Haemocompatiblity of controlled release glass

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There are many medical applications which benefit from the use of soluble biomaterials, including the sustained release of drugs over a precise period of time, or temporary conduits for controlling nerve regrowth. We have manufactured a series of phosphate-based controlled release glasses (CRGs) in which the solubility could be controlled by varying the concentration of CaO and Na_2O. Fibres of the CRG containing iron and cerium were placed into direct contact with human neutrophils and macrophages in tissue culture for 2.5 and 24 h respectively and the responses analysed by scanning electron microscopy (SEM) and confocal microscopy. The supernatants were analysed for the cytokine IL-1β by enzyme-linked immunosorbent assay (ELISA). Disks of CRG of various compositions were placed in contact with whole blood for 30 min and platelet adhesion assessed by SEM. Activation of platelets, granulocytes and complement were quantified by ELISA for β-thromboglobulin, elastase and iC3b. Intrinsic coagulation activation was measured by timing the clotting of recalcified plasma. Only the cerium fibre inhibited IL-1β release from macrophages. No platelet adhesion was observed to any disk composition. Three compositions containing MgO inhibited plasma clotting and showed an insignificant level of complement activation. This study has demonstrated the development of a number of compositions of CRG, which have great potential in a wide variety of biomedical applications.

1. Introduction

Controlled release glasses (CRGs) are a new class of material with exciting potential in the medical field. CRGs are based on calcium and sodium phosphates, unlike commercial glass which has a basic structure consisting of silica. The glass is manufactured in much the same way as conventional soda-lime glass, which is to heat the constituents to temperatures above 1000 °C, then cast the melted material into various forms, such as powder or granules, tubes, fibres and wool. However, CRG differs greatly from commercial glass in that it dissolves completely in an aqueous environment, leaving no solid residue. The solution rate of these inorganic polymers can be predetermined and adjusted by altering the chemical composition, which remains stable and constant throughout the degradation [1].

Previous applications of CRG incorporated various additional metal ions into the basic degradable structure and took advantage of the constant, predetermined solution to create a slow release vehicle. Industries benefiting from such a device include food production, where pesticides, fungicides and animal feeds all require the supply of a range of metal ions to be delivered in this manner [2–5]. In the medical field, CRG has been used for silver release to effect infection control in patients requiring long-term indwelling catheters and wound dressings [1, 6]. The potential for CRG as a bone graft substitute has also been investigated [7, 8].

For blood-contacting applications there is potential for use of CRG in microvascular repair. Tubes with diameters matched with blood vessel size could be used to join severed veins and dramatically reduce surgery time in operational procedures. Fibrous CRG could potentially be woven into a cloth network for use in repair patches. There are situations where the mechanical strength of a medical device is needed only in the short term, such as guides for nerve regrowth. Once the application has been fulfilled, resorption of the device prevents a chronic inflammatory response.

There are of course a variety of potential hazards associated with implanting a dissolving material into the human body. These hazards may become more or less significant when that material is dissolving into the blood stream. Our initial studies of these materials have identified a number of appropriate techniques which should provide us with information about the effects of metal ion evolution into the blood and the potential role of cell/material interactions.

The partial thromboplastin time (PTT) assay is an important aspect of haemocompatibility of these

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glasses. *In vivo* a thrombus is generated after damage to the tissue thromboplastin (tissue factor) which complexes with FVII to initiate the coagulation cascade. This physiological route to thrombogenesis is complemented by the activation of FXII (Hagemen factor) after the contact of blood with an artificial surface. The exact physiological significance of this type of activation is not fully understood but is known as contact phase activation. Hyperactivation of the contact phase proteins causes rapid coagulation. Assessment of the initiation of this cascade complements the knowledge of the effects of a surface on platelet adhesion and activation and so is desirable in the multiparametric assessment of a biomaterial such as a phosphate based glass.

Platelet adhesion was analysed using scanning electron microscopy (SEM) and the quantity and the quality of adhered platelets examined. In general, the use of SEM allows us to assess the reactivity of a surface to platelets. Not only is platelet adhesion estimated, but the degree of platelet spreading will indicate the ability of the surface to activate platelets.

Granulocyte activation is measured by using a heterogeneous enzyme immunoassay for the specific determination of PMN elastase from PMN leukocytes in complex with α_1 anti-proteinase inhibitor in plasma. This means that the elastase released during granulocyte activation is captured by the immobilized antibodies present in the test wells. An enzyme-conjugated secondary antibody when added in conjunction with a substrate will produce a visible colour change which is proportional to the amount of granulocyte activation. The same principle is used for quantifying both complement and platelet activation.

The monocyte/macrophage and the neutrophil are considered to be the initial controlling components of inflammatory and healing responses in the presence of implanted biomaterials [9–11].

This study examines the potential for CRG to be used as a biomaterial in contact with human tissue. Parameters of both haemocompatibility and tissue compatibility were assessed for a variety of CRG compositions.

2. Materials and methods

2.1. Controlled release glass

Eight compositions of glass for blood-contacting application were created (B1–B8). These were cast into disks of 10 mm diameter and 4 mm thickness. All were largely based on Na_2O and P_2O_5. In addition to these constituents, other metal oxides were present in varying concentrations: B3, B4 and B6 contained CaO; B1, B2, B3 and B5 contained K_2O; and all but B4 and B6 contained MgO. For tissue implantation applications, three different fibres were prepared (F1–F3), of approximately 30 μm diameter and 2.5 cm in length. All comprised mainly Na_2O, P_2O_5 and CaO. F1 also contained Fe_2O_3 and NaF; F2 contained Ce_2O_3 and F3 contained Ce_2O_3 and Se. Before biocompatibility assessment, the disks were cleaned by ultrasonication in lipsol (5% vol./vol. in water) for 30 min and the fibres sterlized by dry heat (190 °C for 3 h).

2.2. Collection and manipulation of blood

Whole blood was collected from healthy human volunteers who had refrained from any medication for at least 14 days. Venepuncture was achieved using a 21-gauge needle in a median cubital vein. For monocyte and neutrophil isolation assays, the blood was placed into sodium heparin at a final concentration of 2 units heparin per ml blood. For all other assays, the blood was collected into 3.8% (wt/vol) tri-sodium citrate at a volume ratio of 9 parts blood to 1 part citrate. Platelet poor plasma (PPP) was generated by spinning the blood at 1400 g for 10 min. Platelet rich plasma (PRP) was prepared by spinning the blood at 150 g for 20 min.

2.3. Partial thromboplastin time (PTT) assay

A PTT assay was carried out on each of the eight glass disks. PPP was prepared and 200 μl placed onto the surface of each disk, already equilibrated at 37 °C, and incubated for 1, 3 and 10 min at 37 °C. 67 μl of activated PPP was added to a pre-warmed tube containing 67 μl platelet substitute (Diagen, Diagnostic Reagents, Thames, Oxfordshire, UK) and 67 μl $CaCl_2$ (25 mM in H_2O) and quickly mixed. The turbidity of this mixture was followed over time using a platelet aggregometer (Payton 300BD dual channel, Toronto, Ontario, Canada) linked to a chart recorder. The start of an increase in turbidity indicated the appearance of a fibrin clot and defined the partial thromboplastin time.

2.4. Platelet, complement and granulocyte activation assays

Platelet, granulocyte and complement activation were all quantified by enzyme linked immunosorbent assay (ELISA) of aliquots from the same blood sample. 2 ml of whole blood were incubated with each glass disk at 37 °C whilst agitating on an orbital shaker for 1 h. At the end of the incubation period, 500 μl of blood were withdrawn from each sample and spun at 30 000 g for 30 s in a microcentrifuge. Plasma was aliquoted from the separated blood into separate tubes and frozen at − 80 °C until analysed. The samples taken for complement activation were frozen as a 1 : 1 mixture of plasma and a specimen stabilizing solution (Quidel, San Diego, CA, USA). Platelet activation was assessed by measuring the release of β-thromboglobulin (Boehringer Mannheim, Mannheim, Germany). A measure of alternate complement pathway activation was achieved by quantifying iC3b generation (Quidel, San Diego, CA, USA) and granulocyte activation by an increase in elastase-α_1-antiproteinase complex (Merck, Darmstadt, Germany).

2.5. Platelet adhesion

Platelet adhesion was assessed on three samples: disk B1 (slow solution rate) and disks B4 (medium solution rate) and B8 (faster solution rate). 1 ml PRP was

placed in contact with each sample and incubated statically at room temperature for 3 h. Each sample was then washed with phosphate buffered saline (PBS) and fixed in 2.5% vol./vol. glutaraldehyde in 100 mM sodium cacodylate. After dehydration by graded alcohol exposure (70, 90, 100% methanol), each sample was prepared for SEM viewing by gold sputtering after mounting on an SEM stub.

2.6. Isolation of monocytes and neutrophils

Blood was mixed with dextran and allowed to sediment for 20 min at room temperature until a clear separation between the leukocyte rich supernatant and the red cell fraction was visible. The supernatant was collected and carefully layered onto a lymphocyte separation medium (Gibco BRL, Paisley, UK) and then centrifuged at 400 g for 25 min. From this sample, the monocyte/lymphocyte layer was removed and washed with PBS by centrifugation at 320 g for 5 min.

Also from this separation, the excess plasma and density gradient were removed and all the neutrophils and remaining red blood cells collected and pooled into one tube. 1 ml of water was added to these cells and agitated for 30 s to lyse the red blood cells. The lysing action was stopped by the addition of 9 ml of PBS to this neutrophil rich cell solution, which was then centrifuged at 320 g for 5 min. After centrifugation of each of the monocyte and neutrophil suspensions, excess liquid was removed and each white cell pellet was resuspended by agitation with 10 ml PBS (neutrophils) or culture medium (monocytes).

The cell count, as determined by the use of a haemocytometer, was $5.4 \times 10^5\,ml^{-1}$ macrophages and $8.5 \times 10^5\,ml^{-1}$ neutrophils. Flow cytometric analysis was performed to determine purity of the cell isolations and to confirm cell concentration. 40 µl neutrophil cell suspension was stained with 2 µl CD16b conjugated with fluorescein isothiocyanate (FITC). Monocytes were differentiated by adding 2 µl CD14 conjugated with phycoerythrin (PE) to 40 µl of monocyte suspension. After the monocyte/lymphocyte mixture was cultured with glass fibres (Section 2.7) for 2 h, the supernatant was removed and replaced with fresh culture medium, to remove lymphocytes from the culture. This supernatant was analysed by flow cytometry to characterise the removed cell population. The remaining purity of the macrophages was calculated by comparison of the two sets of flow cytometry data.

2.7. Monocyte/macrophage contact with glass fibres

20 mg of each fibre were placed into 3 cm diameter petri dishes and 1 ml of the monocyte/lymphocyte preparation added. The cells were incubated with fibres at 37 °C in an atmosphere containing 5% vol./vol. CO_2. The culture medium was withdrawn from the petri dish after 2 h of culture to remove unwanted lymphocytes and replaced with fresh culture medium (Gibco BRL, Paisley, UK) containing solubilized amphotericin-B (Sigma, Poole, Dorset, UK) to prevent fungal infections which may arise from contamination from the clean but not sterile method of macrophage isolation.

After 24 h, the supernatant was collected, centrifuged and the non-cellular component frozen at − 70 °C for analysis of the cytokine interleukin-1β (IL-1β). Fibres were removed for cellular morphological analysis and washed twice in PBS. To the remaining fibre samples and macrophages, 1 ml 1% vol./vol. Triton X-100 in PBS was added and lightly agitated for 5–10 min. This mixture was then centrifuged and the supernatant collected and again frozen at − 70 °C for ELISA analysis. This formed the positive internal control, providing data on the full potential of available cytokine that could have been released by the cells. A negative control of cultured macrophages with no fibres present was also performed. The supernatant was analysed for concentrations of IL-1β by ELISA (Biosource Europe S.A., Fleurus, Belgium).

2.8. Neutrophil contact with glass fibres

1 ml of isolated neutrophils was placed into contact with 20 mg of each fibre in 3 cm diameter petri dishes and incubated for 2.5 h at 37 °C in an atmosphere containing 5% vol./vol. CO_2. The supernatant was collected and frozen for IL-1β analysis as in Section 2.7. Fibres were removed and immediately washed in PBS for cellular morphological examination. Negative and positive controls were performed as in Section 2.7. The IL-1β assay was performed to check mononuclear contamination. It was not expected to observe IL-1β release from neutrophils.

2.9. Macrophage and neutrophil morphology

Cell attachment onto fibres, cell alignment and spreading were evaluated on each fibre composition. Fibres were fixed in 2.5% vol./vol. gluteraldehyde in PBS for 30 min and washed twice in PBS, 10 min each time, then dehydrated in graded methanol: 70, 90 and 100%, 15 min for each incubation and twice for each concentration. The prepared samples were then mounted on stubs and gold sputtered, before viewing by SEM.

Fluorescent staining of F-actin allowed examination of the microfilament structure of the cytoskeleton of the cells attached to the fibres. Each sample was then fixed in PBS containing 4% vol./vol. formaldehyde and 2% weight/vol. sucrose for 10 min. After washing once with PBS, 1% vol./vol. Triton X-100 in PBS was added to the fibres for 3 min before washing again with PBS. The fibres and cells were stained with rhodamine phalloidin (Molecular Probes, OR, USA) (5 µl in 200 µl PBS) for 20 min before a final wash with PBS. The fibres were then mounted with florostat before viewing with a confocal laser scanning microscope (Zeiss, Welling Garden City, UK).

3. Results

Three of the glass disk formulations, B3, B6 and B8 appear to inhibit coagulation of plasma, after

TABLE I Plasma clotting times (s) in partial thromboplastin time assay after contact of platelet poor plasma for 10 min at 37 °C

Disk number	$n = 1$	$n = 2$	$n = 3$	$n = 4$	$n = 5$
B1	140	180	60	120	130
B2	270	110	116	134	110
B3	*	*	*	*	*
B4	*	180	154	240	240
B5	*	420	728	540	420
B6	*	*	*	*	*
B7	*	540	*	*	300
B8	*	*	*	*	*

*represents no clotting at all.

TABLE II Plasma clotting times (s) in partial thromboplastin time assay after contact of platelet poor plasma for 3 min and 1 min at 37 °C

Disk number	3 min activation		1 min activation		
	$n = 1$	$n = 2$	$n = 1$	$n = 2$	$n = 3$
B1	140	120	218	200	–
B2	110	140	140	100	–
B3	*	*	568	*	*
B4	160	120	110	146	–
B5	210	220	240	280	–
B6	*	*	*	768	*
B7	420	330	330	*	360
B8	*	*	*	300	*

* represents no clotting at all.
– represents no test performed.

activation times of 3 and 10 min as can be seen in the PTT data in Tables I and II. The plasma did not clot after an observation time of 12 min. Indeed, even when viewed after 12 h, the plasma was still fluid. After an activation period of 1 min, two times out of three, no clotting occurred in these three samples. When the plasma was seen to clot after 1 min activation time, with disks B3 and B6, it did so after approximately 700 s. This demonstrates the effect of the released ions into the blood, as unactivated blood clotted after approximately 200 s. Disks B5 and B7 also extended the clotting time beyond the unactivated control, sample B7 clotting later than B5. B4 provided a typical clotting time, similar to unactivated plasma (approximately 200 s). Only disks B1 and B2 clotted within the observation period every time. The clotting times equated to 126 ± 43 s for sample B1, 148 ± 69 for sample B2 (mean $\pm$ standard deviation, sample size $n = 5$), indicating that sample B1 is probably not as haemocompatible as B2. Statistics could not be performed on the other samples since the inclusion of ∞ (due to the samples which prevented any clotting at all, therefore time to coagulation $= \infty$) into a statistical calculation is meaningless. The inhibition caused by samples B3–B8 is almost certainly due to the type and quantity of ions being released, disrupting the complexes Ca^{2+}–FVIIIa–FIXa and Ca^{2+}–FVa–FXa, thus preventing coagulation.

SEM analysis showed that no adhesion of platelets to the surface of any of the disk compositions had occurred. The surface topography is seen to be changed after 30 min contact with blood but this is due to the dissolving glass, explaining why the platelets are not adhered. The interface is so mobile any particular platelet has too short a residence time for adhesion.

When preparing the blood samples for the iC3b, elastase and β-TG assays, it was noted that after 30 min activation with disks B2, B4, B5 and B7, (for which period the blood was contacted), the blood had haemolysed, the contact with the different formulations of glass causing a disruption in the red blood cell membrane resulting in the release of haemoglobin. This reaction was not seen however, with disks B3, B1, B6 and B8 (or with the control sample which contained no disk), suggesting that this was a chemical/biochemical rather than a purely physical phenomenon due to shaking.

There was an insignificant level of complement activation in disks B3, B6 and B8 compared with the control sample as can be seen in Fig. 1. Disk B2 had the largest activation, followed by disks B7, B5 and B4 respectively. However, the standard deviations were very large in these last four samples and so the significance relating to the differences between them are hard to determine. It is possible that the large standard deviations were due to the samples being tested with blood from different sources. Disks B3, B5 and B8 had the lowest granulocyte activation with no differences between them as can be seen in Fig. 2. B2, B4, B6 and B7 also had similar activations to each other but larger than the former group. There were no significant differences between any of the samples in the platelet activation assay as can be observed in Fig. 3. This may be due to the high levels of platelet activation caused by each disk being at the high range of the sensitivity of the assay. In any case, massive platelet activation was observed in all samples, including the control.

The mean purity was 47% neutrophils in the polymorph isolation, the other 53% of contaminating cells being red blood cells as determined by the flow cytometer and from this the neutrophil count was found to be 1.2×10^6 ml^{-1} neutrophils. The results

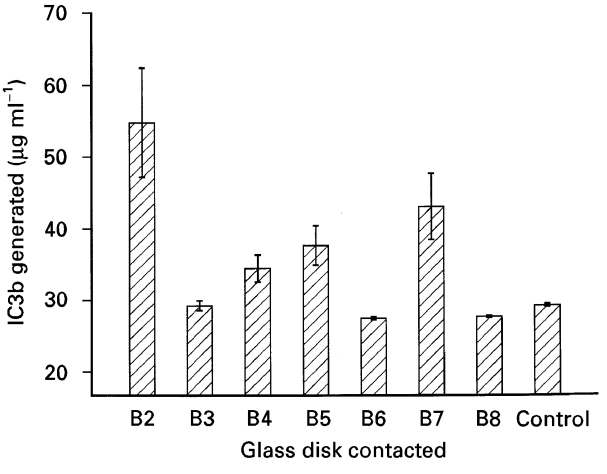


Figure 1 Complement activation after a 1 h contact, 37 °C, $n = 5$. Data is plotted as mean and standard error of mean.

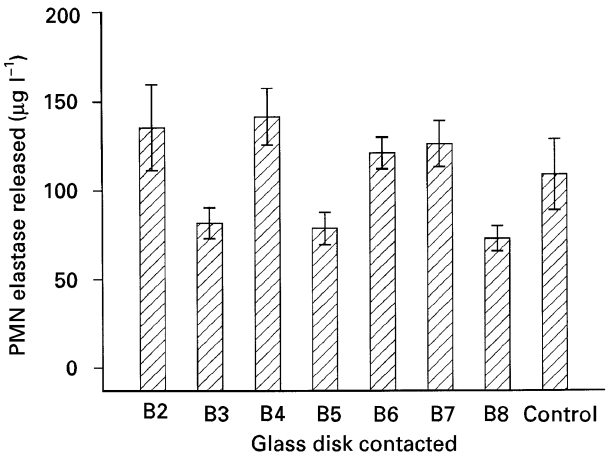


Figure 2 Granulocyte activation after a 1 h contact, 37 °C, $n = 5$. Data is plotted as mean and standard error of mean.

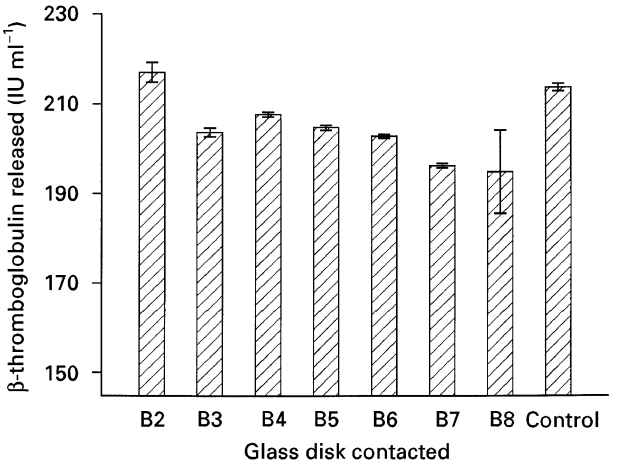


Figure 3 Platelet activation after a 1 h contact, 37 °C, $n = 5$. Data is plotted as mean and standard error of mean.

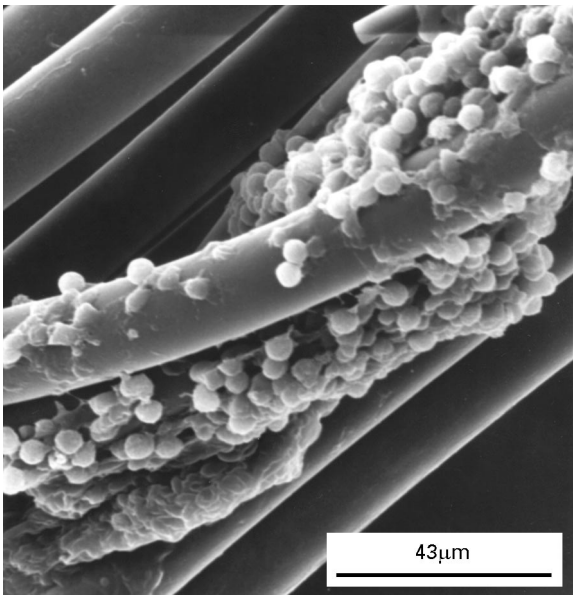
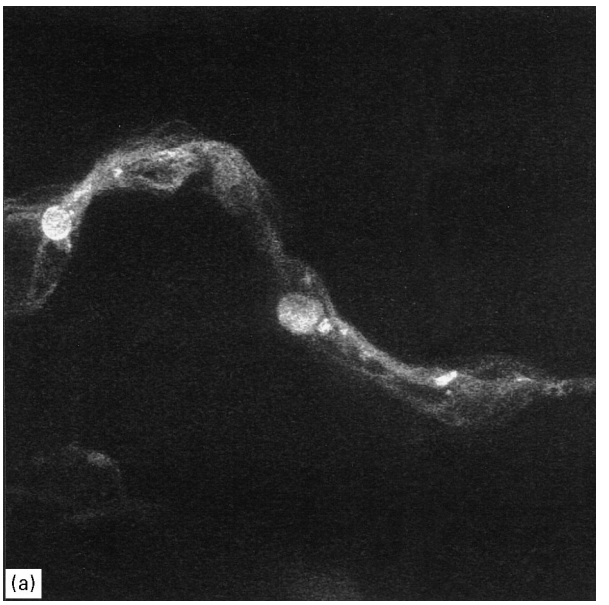


Figure 4 SEM image of neutrophils adhered to fibre F2 magnification ×700.

from the flow cytometry data on the 2 h supernatant from the macrophage contact experiments showed that 95% of the leukocytes were removed from the culture. The flow cytometry analysis also revealed that $42 \pm 7\%$ of the monocyte population is also removed during this change in culture medium. This was a significant increase compared to culturing monocytes on normal tissue culture plastic (negative control) where the percentage of non adhered macrophages is significantly less (20%). With these results, it was found that changing the culture medium after 2 h, the macrophage cell count was reduced from 3.5×10^5 ml^{-1} cells to 1.47×10^5 ml^{-1} macrophages.

As indicated in the SEM photograph and the confocal microscopy (CM) picture in Figs. 4 and 5 (a and b) all of the fibre samples had adherent neutrophils and macrophages on the material surface for each period of contact, although F2 appeared to have more



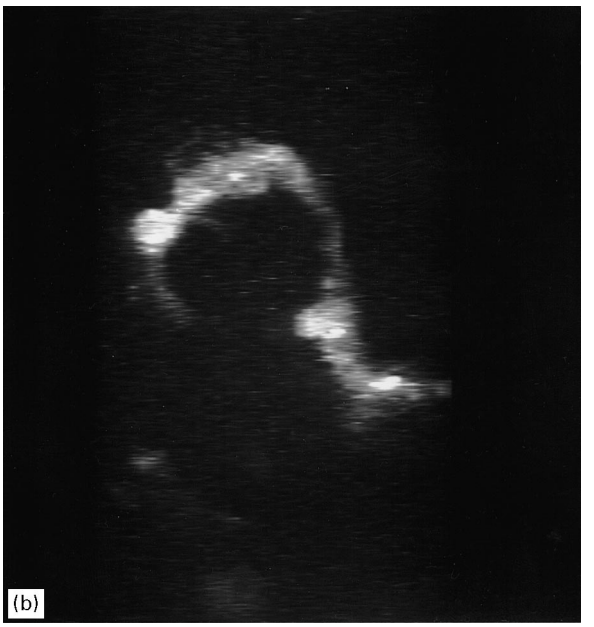


Figure 5 (a) CM image of macrophages wrapped around fibre F1 magnification ×130 and (b) CM image of macrophages wrapped around fibre F1. Rotated through 75° magnification ×130.

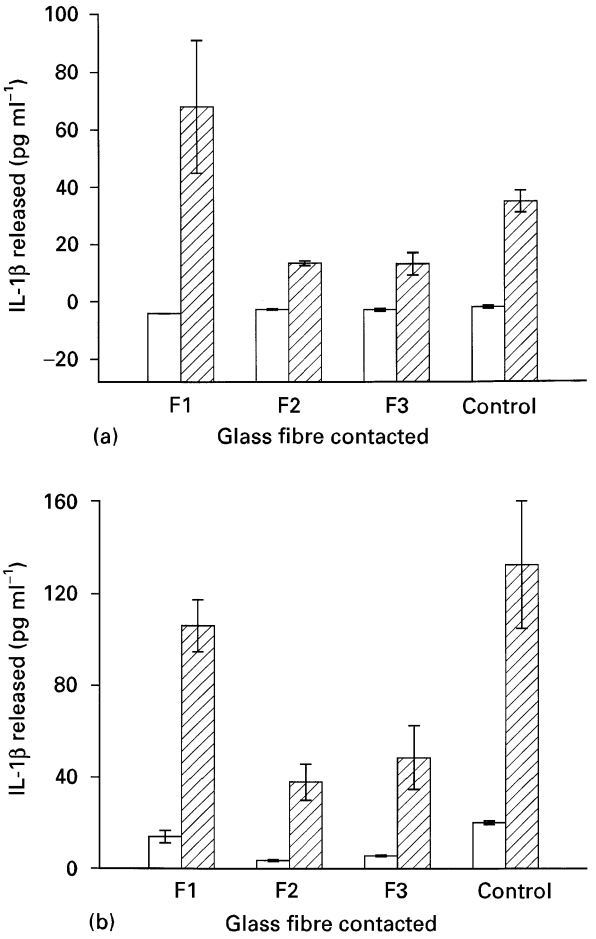


Figure 6 (a) IL-1β concentrations after: (□) a 2.5 h neutrophil contact and (▨) a 24 h macrophage contact for fibres F1, F2, F3 and a negative control. $n = 3$. Data is plotted as mean and standard error of mean and (b) IL-1β concentrations for positive control (lysed cells): (□) after a 2.5 h neutrophil contact and (▨) a 24 h macrophage contact for fibres F1, F2, F3 and a negative control, $n = 4$. Data is plotted as mean and standard error of mean.

adhered cells than either F1 or F3. The neutrophils as seen on F2 in Fig. 4, were clustered together. The CM images in Fig. 5(a and b) show macrophages wrapped around fibre F1. The image in Fig. 5a can be seen rotated in the z-plane by 75° in Fig. 5b. The 3D image produced by the CM shows the macrophages orientating along the length of the fibres as well as wrapping around the circumference as seen in Fig. 5 (a and b).

The IL-1β ELISA performed, produced results as shown in Fig. 6 (a and b). As can be seen in the two graphs in Fig. 6 (a and b), the activation of the neutrophils and the macrophages in contact with the three fibre samples was low in comparison to the negative control (cells in contact with no fibres). F2 in particular, had a significantly low macrophage IL-1β concentration in the supernatant, almost a third of that of the control. All fibres and the control had an insignificant value of IL-1β concentration in the neutrophil supernatant after 2.5 h, as expected. The IL-1β concentrations of the supernatant of the Triton X-100 lysed cells can be seen in Fig. 6b. This shows the maximum possible IL-1β concentration possible from the cell population in each petri dish. The macrophage IL-1β assay showed high concentrations of IL-1β in the positive control in Fig. 6b when the cells were lysed. When the neutrophil isolation was lysed, some IL-1β was seen to be present. This is not as expected, and it is fair to assume that this small amount of IL-1β is due to the contaminating leukocytes (5% of the cell population).

4. Discussion and conclusions

Glass disks B3, B6 and B8 all have been shown to prevent, rather than encourage, coagulation of plasma. These particular compositions also performed well in the granulocyte and complement activation assays performed. This indicates that the potential use of the B3, B6 and B8 glass compositions in a short term blood contacting implant is possible. It is necessary however, to perform more extensive *in vitro* experiments to confirm these conclusions and determine the exact mechanism of the inhibition of coagulation.

The adherence of the neutrophils and the macrophages to all three fibre compositions is an interesting phenomenon. The potential use of these compositions, especially in fibre form perhaps lies more with a tissue engineering role rather than a blood contacting implant area.

The adherence of neutrophils and macrophages to the CRG surface is important, however also of significance is the activation and subsequent release of secretory products by these inflammatory cells. The macrophage may be considered as the control cell in inflammatory reactions involving implanted biomedical polymers. IL-1β is a cytokine that has a fundamental role in the modulation of an inflammatory response by affecting many cell types [12, 13], influencing fibroblast growth and proliferation [14, 15] as well as neutrophil adhesion to endothelial cells [16] to name only two of the attributed effects of IL-1β release. The results of the ELISA on the three fibres *in-vitro* studies indicate that the fibre presence did cause the macrophages to release IL-1β.

Samples B3 and B8 stimulated low complement, granulocyte and platelet activations compared with the negative control and also prevented the coagulation of plasma. Sample B6 also lengthened the plasma coagulation time considerably and performed well in the complement and platelet activation assays. Sample B1 was not evaluated in the platelet, granulocyte and complement activation studies, because it showed substantial clotting in the PTT assay after approximately 126 s. These views must, however, be tempered by the fact that a more in depth study of platelet activation, possibly by flow cytometry, must be performed before final conclusions are drawn.

The calculated percentages of the amount of IL-1β released by the macrophages in direct contact with the fibre samples compared with the positive control give indications of the extent of macrophage activation. Fibres F2 and F3 had an effect on the IL-1β concentration similar to that of the negative control. F2 stimulated approximately 36% of the potential maximum amount of IL-1β. F3 and the negative control both stimulated 26%, F1, however, stimulated a higher response (64%) of the total IL-1β. The tendency for

the neutrophils and the macrophages to adhere to and orient along the fibre surface demonstrates the potential for these materials to be employed in a tissue regeneration application. It is clear that small changes in chemical composition have a considerable effect on the tissue responses to this group of materials. Our future studies will concentrate on identifying how these changes can be used to customize the biomaterial to a particular application, either stimulating or inhibiting the required response.

This initial haemocompatibility study on a novel biomaterial has indicated that selected and controlled compositions of these materials may provide good blood-contacting surfaces. Furthermore, the adaptability in form and shape of the material and the extremely promising cell/material interactions highlighted demonstrate that controlled release glasses have a considerable potential in many biomaterial applications.

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