Inhibition of cell adhesion by a synthetic polymer adsorbed to glass shown under defined hydrodynamic stress

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Summary

A co-polymer with hydrophobic and hydrophilic segments was allowed to adsorb from aqueous solution onto glass previously made hydrophobic by derivatization with octadecyl dimethylchlorosilane. The polymer is thought to adsorb via its hydrophobic segments, leaving the hydrophilic segments free to extend into the water. After allowing cells to settle on the treated surface, the shear stress at the chamber wall required to remove red blood cells, Dictyostelium discoideum amoebae and Escherichia coli was determined in a calibrated laminar flow chamber. On octadecyl glass a shear stress of 2–3 N m⁻² evicts 50% of adherent red cells and E. coli. No D. discoideum amoebae could be removed at 5 N m⁻². In striking contrast, the lowest experimentally obtainable shear stress of 0·03 N m⁻² removes 97·0–99·5 % of cells of all three types from the polymer-treated surface, even after a cell residence time of 1 h without flow in the absence of free polymer. The minimum shear stress of 0·03 N m⁻² corresponds to only ~20 times the force of gravity on a red cell. The mechanism of action of the polymer and the implications of the results are discussed.

Key words: cell adhesion, blood compatible synthetic polymer.

Introduction

The prevention of cell adhesion to surfaces under controlled conditions has long been the goal for the development of surfaces that are non-adhesive to blood components, marine microorganisms and bacteria. The search has been for a material that would have minimal attractive forces for cells and proteins, and that would possess maximal repulsive forces. This implies that the combined energies of van der Waals' attraction, entropic attraction (i.e. ‘hydrophobic bonding’) and hydrogen bonding should be smaller than the sum of electrostatic repulsion, hydration repulsion and entropic repulsion due to thermal motion of flexible molecular chains. It should, in addition, be mechanically and chemically stable. The search for such a material has largely concentrated on synthetic polymers (see Andrade, 1985a; Norde et al. 1986) and since their surface properties in a biological milieu will be modified by protein adsorption, the latter process has been extensively studied (see Andrade, 1985b).

The relationship between surface physicochemical properties and biocompatibility is not a trivial problem and is still much disputed after more than two decades (see the critical discussion by Ruckenstein & Gourisan-kar, 1984). In this period only limited progress has been made towards the goal of a blood-compatible interface for vascular prostheses or kidney dialysis equipment (Brash, 1983).

Of the large number of polymers that have been assessed for blood compatibility, polyethylene oxide (PEO), either alone or as a component of a co-polymer, has been found to have notable anti-adhesive properties. George (1972) described a dramatic reduction in the adhesion of platelets to glass in the presence of PEO. Earlier, Hiatt et al. (1971) had found that PEO prevented the adsorption of rabies virus to glass. Whicher & Brash (1978) and Brash & Uniyal (1979) showed that little protein adsorption occurred on a solid co-polymer composed of PEO and polyurethane. Thrombin and platelet adsorption to similar co-polymers was studied by Sa da Costa et al. (1980,
et al. (1982) described the striking in vitro adsorption by scanning electron microscopy (SEM) after one day under in vitro conditions. They made and tested polymers with PEO side-chains (arranged herringbone fashion on a backbone of PVC) of various lengths (5–100 monomers) and found the longest to be the most blood-compatible.

Against the background of these results, we describe a relatively simple and well-defined water-soluble polymer consisting of two polyethylene oxide segments spaced with a relatively hydrophobic polypropylene sequence. The polymer adsorbs strongly to a hydrophobic glass surface, where it almost totally prevents three very different cell types from adhering, as judged by the criterion of liquid shear in a calibrated flow system.

**Materials and methods**

**Materials**

The bifunctional polymer Pluronic (F108) was obtained from ICI Plc. Penicillin–streptomycin (5000IU) from Gibco Chemicals, Middlesex, UK was stored below 0°C. Concanavalin A (ConA) conjugated with tetramethylrhodamine B isothiocyanate was purchased from Sigma Chemical Co., Poole, UK. A stock solution 0.5 mg ml⁻¹ prepared in Tris buffer at pH 7.4 was stored in 0.2 ml samples below 0°C until required. Bacteriological peptone and yeast extract were obtained from Oxoid Chemicals Ltd, Basingstoke, Hampshire, UK. Disodium hydrogen orthophosphate, sodium dihydrogen orthophosphate, sodium chloride and D-glucose were all of AnalaR quality from BDH Chemical Co., Poole, UK. The procedure for obtaining surface chemically pure water by distillation from alkaline potassium permanganate will be described elsewhere (Owens et al. 1987). Microscope coverslips (40 mm x 22 mm x 0.17 mm) from Chance Proper Plc, Smethwick, UK were rendered hydrophobic by overnight immersion in a 2% solution of octadecyl dimethylchlorosilane (Sigma, Southampton, UK) in chloroform. The treated coverslips were first rinsed with chloroform, then in distilled water to remove HCl. If required for later use they were stored in chloroform. Precision micrometer flow valves (Nupro Co., types S and M) were purchased from North London Valve & Fitting Co., London, UK.

**Cultures**

Vegetative cells of Dictyostelium discoideum (Ax2 strain) were grown to the fully differentiated spore fruiting stage at 22°C on 2% nutrient agar inoculated with Escherichia coli B/r. The dormant spores were germinated in glucose-free axenic medium containing 0.5 ml penicillin–streptomycin for 72 h in a rotary incubator at 22°C. This was followed by a growth cycle in axenic medium supplemented with 86 mm-glucose. Ax2 amoebae were isolated from shaken cultures during log-phase growth (1 x 10⁶ cells ml⁻¹) by allowing the cells to settle onto a clean glass surface rather than the usual centrifugation routine. In so doing we avoided any tendency for the cells to rupture during harvest. The adherent cells were first washed with 20 mM-NaCl and then resuspended in the same electrolyte using gentle fluid flow from a Pasteur pipette to dislodge cells from the glass surface. Streaming flow experiments were done in unbuffered 20 mM-NaCl at the pH of our distilled water (pH 5.8–6.2). The work was done in a temperature-controlled laboratory at 20 ± 0.5°C.

Human red blood cells were obtained by venipuncture into citrate buffer, pH 6.8. The cells were washed three times by centrifuging with Dulbecco A phosphate-buffered saline (PBS), pH 7.4 (Oxoid, UK) at 400 g. A 10 µl sample of the packed cells was diluted with similar PBS to give a cell density of 8 x 10⁶ ml⁻¹. This was kept at room temperature until required. Streaming flow experiments were also done in PBS at pH 7.4.

E. coli B/r were incubated in nutrient broth no.2 (Oxoid) for 48 h at 37°C. A 30 ml suspension of bacteria was pelleted by centrifugation at 1000 g for 10 min. Bacteria were washed by four successive 10-min centrifugations at 1000 g using 30 ml samples of PBS at pH 7.4. The washed cells were resuspended and stored in PBS until required. Streaming flow experiments with E. coli were conducted in PBS, pH 7.4.

**Methods**

A laminar flow of electrolyte from an elevated reservoir was passed through a rectangular conduit in which a derivatized glass coverslip formed the upper wall of a closed parallel plate channel 388 µm wide. The wall shear τw exerted by the flow against the solid surface was calculated for varying rates of flow from the equation:

\[ \tau_w = \frac{dP}{dz} \cdot \frac{b}{t}, \]

(1)

where P is the hydrostatic pressure drop across the conduit of length l and wall separation 2b. The conduit design, flow system and calibration procedure are described in detail elsewhere (Owens et al. 1987) so only the salient features are given here.

The components of the conduit are illustrated in Fig. 1. A glass coverslip C was held in a recess on the upper face of a polycarbonate block D by a stainless steel frame A. This was secured by screws that passed through a silicone rubber gasket B into a stainless steel baseplate E. The flow conduit was formed between the coverslip and a flat-bottomed channel across the polycarbonate block D. Fluid enters through a cylindrical pipe integral with the end wall of block D leading into a confusor of elliptical section and then into the conduit. Fluid exit is via a symmetrical arrangement.
The baseplate E, which has a central hole for transmitted illumination, locates the assembly on the microscope stage. A flowline of glass and silicone rubber tubing was connected to the chamber and the whole system filled with electrolyte, care being taken to exclude any air bubbles. Exchange of electrolyte for 0.1% polymer solution followed, to ensure that the polymer-coated glass did not become exposed at any stage to air. After 2 h the system was flushed with electrolyte and the cells were injected into the inverted chamber on an inverted microscope through a silicone rubber diaphragm in a side arm, using a hypodermic syringe with an 8 cm needle. Sedimentation of cells onto the derivatized glass wall was essentially complete after 20 min. Cells were observed with a Zeiss 25X oil-immersion objective lens under bright-field and IRM illumination. The flow rate through the conduit was varied by means of precision micrometer valves mounted in parallel in the flowline. On polymer-treated surfaces, cells were exposed to a minimal flow rate of 0.01 ml s⁻¹ for 30 s in contrast to those on control surfaces where the flow rate was extended to 2.0 ml s⁻¹ over the same period. Cell-substratum interactions under hydrodynamic shear were recorded on closed-circuit TV using a Chubb surveillance camera connected to a video recorder (Sony U-Matic) and monitor. Later experiments utilized a sensitive 'Falcon' SIT camera (Custom Camera Devices Ltd, Wells, UK).

Calibration of flow system

For a flow chamber of the type used, the Reynold's number is given by

\[ R = \frac{32 V_{\text{max}} b}{\eta} \]

where \( V_{\text{max}} \) is the maximum velocity of flow (along the central axis of the chamber), \( b \) is the separation of the flat parallel walls and \( \eta \) is the absolute viscosity. The critical Reynold's number \( R_c \) corresponds to a critical value of \( V_{\text{max}} \) below which flow is laminar and above which it is turbulent (Eskinazi, 1975, p. 384). For a rectangular cuvette like ours where length > width > depth, \( R_c = 2300 \). In our system at maximum flow \( R = 200 < R_c \) so that flow is laminar under all our experimental conditions.

Although flow in the cuvette will always be laminar, the liquid must pass a certain distance along the channel before a constant parabolic velocity profile is established. This establishment length \( L_e \) is a function of chamber shape and Reynold's number, \( L_e = 0.013 bR \) (Sparrow, 1955). For our system \( L_e < 1 \% \) of the conduit length so flow will be fully developed over virtually the entire length of the conduit. This conclusion is consistent with the findings of Van Wagenen & Andrade (1980) for the measurement of streaming potentials generated by a laminar flow of electrolyte between parallel glass plates. Fig. 2 shows a linear relationship between the wall shear, calculated from equation (1), and the applied pressure difference across the chamber (\( P \)).

Results

Numerical results for the behaviour of red blood cells, \( D. \) discoideum and \( E. \) coli under hydrodynamic stress on control octadecyl glass and F108-treated octadecyl glass are shown in Table 1. On F108-treated glass between 97-0 and 99-5% of cells that were initially in contact with the surface were removed at a wall shear stress \( \tau_w = 0.03 \) N m⁻². On the control surface 100-0%...
Table 1. Behaviour of cells under hydrodynamic stress

<table>
<thead>
<tr>
<th></th>
<th>Number of expts</th>
<th>Before flow</th>
<th>After flow (0-03 N m⁻²)</th>
<th>% Cells removed</th>
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<td><strong>A. Polymer-treated glass</strong></td>
<td></td>
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<tr>
<td>RBC</td>
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<td>1361</td>
<td>17</td>
<td>98.8</td>
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<td>E. coli</td>
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<td>1070</td>
<td>5</td>
<td>99.5</td>
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<td>137</td>
<td>4</td>
<td>97.0</td>
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<table>
<thead>
<tr>
<th></th>
<th>Number of expts</th>
<th>Before flow</th>
<th>After flow (N m⁻²)</th>
<th>% Cells removed (N m⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B. Control octadecyl glass</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RBC</td>
<td>2</td>
<td>186</td>
<td>186</td>
<td>0</td>
</tr>
<tr>
<td>E. coli</td>
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<td>360</td>
<td>360</td>
<td>126</td>
</tr>
<tr>
<td>D. discoideum</td>
<td>3</td>
<td>29</td>
<td>29</td>
<td>29</td>
</tr>
</tbody>
</table>

For 50% removal in 30s: red blood cells (RBC), 2.3 Nm⁻²; E. coli, 3.0 Nm⁻².

Discussion

The parallel plate hydrodynamic shearing technique that we have used for assessing cell-to-substratum adhesion is well known. It has been extensively employed to investigate the adsorption of cells from flowing suspensions onto the chamber walls as well as the removal of attached cells (Doroszewski et al., 1977, 1979; Forrester & Lackie, 1984; Hochmuthe et al., 1973; Mohandas et al., 1973, 1974). The hydrodynamic removal force on a cell attached to the chamber wall can be determined from the wall shear stress \( \tau_w \) and there is reason to believe that this force is not significantly dependent on the details of cell shape and the degree of spreading (Hubbe, 1981) for any given cell type.

Our data for red cells on siliconized glass (Table 1) can be directly compared with the work of Mohandas et al. (1974) for the removal of red cells by hydrodynamic shear in a saline solution. These authors report that cells are removed during 300 s flow only when \( \tau_w > 0.35 \) N m⁻². Cell removal was found to depend on both \( \tau_w \) and time. Their fig. 8 shows that half the cells are removed in 300 min on exposure to 1 N m⁻² and all are gone in the same time at 1.5 N m⁻². We found that in 30 s half the initially adherent cells are removed from octadecyl glass at 3 N m⁻² and that all are removed at treated glass before flow, taken from videotape. The uneven background is due to light transmitted by the non-optical plastic base of the chamber. Fig. 3D,E,F shows typical corresponding fields at minimum flow, giving 0.03 N m⁻² wall shear stress. The 0.5 s photographic exposure time shows cells that have been removed by flow as streaks or blurs, while the few static cells are sharply focused. The behaviour of cells on the control surface of octadecyl glass, before and after flow, is shown in Fig. 4. We also made two tests on the polymer-treated glass to assess its durability. In the first, physiological saline was allowed to flow through the chamber at full rate for 1 h. After this the surface was as anti-adhesive to red blood cells as it was before exposure to prolonged flow. In the second test the polymer-coated glass was allowed to air dry for 30 min, before being re-hydrated in physiological saline for 30 min. The prevention of cell adhesion by this surface was indistinguishable from that of polymer-treated glass kept rigorously out of contact with air.

We found that it was possible to define the boundary zone between polymer-treated and untreated glass using a fluorescent dye adsorption test. Rhodamine-conjugated ConA was shown to adsorb strongly to hydrophobic glass but failed to adsorb to glass treated with F108, thus both providing a convenient practical assay for polymer adsorption and showing that F108 vetoes protein adsorption as well as cell adhesion.
Fig. 3. Cells on F108-treated glass before flow (left column) and after flow at the minimum rate (right column). A,D. Red cells; B,E, Dictyostelium; C,F, E. coli. Wall shear stress and scale inset.

$5 \text{ N m}^{-2}$. Since our flow times differ substantially from those of Mohandas et al. (1974) we can only state that there is no evidence that the two sets of results are inconsistent. In view of our results with F108, it is of interest that Mohandas et al. (1974) found a large decrease in percentage adhesion in the presence of
fibrinogen. Unfortunately they did not measure protein adsorption to siliconized glass and give no shear stress data. The analysis of red cell removal from octadecyl glass is considered in more detail elsewhere (Owens et al. unpublished data).

Our data for bacterial adhesion can be compared with those of Fowler & MacKay (1980), who used a radial flow chamber to examine the growth of unidentified cocci in protein-free media. Although cell counts are not given, cells are stated to be unable to attach and multiply where the wall shear stress exceeds 2·6 N m⁻² (units of τ in their Tables 7.1 and 7.2 are not correctly expressed). This is very similar to our value of 3·0 N m⁻² for 50% removal of E. coli, but owing to the very different conditions employed, the similarity of the two sets of results may be fortuitous. Taking the dimension of E. coli as a cylinder of 0·5 μm radius and 3 μm long, the bacterium is equivalent to a sphere of 0·8 μm radius. Consequently, using \( F = 32\tau r^{-2} \) (Hubbe, 1981) we obtain a characteristic removal force of 1×10⁻¹⁰ N per cell at 3 N m⁻².

In the case of F108-treated glass, the minimal wall shear stress used in our system, 0·03 N m⁻², removes between 97·0 and 99·5% of attached cells, according to cell type. In view of the widely different surface compositions of these cells (prokaryote, eukaryote and slime mould) this result argues persuasively that F108 is a very potent general anti-adhesive. Therefore its mode of adsorption and mechanism of action are of considerable interest.

Pluronic F108 consists of two hydrophilic polyoxyethylene chains separated by a central hydrophobic polyoxypropylene chain and has the general formula:

\[
H - (\text{OCH}_2\text{CH}_2)_a(\text{OCH}_2\text{CH(}\text{CH}_3))_b - (\text{OCH}_2\text{CH}_2)_a\text{OH}
\]

where \( a \) is approximately 130 and \( b \) is approximately 45. The molecule adsorbs on hydrophobic surfaces in such a way that the polyoxyethylene chains are oriented

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**Fig. 4.** Cells on control hydrophobed glass before flow (left) and after flow (right). A,C. Red cells; B,D, Dictyostelium.
Steric repulsion is to be expected because of the extension of the hydrophilic arms into the water. If so, repulsion should begin when the glycocalyx of an approaching cell begins to interdigitate with the hydrophilic segments of the adsorbed polymer, thus reducing the available modes of gyration of the polymer chains and decreasing entropy. A direct measurement of intermolecular repulsion associated with PEO chains has been obtained by Klein & Luckham (1982) using the Israelachvili technique. They measured the force between mica plates with adsorbed PEO in 0.1 M KNO₃. Repulsion began at a mica–mica separation of 6R (R = radius of gyration of the polymer) and rose monotonically as the separation was reduced. Little evidence of desorption over 72 h was found. Thus the effective length of the individual polymer chains when they began to repel mutually is 3R. This corresponds to 18–20 nm for one polymer (Mₑ = 40,000; degree of polymerization, n ≈ 900) or 33–38 for another (Mₑ = 100,000; n ≈ 2300). Under conditions where PEO can simultaneously adsorb to both mica plates, cross-bridging forces could be measured (Klein, 1986). These results suggest that repulsion in the case of the PEO co-polymer that we used (n ≈ 130) might begin at a comparatively small effective extension distance, say 3 nm.

Evidence for thermal flexibility of PEO chains in a mixed polymer was obtained by Mori et al. (1982) who related the width of the peak ¹³C nuclear magnetic resonance (n.m.r.) signal to the degree of polymerization (n) of the PEO moiety. They found that increasing n to 100 decreases the width of the signal peak, indicating high flexibility (no graph shown).

The other probable source of repulsion, water structuring, as an important component of interaction forces is a relatively recent discovery (Le Neveu et al. 1977; Parsegian et al. 1979; Parsegian & Rau, 1984; see also Israelachvili, 1985). The electric field at a surface (due to adsorbed ions, ionogenic groups or possibly dipoles) alters the orientation and thus the structure of the nearby water. The result is that water molecules within about 2 nm of a surface are to some extent associated with the surface. Although the individual binding energy per molecule is extremely small, the energy needed to remove large numbers of them from any macroscopic region between approaching surfaces is very substantial. Between the lamellae of lecithin liquid crystals the hydration pressure at 1 nm separation exceeds 10⁷ N m⁻² and rises to near 10⁹ N m⁻² or 10⁴ atmospheres at 0.2 nm (Parsegian et al. 1979). However, until the extent of water structuring associated with F108 is measured it will not be possible to appreciate fully how the PEO exerts its remarkable anti-adhesive effects.

**Fig. 5.** Diagram of a molecule of the bifunctional polymer F108 adsorbed at the interface between water and hydrophobed glass.

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The authors thank British Petroleum Plc for financial support and for permission to publish this paper. D.G. thanks the Wellcome Trust and the SERC for financial support.

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(Received 23 December 1986 – Accepted, in revised form, 7 April 1987)