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In vitro host response assessment of biomaterials for cardiovascular stent manufacture

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The deployment of a vascular stent during angioplasty has greatly reduced the risks of restenosis. However, the presence of the device still induces a host response as well as a mechanical action on the blood vessel wall and an alteration of the haemodynamics. Platelet and inflammatory cells can adhere on the stent surface and be activated to produce biochemical signals able to stimulate an excessive proliferation of the smooth muscle cells with the consequent obstruction of the vessel lumen. For these reasons, the host response to two of the materials used in stent manufacture, stainless steel and diamond-like carbon, was investigated *in vitro*. The data showed that stainless steel induced a higher level of host response both in terms of platelet aggregation and macrophage activation. However, the spreading of inflammatory cells was more accentuated on diamond-like carbon. The inflammatory cells produced levels of platelet-derived growth factor, a key signal in smooth muscle cell proliferation, similar to stainless steel thus suggesting that carbon coatings may not be able to prevent restenosis.
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Introduction

Blood circulation in vertebrates is supported by the fine architecture of the blood vessels. These vessels do not exert a mere conduit function, but also participate to the haemodynamics and haemostasis [1]. The contractile peristaltic movement favouring the blood flow is ensured by a layer of smooth muscle cells (SMC) able to contract and relax periodically. Endothelial cells coat the inner surface of the vessel with a highly hydrophilic, smooth layer which inhibits the formation of blood clots [1]. Furthermore, the endothelial cells regulate the infiltration of plasma components and inflammatory cells from the blood to the surrounding tissue when this is required to tackle injuries or infections [2].

In some pathological circumstances, however, this delicate balance of functions is altered and an unwanted accumulation of inflammatory cells takes place in the space separating the endothelial cell layer (intima) from the SMC layer (media). The highly active inflammatory cells nestling in this space send biochemical signals to the SMC which start to proliferate invading the lumen of the vessel [3]. As consequence of this, a partial or complete occlusion (stenosis, from the Greek = obstruction) of the vessel develops. This occlusion may become particularly dangerous when taking place in the small vessels supplying blood to the cardiac muscle [4].

Cardiovascular techniques have been optimised in the last three decades to reduce the occlusion of these vessels. Angioplasty, a technique performed by the endoscopical insertion of an expandable balloon into the vessel is the method preferred by the surgeons to eliminate the occlusion [4]. However, this method is not always able to avoid the recurrence of the occlusion. For this reason, a modification of the technique has been adopted where a metal stent is deployed at the time of the balloon expansion to keep the lumen of the vessel pervious [5, 6].

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Stents are tubular meshes made of either stainless steel (St, 316L) or nitinol, a nickel (55%)-titanium (45%) alloy [6–9]. These metals, although, ensuring the required mechanical performances still suffer of biocompatibility problems [10].

The poor biocompatibility is due to: (i) the activation of the coagulation cascade, (ii) the triggering of an excessive wound healing response, (iii) the corrosion of the metal with the release of metal ions which are potentially toxic for the cells. The reaction of the body to the foreign metal is aggravated by other factors such as the alteration of the haemodynamics and the mechanical stress exerted by the device on the vessel wall [11].

The poor biocompatibility of the materials employed in the manufacture process leads to clinical complications which are (i) the development of thrombus (consequence of the clotting system activation) and (ii) re-stenosis (consequence of the excessive intima growth) [4].

Many strategies have been adopted to reduce the risks of thrombosis and restenosis caused by stents [10]. The coating of stents with more biocompatible materials is the common denominator to all of them. Stents are already available on the market with coatings based on pyrolitic carbon (Sorin, Italy) and a biomimetic polymer (Biocompatible Ltd, UK). Many other types of non-degradable and biodegradable coatings have been proposed [10]. These include, for example, polyurethanes, polydimethylsiloxane, polyethylene terephtalate (Dacron) and polyglycolic/polylactic acid block co-polymer systems. Some of these polymeric coatings have also been used to deliver drugs able to minimise restenosis [10].

In the present paper, the effect of the surface physicochemical properties of the material on thrombosis and restenosis is evaluated *in vitro* in clinically reflective conditions which assess sequentially protein adsorption, platelet aggregation and inflammatory cell activation. These models were applied to test St, the most used materials in stent manufacturing, and diamond-like carbon (DLC) a typical stent coating material, to compare their potential to elicit the host response.

Materials and methods

Specimen preparation

316L St (10 and 15 mm diameter) were purchased from Goodfellow, UK. St discs were also coated with DLC by Teer Coatings Ltd, UK by a standardised magnetrone coating system. All the discs were washed in 1 : 10 diluted 7X-PF detergent (ICN, UK) and extensively rinsed in deionised water before use.

Protein adsorption

Human citrated platelet poor plasma was spiked with radiolabelled ^{125}I- fibrinogen (Amersham, UK) with a final activity ranging between 0.734×10^6 and 1.406×10^6 cpm/ml. Each disc was equilibrated in phosphate buffered saline pH 7.2 (PBS, Oxoid, UK) for 15 min and then incubated in spiked plasma (0.25 ml) for 1 h, 37 °C. The discs were washed five times with 1 ml of PBS and the adsorbed proteins eluted with 2% (w/v) sodium dodecyl sulphate/0.05 M Tris/HCl pH 6.5 buffer without reducing agents for 30 min, 37 °C. The radioactivity of the eluted proteins was measured using a Wallac Wizard, 1480 Automatic gamma counter (LKB-Wallac, Sweden). The levels of fibrinogen were calculated from the specific radioactivity of the spiked plasma. Six samples for each material were tested and the data expressed as mean $\pm$ standard deviation.

Platelet adhesion

Platelet-rich plasma was prepared from fresh human peripheral blood. The citrated blood was centrifuged at $200 \times g$ for 30 min. The plasma was gently collected by a glass pipette and used for the experiment. St and DLC discs were incubated with 0.5 ml of plasma at 37 °C for 1 h under a 95% air, 5% CO_2 flow in humidified atmosphere. The samples were washed three times in PBS and fixed in 2.5% (w/v) glutaraldehyde in 0.1 M cacodilate buffer pH 7.2 at ambient temperature for 1 h. The specimens were then washed in deionised water and stepwise dehydrated by ethanol/water mixtures (25%, 50%, 75% and 90% by volume). Freeze-dried samples were sputter-coated by palladium and analysed by scanning electron microscope (SEM, 6310 Jeol Instruments, UK) at 5 keV.

Inflammatory cell adhesion and activation

Mononuclear cells, a heterogeneous cell population including monocytes and lymphocytes, were isolated by Boyum's method [12]. Briefly, the heparinised blood was layered on to a polysucrose gradient (Histopaque 1077, Sigma, UK) and centrifuged at $400 \times$ g for 30 min. The mononuclear cell ring formed at the plasma/gradient interface was withdrawn and washed three times in PBS. The cells were finally re-suspended in RPMI-1640 medium (Sigma) and counted by haemocytometer.

The early monocyte oxidative burst initiated by the tested materials was evaluated by the means of a chemiluminescence method previously described [13]. Briefly, 10^5 cells were incubated with a 10-mm disc of the tested materials in presence of 1 mM luminol (Sigma) and 1 μM phorbol myristate acetate (PMA, Sigma) and 10% (v/v) human plasma. The production of free radicals by the cells was followed by luminometer (BioOrbit 1251, Labtech, UK) in the first 5 min of incubation. Cells incubated with PMA, but not exposed to any material were considered as control. The integral of the peak area was calculated and data expressed as mean $\pm$ standard deviation of the percent of activation of the control from $n = 10$.

In a separate set of experiments the adhesion and activation of the mononuclear cells was assessed after 3 h incubation with the materials. Discs as well as control tissue culture plates (TCP) were preconditioned for 30 min with freshly isolated human plasma obtained by the same mononuclear cell isolation procedure described above. The discs were washed three times in PBS to remove the excess of proteins and incubated with 10^5 cells/ml in RPMI-1640 medium for 3 h at 37 °C, under a 95% air, 5% CO_2 flow in humidified atmosphere. The adhering cells were washed, fixed and prepared for SEM following the same procedure used for the platelet

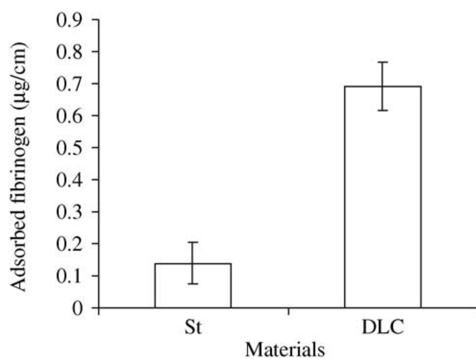


Figure 1 Levels of adsorbed plasma fibrinogen on the biomaterial surfaces.

adhesion studies. The supernatants from the cell adhesion experiments were collected, centrifuged at $1000 \times g$ to remove unbound cells and the supernatants stored at $-70\,^{\circ}$C until use. One hundred microlitres of each supernatant were tested for tumour necrosis factor alpha (TNFα) and interleukin-1β (IL-1β) using Amersham kits (catalogue numbers RPN2788 and RPN2781). Tumour growth factor β (TGFβ) levels were also tested using a DRG Diagnostics kit, Germany (catalogue number EIA-1864). Material samples were tested in duplicate on different days with cells isolated from three different blood donors. Data were expressed as mean pg per ml from $n = 3$

The supernatants of the mononuclear cell cultures onto the different biomaterials were also tested for platelet derived growth factor (PDGF) by Brunette's method [14]. Samples were diluted 1 : 2 with non-reducing ''sample buffer'' for electrophoresis (Biorad, UK) and (15 µl) loaded onto a 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis. A purified PDGF standard from Sigma was loaded as control. The electrophoresis was performed at 100 V constant voltage in a 3P Protean kit (Biorad). The separated proteins were then transferred onto a nitrocellulose membrane by horizontal electrophoresis for 1 h at 100 V by a Biorad Transblot unit. After blocking the membrane with 2% (w/v) skimmed milk in PBS, an anti-human mouse PDGF primary antibody (Sigma), previously diluted 1 : 1000 in 0.1% (w/v) skimmed milk in PBS, was added overnight under shaking. The membrane was extensively washed in PBS and incubated for 1 h in 1 : 2000 diluted anti-mouse goat IgG conjugated with horse radish peroxidase, washed again and the PDGF specific band detected by adding 0.3% (w/v) hydrogen peroxide and 1-chloro-4-naphthol as enzyme substrates.

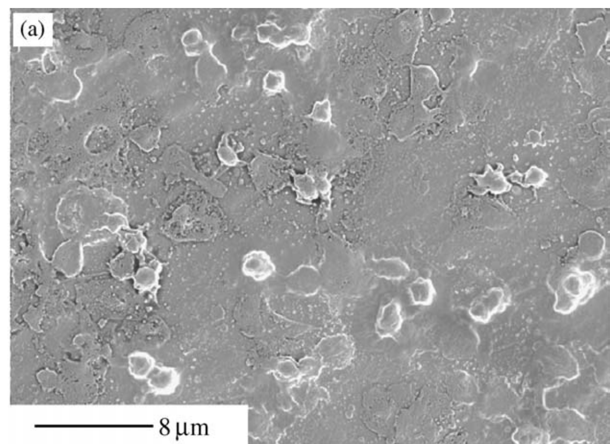
Results

Protein adsorption

Fig. 1 shows the levels of fibrinogen adsorption. DLC adsorbed fibrinogen amounts on its surface higher than St.

Platelet adhesion

The study of the morphology of the adhering platelets showed that St promoted relatively high degree of platelet adhesion, spreading and aggregation (Fig. 2(a)), whereas only modest spreading and no significant aggregation were observed on DLC surfaces (Fig. 2(b)).



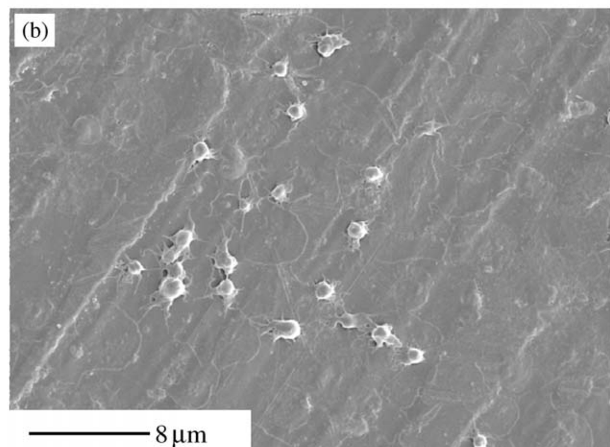


Figure 2 SEM of adhering platelets. (a) St and (b) DLC.

Inflammatory cell adhesion and activation

Fig. 3 shows the early activation of the monocytes when in contact with the tested materials. St and DLC discs did not induce any significant activation of the inflammatory cells. However, when the activation of the monocytes/macrophages was evaluated at a longer incubation time (3 h) as release of biochemical signals into the medium a different pattern emerged (Table I). TNFα and TGFβ released levels were higher when the cells were incubated with St than DLC. IL-1β levels were low for all the tested substrates.

Both the materials showed comparable levels of secreted PDGF (Fig. 4, lanes 3–4). The PDGF appeared released in aggregated form since its molecular weight was higher than the purified standard (lane 2).

SEM analysis of cell adhesion showed a different behaviour of the monocytes/macrophages when in contact with the different surfaces (Fig. 5 (a)–(c)). St

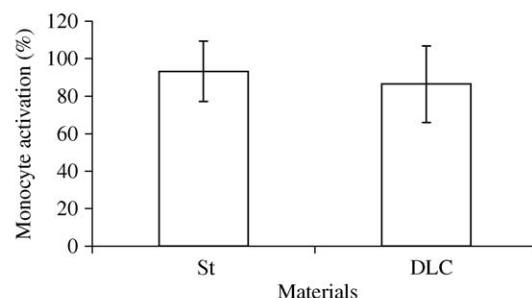


Figure 3 Oxidative burst of monocytes elicited by the tested materials.

TABLE I Levels of biochemical signals released by the monocytes/macrophages during their incubation with the tested biomaterials

Sample ($n = 3$)	Biochemical signals (pg/ml) (range)		
	IL-1β	TNFα	TGFβ
TCP	0.2 (0.0–0.5)	1.6 (0.5–2.9)	5.4 (0.0–10.9)
St	4.8 (1.6–10.4)	10.1 (4.5–15.3)	763.6 (703.6–808.2)
DLC	3.1 (1.7–5.1)	3.3 (0.6–6.9)	135.4 (71.8–256.4)

induced a low degree of cell spreading showing a rough plasmalemma typical of an activated state (Fig. 5(b)). A comparable high degree of cell spreading was observed on TCP (Fig. 5(a)) and DLC (Fig. 5(c)) where the macrophages showed a tendency to fuse into giant cells.

Discussion

The adsorption of plasma proteins on the implant surface is considered a key event in the regulation of the host response towards the foreign material [13, 15–18]. Depending on their amounts and/or conformational changes at the material surfaces, proteins such as fibrinogen with a receptor role for platelets and inflammatory cells drive the wound healing either towards a mild process or towards a protracted host response with an altering of the normal tissue repair [19, 20]. The protracted secretion of cytokines and growth factors can induce excessive SMC proliferation thus leading to restenosis [21].

To minimise this risk, stents coated with drug-eluting polymers have been recently used [10]. However, the costs related to the manufacturing of these stents, the difficulty related to the control of the drug release and the development of drug-resistant cells rise concerns about the validity of this approach [10]. DLC-coated stents have also been made available on the market on the basis of their recognised haemocompatibility [22].

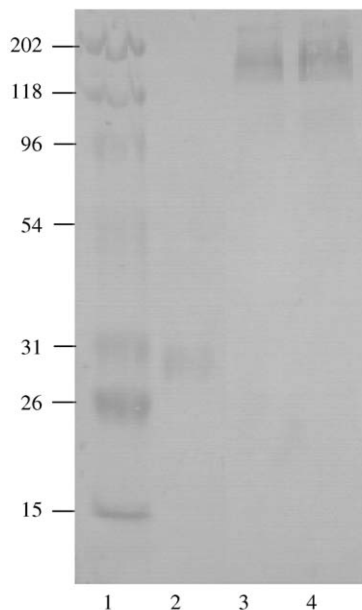


Figure 4 Western blot of the PDGF release by monocytes/macrophages when in contact with the tested materials. Lane 1 = Molecular weight standards (kDa), Lane 2 = PDGF standard, lane 3 = St and lane 4 = DLC.

In the present study, the interactions of DLC surfaces with key elements of the host response have been analysed and compared with St, the material of choice for the stent manufacture. St seemed to induce a more accentuated host response re-enforcing the hypothesis about its role in restenosis. Conversely, the effect of DLC on the aggregation of the platelets and on the secretion of biochemical stimuli by the inflammatory cells seemed to be relatively mild. These findings, associated with the known lack of corrosion of carbon materials, would encourage the use of DLC coatings in stent manufacturing.

These general observations, however, may be misleading since they do not take into account the complex

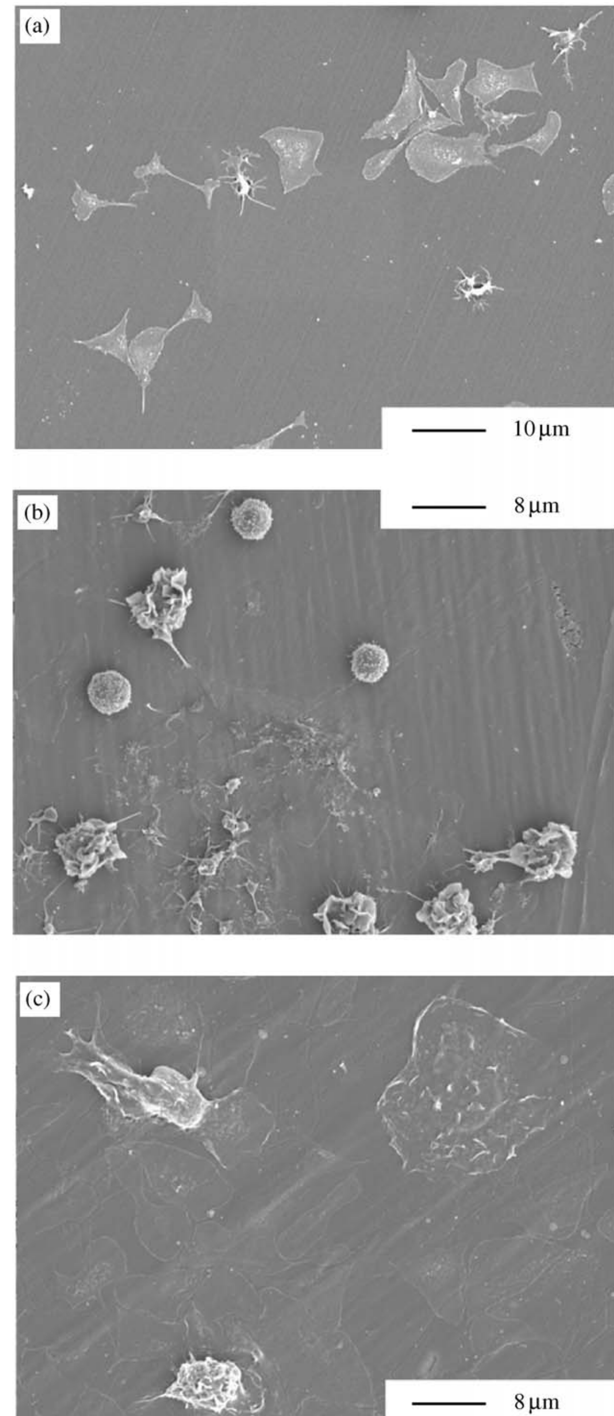


Figure 5 SEM of adhering monocytes/macrophages. (a) TCP, (b) St and (c) DLC.

and different cellular and biochemical patterns induced by these two materials. St and DLC did not change the early monocyte oxidative burst generated by the phorbol ester stimulus suggesting that *in vivo* the two materials may not alter the normal monocyte free radical secretion generated by the inflammatory status [13]. However, at longer incubation time the plasmalemma of monocytes/macrophages adhering onto St showed a rough morphology typical of activated cells. This activation was confirmed by the analysis of a key pro-inflammatory cytokine (TNFα) and of two growth factors (PDGF and TGFβ). The higher levels of adsorbed fibrinogen levels on DLC may be responsible for the high degree of spreading and fusion of the monocytes/macrophages on this type of surface. The cells seemed also to be switched to a different biochemical pathway where the production of growth factors was stimulated and the secretion of key pro-inflammatory cytokines was inhibited. Previous studies corroborate the relatively high degree of macrophage adhesion on DLC [23]. The relative inertness of St towards plasma protein adsorption (data not shown) may explain the relatively lower degree of cell spreading on this material. However, the different cell behaviour may be caused also by the adsorption of other host response proteins not investigated in this study [17].

Therefore, although the overall picture would suggest that DLC is a material more inert than St, concerns still arise about the ability of this type of coating to support macrophage adhesion and growth factor synthesis. In particular, the stimulation of PDGF, a growth factor with a recognised role in inducing SMC proliferation [21], may indicate that DLC may not prevent restenosis. The data collected in this work also show that PDGF is not released into the medium as free, low molecular weight molecule, but as high molecular weight complex. Indeed, PDGF can form complexes with other cytokines such as IL-1β thus may explain the low levels of IL-1β detected in the tested supernatants.

Conclusions

Stainless steel activates both platelets and macrophages, which release pro-inflammatory cytokines and growth factors in the surrounding medium with the risk of a protracted inflammation and consequent restenosis. The DLC coating seems to reduce the host response thus suggesting that tissue healing can take place around this biomaterial without suffering significant alterations. However, the relatively high degree of macrophage spreading and their relatively high PDGF release induced by DLC still pose doubts about the real benefit that might be achieved *in vivo* by stents coated with this material.

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