

Medical grade and non-medical grade materials were assessed for their ability to induce intrinsic coagulation. Marked differences were observed between all the materials assessed, except for LDPE and PVC. The results were not consistent with expectations from clinical indications. It is concluded that contact phase activation is just a part, but a necessary element, of a multi-parametric approach to biomaterials assessment which is necessary for the successful design of materials for blood-contacting medical devices. Significant differences were shown to exist between two different heparinization processes and the non-heparinized version. No difference was observed between the two heparinized surfaces. The assay demonstrated that the heparinization process not only improved the thrombogenicity of a biosensor lead but also that some local anticoagulation was conferred upon the surrounding plasma, presumably at the level of factor Xa, by its deactivation. It is concluded that this assay would also be effective in the screening of heparinized materials in their efficacy of heparinization, which is a necessary element of the design of non-thrombogenic biomaterials.

ACKNOWLEDGEMENTS

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REFERENCES