ADHESION OF RED BLOOD CELLS TO CHARGED INTERFACES BETWEEN IMMISCIBLE LIQUIDS. A NEW METHOD

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SUMMARY

We have devised a method of making a flat oil/water interface which remains flat on inversion. Cell adhesion to the interface can be observed microscopically.

Glutaraldehyde-fixed human red blood cells adhere to the interface between physiological saline and hexadecane containing surface-active behenic acid at pH values below about 7.5. At high pH values, cells are prevented from adhering due to dissociation of the carboxyl groups of behenic acid oriented in the interface. The negative red cells are driven away electrostatically.

Adherent and non-adherent cells remain on the aqueous side of the interface and do not appreciably deform it when adherent. Cells are electrostatically attracted to a similar interface containing positively charged octadecyltrimethylammonium ions. Cells also adhere to an interface containing octadecanol, which carries no charge.

Underlying both electrostatic repulsion and attraction between red cells and oil/water interfaces is an attractive force which may be of electrodynamic (van der Waals) origin.

INTRODUCTION

A new method is described for investigating the adhesion of cells to liquid/liquid interfaces. We chose the hexadecane/water interface since the phases are mutually immiscible. Water-insoluble behenic acid \([\text{CH}_3(\text{CH}_2)_{17}\text{COOH}]\) was dissolved in the oil and the density of negative electrostatic charge thus imparted to the interface was adjusted by changing the pH of the aqueous phase. Cell adhesion was studied as a function of pH and consequently of interfacial charge density.

Interfaces between immiscible liquids are uniform, and except for thermal or mechanical vibrations they are molecularly smooth. These features offer distinct advantages over commonly used substrates such as glass or plastic for cell adhesion studies. Spatial uniformity of the surface implies that if some cells stick when others do not then heterogeneity of the cell population rather than surface heterogeneity is responsible. In addition, water and hydrocarbon oil have reasonably well defined absorption spectra making it possible to compute electrodynamic (van der Waals) attractive forces between cells and the interface (Parsegian & Gingell, 1973) and perhaps compare theory with experiment when the electrostatic charge at the interface is known.

Spreading of cells at such an interface was first described by Rosenberg (1964). He
allowed cells to settle under gravity on to fluorocarbon/water interfaces and observed cell spreading and locomotion. This strikingly original approach does not appear to have been developed further. The main objection to Rosenberg's technique for adhesion studies lies in the difficulty of assessing adhesion, other than by making judgements based on cell shape, since a dish of oil and water cannot be bodily inverted to see whether the cells fall off.

We have devised a simple technique whereby a small oil/water meniscus can be flattened. The interface can then be inverted and observed in a microscope.

**PRINCIPLE OF THE METHOD**

Imagine a narrow glass tube with a stopper at each end. The tube is half filled with water then topped up with hydrocarbon oil. If the tube is sufficiently narrow, inverting the tube will not cause the two immiscible phases to flip over and change ends. Cells then added at the (uppermost) aqueous end settle to the meniscus and adhesion to the interface can be subsequently assessed by re-inverting the tube. This can be done with red cells and the result is just visible to the unaided eye. However, cells which stick to the interface under appropriate conditions cannot be counted under a microscope because of their non-uniform distribution caused by the sharply curved meniscus, as well as the excessive working distance. The device we have made obviates both these difficulties by producing a flat meniscus in a short tube. The way this is done is simple and seems not to have been described before. If an interface between two immiscible liquids or a liquid and a gas lies within a horizontal torus separating upper and lower phases it is possible to adjust the level of the interface such that the meniscus is completely planar. This can be achieved whatever the angle of contact between the liquids and the material of the torus. In practice, it is desirable to keep the liquid junction at a fixed level and raise or lower the torus. In Fig. 1 it is shown that for a contact angle $\theta$ the meniscus will lie flat in a torus of minor radius $r$ when the liquid junction is a distance $y = r \sin \theta$ above a plane ($p$) dividing the torus into equal halves. The device we have constructed for our experiments is shown in Fig. 2. It is essentially a pill-box with a window ($W_1$, $W_2$) in each end, held in place by removable plates ($R_1$, $R_2$). The lid ($L$) rotates upon the base ($B$) and the two parts are held together with spring ball assemblies ($S$) engaging in a V slot cut in the wall of the base. The torus ($T$) is a tight fit in a carrier ($C$) which runs on a screw thread tapped in the inner wall of the base. The carrier, made of PTFE to reduce friction, receives pins ($P$) fixed to the lid. Rotation of the lid thus impels the torus to move up or down on the pins. The liquid volume of the chamber...
Red cell adhesion to oil/water interface

remains constant and the torus moves up or down with respect to the liquid interface, thereby varying the curvature of the meniscus. A suitable material for construction is EN58J stainless steel, passified in 70% nitric acid for 2 h at room temperature. After this treatment, the metal resists attack by 0.15 M NaCl. Observation of the interface was carried out on a Wild microscope (which can accommodate a sufficiently bulky object on the stage) using a Zeiss UD-40 long working distance objective. This allows about 8 mm between the end of the objective and the meniscus. Photographs were taken using a Zeiss Ukatron UN 60 microflash apparatus. Smooth inversion was facilitated by a cradle mounted on the microscope stage.

Mode of operation

After filling the lower chamber with saline roughly up to the centre of the torus, oil is added to fill the upper chamber. The meniscus is adjusted to flatness by noting when an object viewed along the optic axis ceases to be distorted. The upper window (W₁) is then clamped in place and the meniscus readjusted if necessary. Next, the chamber is inverted and the window (W₂) is temporarily removed to add the cell suspension. The chamber is then held water uppermost to allow cells to settle on to the liquid/liquid interface, then turned over after 10 min to observe whether they remain adherent to the interface under the force of gravity. The chamber is not filled directly with cell suspension in order to avoid cell contact with the air/water interface. Between experiments the chamber was cleaned by sonication alternately in absolute ethanol and pure diethyl ether.

Reagents

Fluka spectroscopically pure hexadecane was purged of surfactants by passage through a column of aluminium oxide. It gave a consistent interfacial tension value of 5.16 x 10⁻² N m⁻¹ at 20 ±0.1 °C against 4 times glass-distilled water in a falling drop apparatus. Behenic acid (Sigma docosanoic acid, approx. 99% pure) used without further purification, was dissolved to give a 2 x 10⁻⁵ M solution in hexadecane. The acid is not readily soluble and requires warming. Fluka octadecanol and C₁₈TAB (octadecyltrimethylammonium bromide) were a gift of Dr J. Mingins and were used without further purification. Water was distilled four times in an all-glass apparatus, the last two distillations being from alkaline potassium permanganate and then from orthophosphoric acid according to the usual recipe. The interfacial tension
against air was $7.3 \times 10^{-2}$ N m$^{-1}$ at 20 ± 0.1 °C. Analar NaCl was used to prepare the salt solutions and 1 mM EDTA was added to remove any traces of Ca$^{2+}$.

**Cells**

Human red cells obtained by venipuncture were washed 4 times by centrifugation in normal saline then fixed in 3.3% glutaraldehyde in normal saline at 4 °C for 18 h. Aldehyde fixation has little effect on red cell charge (Seaman & Cook, 1965) and was done to avoid protein contamination of the interface. The cells were then washed in saline by centrifugation and suspended in 139 mM NaCl containing 10 mM NH$_4$OH/HCl buffer and 1 mM EDTA at pH values within the range pH 7-9. Measurements outside this range were made by directly adjusting the pH with HCl.

![Graph showing adhesion of red blood cells to an electrostatically charged interface between oil and water.](image)

**RESULTS**

As seen in Fig. 3, cell adhesion was complete at pH 6.0. At pH 7.7 approximately 90% of the cells adhered to the interface, but increasing the pH only 0.5 units to 8.2 resulted in 90% of the cells falling off upon inverting the interface. At pH 9.0 no cells remained adherent to the interface. The inflexion point in the cell adhesion curve is at pH 7.95 where 50% remain stuck. These results were obtained allowing 20 min for behenic acid to equilibrate at the interface, followed by introduction of cells which were allowed to settle for 10 min. Increasing the settling time to 1 h gave the same results, showing that adhesion is not appreciably limited by viscous drainage of water between the oil and an approaching cell. Near pH 9.0 where the interfacial tension is very low the meniscus can become unstable, making measurements difficult (see Appendix B).
Red cell adhesion to oil/water interface

Fig. 6 shows cells which have settled on to the interface at pH 8-25. The photograph was taken within about 30 s of inversion, during which time unattached cells fall a negligible distance. The following photograph (Fig. 7) shows some of the cells falling away from the surface and going out of focus at 1 min after inversion. Fig. 8 shows the same field after 5 min when all unattached cells have fallen off. Photographs in Figs. 9 and 10 were taken at pH 7-0, 30 s and 5 min after inversion, respectively. This method of recording by taking photographs only after inversion obviates counting difficulties associated with non-random settling of cells on to the interface, which can occur due to the method of adding the cells. Careful comparison of photographs taken before and immediately after inversion show that the method is reliable. The number of cells counted at the interface directly after inversion varied from 400 to 1400 except at pH 7 where only 200 were counted.

These results show that increasing the pH progressively prevents fixed human red cells from adhering to a behenic acid-hexadecane/saline interface. The statistical adhesive behaviour seen is thought to reflect a distribution of surface properties of the red cells since the liquid interface is homogeneous.

Cells at the behenic acid-hexadecane/saline interface were viewed in a direction parallel to the interface in a glass cuvette (Fig. 11). At pH 6-4 cells could be seen settling on to the interface: those landing edge-on and remaining so seemed to make a point contact without visible distortion of either cell or interface. Sometimes edge-on cells would suddenly 'snap' down face-on to the interface, but they always remain on the aqueous side. On inverting the cuvette, so that the oil phase was uppermost, cells remained attached and edge-on cells maintained exactly the same relationship with the interface as before.

Observations were also made on vertical interfaces. Cells were allowed to settle on to the behenic acid-hexadecane/saline interface at pH 7 and pH 8-3. Those remaining adherent to the inverted interface were observed after tilting the whole microscope and chamber through a right angle. At both pH values all cells which stuck to the horizontal interface remained attached when the interface was tilted vertically. They do not measurably slide down the interface during a period of approximately 10 min. The cells therefore behave as if they were adherent to a rigid interface.

We also observed the behaviour of cells at the hexadecane/saline interface in the presence of other surfactants. Octadecytrimethylammonium bromide ('C18TAB', CH₃(CH₂)₁₇N+(CH₃)₃Br⁻) was dissolved in hexadecane to give a 2 x 10⁻⁴ M solution. The C₁₈TAB-hexadecane/saline interface was left for 20 min at pH 4-5 to equilibrate, then cells were allowed to settle for 10 min. On inverting the chamber the cells proved to be 100%, adherent to the interface. In another experiment C₁₈TAB was spread by the crystal method: a few crystals of C₁₈TAB were dropped through the aqueous phase on to a clean hexadecane/saline interface: C₁₈TAB spreads immediately to give an interfacial monolayer and violent fragmentation of the crystals can be observed microscopically at the interface. The interfacial tension is greatly lowered after spreading from a crystal, reflecting the high interfacial molecular density obtained, and such interfaces are mechanically unstable (Appendix B). However, it was clear that 100% of the cells were adherent to this interface.
Octadecanol was made up as a saturated solution in hexadecane. The interface with saline at pH 4.5 also has to be treated carefully because of its low surface tension. Following the normal routine, cells were found to be 100% adherent to the interface.

**DISCUSSION**

Consider a solute such as behenic acid dissolved in oil (in which it is soluble in an unionized state only) interfaced with water, in which the acid is highly insoluble. The interface will contain an excess of behenic acid molecules which is a function of the bulk activity of behenic acid in the oil phase, as expressed by the Gibbs adsorption isotherm. Behenic acid \([\text{CH}_2(\text{CH}_3)_n\text{COOH}]\) is surface active, orienting with its carboxyl group in the aqueous phase and its hydrocarbon chain in the oil. As the pH falls below the pKa of the carboxyl group the acid will be progressively associated, while above the pKa dissociation will impart a negative charge to the interface which will repel the negatively charged red cells. The pKa of free monocarboxylic acids asymptotes towards ~6 as chain length increases. But in a negatively charged monolayer the pKa is progressively shifted to more alkaline values as the surface density of acid molecules increases, up to the limit of a confluent monolayer. A value for this effective pKa between 8.5 and 8.9 has been reported for a scraped behenic acid film analysed by infrared spectroscopy (Bagg, Haber & Gregor, 1966). Since at all pH values above ~4 the sialic acid groups responsible for the negative charge on the red cell are fully dissociated, the sudden decrease in the fraction of cells adherent to the interface near pH 8.2 as the solution is made more alkaline does not result from any change in red cell charge but is due to ionization of behenic acid at the interface.

The observation that cells adherent to the behenic acid–hexadecane/saline interface at pH 6.4 remain on the aqueous side is not surprising since they still carry a full dissociated sialic acid charge at this pH. The lack of visible distortion of the interface is also as expected since the calculated energy of contact \((\sim -10^{-7} \text{ J m}^{-2} \text{ in the secondary minimum, or } \sim -10^{-4} \text{ J m}^{-2} \text{ in the primary minimum, Parsegian & Gingell, 1973})\) is small compared with the energy required to increase the oil/water surface area by contact deformation. A simple method, given in Appendix A, allows us to make an assessment of the interaction energy as a function of the equilibrium depth of immersion of the cell in the interface. The behenic acid–hexadecane/saline interfacial tension at pH 6 where all the behenic acid is associated is about \(5 \times 10^{-2} \text{ N m}^{-1}\). If cell/water interfacial tension is \(10^{-4} \text{ N m}^{-1}\), we find that an equilibrium depth of immersion of \(100 \text{ nm}\) implies an interaction energy of \(-10^{-3} \text{ J m}^{-2}\) of cell/oil contact while 10-nm immersion implies \(-10^{-4} \text{ J m}^{-2}\). Therefore we conclude that the lack of visible deformation is indeed commensurate with a contact energy of \(-10^{-4} \text{ J m}^{-2}\) or less. It is also possible to show that the oil/water interface is ‘hard’ as seen by an approaching cell. If we assume the fixed cell to be rigid, using the values above and assuming an equilibrium immersion depth of 50 nm, it can be calculated that a force of \(3 \times 10^4 \text{ g}\) would be required to push the cell a further 50 nm into the oil. These calculations are also of wider interest since they allow interaction energy to be calculated from immersion depth and measurable surface tensions.
Red cell adhesion to oil/water interface

We also observed the behaviour of cells on vertical interfaces. Cells adherent to behenic acid-hexadecane/saline interfaces between pH values 7-0 and 8-25 were watched after swinging the microscope and chamber through a right angle. To our surprise the cells did not slide down the vertical interface. Photographs taken 1 min apart showed no discernible movement, at a magnification of 400 x. This shows that the interface is rather rigid, for the following reason. The rate of settling in bulk water was measured in a cuvette using a horizontal microscope and found to be 130 μm per min. In hexadecane the rate would be approximately 40 μm per min. If the interface is not rigid so that fluid motion due to a cell settling under gravity on the aqueous side of the interface can cause fluid motion in the oil, the rate of settling should be between 40 and 130 μm per min. This is not observed, so the interface is more viscous (rigid) than either bulk oil or bulk water. While we cannot at present rule out protein contamination, this seems unlikely because of glutaraldehyde fixation and careful washing of the cells and because the non-sliding is apparently not a function of pH whereas protein adsorption would be.

We can calculate the rate of sliding which would be expected were the interface completely rigid. If the cell is separated from the interface by a distance of say 5 nm (Parsgian & Gingell, 1973) in a secondary minimum adhesion the only force resisting sliding is viscous drag. If each cell has a contact area of 1 μm² and the 5-nm gap contains water of bulk viscosity the cells would be expected to settle at approximately 0.2 μm per min. On the other hand if the cell were in a primary minimum on a rigid surface it probably would not slide at all. Our present observations do not allow us to distinguish between these two possibilities.

The observation that cells adhere completely to the C_{18}TAB-hexadecane/saline interface at pH 4.5, where the cationic detergent is completely ionized, giving a positive charge, shows that electrostatic attraction between the interface and the negative cell surface assists adhesion. It also suggests that a reduction in interfacial tension alone is not sufficient to prevent cell adhesion. This conclusion is supported by the fact that cells also adhere completely to the uncharged octadecanol-hexadecane/saline interface. Cell adhesion to the uncharged interface requires an attractive force to overcome the electrostatic repulsion; this arises because the cell’s double layer becomes restricted as it approaches the uncharged surface. We therefore conclude that negatively charged red cells tend to be electrostatically repelled from negative (as well as uncharged) interfaces but are attracted when the interface bears a positive charge. Underlying both attractive and repulsive electrostatic forces is an attractive force which may be electrodynamic (van der Waals force). These results are fully in accordance with the demonstration that mutual electrostatic repulsion is responsible for keeping red cells from aggregating (Gingell & Todd, in preparation). Further work is in progress to put the adhesion of cells to charged oil/water interfaces on a quantitative footing.

* There is no evidence that cells prepared in this way lose proteins or other molecules which could contaminate the interface. This has been shown in the course of experiments on red cell adhesion to a polarizable metal electrode whose surface cleanliness can be monitored with precision by measuring its differential capacity (Gingell & Fornes, in preparation).
Initial observations on inverted interfaces were made in the laboratory of Dr I. R. Miller at the Weizmann Institute of Science, Rehovot, Israel. One of us (D.G.) wishes to express his thanks to EMBO for support at that time, and also to Dr N. Gershfeld of the National Institutes of Health, Bethesda, Maryland, for generous extension of laboratory facilities. The work could not have been done without the patience and superlative engineering skill of Mr David Ubee, who built the inversion chamber. We would also like to thank Dr Jim Mingins and Dr Julian Lewis for their valuable criticisms of the manuscript.

REFERENCES


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APPENDIX A

The equilibrium depth of penetration of a fixed cell (modelled as a hard sphere) into an interface between oil and water can be approximately calculated in the following way.

Consider a sphere of radius $R$ partially immersed in the interface at a depth $x$ (Fig. 4). The area of cell/oil contact is $S(x) = 2\pi Rx$. The area of the plane formed by an imagined extension of the flat oil/water interface through the cell is $A(x) = \pi(2Rx - x^2)$. Writing $\gamma_{ow}$, $\gamma_{ow}$, $\gamma_{ow}$ as specific interfacial free energies of the cell/oil,
Red cell adhesion to oil/water interface

cell/water and oil/water interfaces respectively, the change in energy at a depth of immersion $x$ is

$$G = \gamma_{co} S(x) - \gamma_{cw} S(x) - \gamma_{ow} A(x),$$  \hspace{1cm} (A 1)$$

$$G = -2\pi R(x^2 - x^2) \gamma_{ow}. $$  \hspace{1cm} (A 2)$$

The force is

$$F = -\frac{dG}{dx} = -2\pi [R(\gamma_{cw} - \gamma_{co}) + (R-x) \gamma_{ow}].$$  \hspace{1cm} (A 3)$$

Equilibrium exists when $F = 0$ and $x = \bar{x}$, i.e. from (A 3),

$$\gamma_{co} = \gamma_{cw} + \gamma_{cw} - \gamma_{ow} R.$$  \hspace{1cm} (A 4)$$

Consequently, since $\gamma_{cw} \ll \gamma_{ow}$, $\bar{x}/R \sim 0$ implies $\gamma_{co} \sim \gamma_{ow}$. It is a stable equilibrium since

$$\frac{d^2G}{dx^2} = 2\pi \gamma_{cow} > 0.$$  \hspace{1cm} (A 5)$$

The condition for total engulfment of the cell by the oil can be obtained from (A 4) putting $\bar{x} = 2R$,

$$\gamma_{co} = \gamma_{cw} - \gamma_{ow}.$$  \hspace{1cm} (A 6)$$

The above treatment neglects the effect of gravity, which is easily shown to be negligible. The model assumes that the oil/water interface is flat right up to the cell. This is correct since it is the shape which gives minimal oil/water surface area for a given depth of immersion.

APPENDIX B

At very high pH values near 9.0 measurements of cell adhesion in the torus apparatus are frustrated by instability of the oil/water interface when water is uppermost. The reason seems to be that the oil tends to rise through the water and the water tends to fall into the oil due to their different densities (density of hexadecane $\rho_d = 0.77$). This spontaneous tendency is offset by the work which must be done to increase the surface area, since the planar interface has minimal area. But at sufficiently low surface tension the phases can exchange. This is shown in Fig. 5. The smooth curve shows the rising droplet of oil and descending bulge of water which can be observed at high pH. The step function is used as an approximation to this curve for a simple order-of-magnitude calculation valid for large deformations of the interface.

The surface energy change due to deformation of the interface is

$$G_y = 2\pi \gamma [r_0(h_0 + h_w) + r_w h_w],$$  \hspace{1cm} (B 1)$$

where $\gamma$ is the interfacial energy per unit area.

The gravitational potential change due to fluid displacement is

$$G_g = -\frac{\pi}{2} \Delta \rho g [h_0^2 r_0^2 + h_w^2 (r_w^2 - r_0^2)],$$  \hspace{1cm} (B 2)$$
where $\Delta \rho = \rho_w - \rho_o$, the difference in density of the 2 phases. Since the total volume of oil and water is constant the net displacement is zero,

$$h_o = h_w \left( \frac{\tau_{w}^2 - \tau_{o}^2}{\tau_{w}^2} \right). \quad (B\ 3)$$

From (B 1), (B 2) and (B 3) the net energy change is

$$G = \pi h_w \left( 2\gamma R_1 - \Delta \rho g \frac{h_w}{2} R_2 \right), \quad (B\ 4)$$

![Diagram](image)

**Fig. 5. Instability of an oil/water interface in the face of gross deformation.**

where $R_1 = \frac{\tau_{w}}{\tau_{o}} (\tau_{w} + \tau_{o})$ and $R_2 = \frac{\tau_{w}}{\tau_{o}} \left( \frac{\tau_{w}^2}{\tau_{o}^2} - 1 \right)$.

The force tending to change the shape of the interface for any given displacement $h_w$ is

$$- \frac{dG}{dh_w} = \pi (\Delta \rho g h_w R_2 - 2\gamma R_1). \quad (B\ 5)$$

Equilibrium exists when

$$- \frac{dG}{dh_w} = 0,$$

so that the equilibrium displacement is

$$h = \frac{2\gamma}{\Delta \rho g} \frac{R_1}{R_2},$$

$$h = \frac{2\gamma}{\Delta \rho g} \frac{\tau_{o}}{\tau_{w} (\tau_{w} - \tau_{o})}. \quad (B\ 6)$$
Red cell adhesion to oil/water interface

\[ \frac{d^2G}{dh^2} = -\pi \Delta \rho g R_2 < 0 \quad \text{since} \quad r_w > r_o \quad \text{always}. \]

This is the condition for an unstable equilibrium: the extremum (equation B 6) is a maximum not a minimum. If the interface is perturbed by an amount \( h < h \) there is a return to the flat condition. But if the perturbation \( h > h \) the phases exchange. In the chamber a perturbation may be due to inadvertently bulging the meniscus while adjusting its shape.

For example, if \( r_w = 0.002 \text{ m}, r_0 = 0.001 \text{ m} \) (approximate values for the chamber used) \( \Delta \rho = 0.23 \) we find from equation (B 6) that when \( \gamma = 10^{-2} \text{ J m}^{-2} \), \( h_{\text{wp}} = 4400 \mu\text{m} \). When \( \gamma = 10^{-3} \text{ J m}^{-2} \), \( h_{\text{wp}} = 440 \mu\text{m} \). Thus except at very low interfacial tension the interface is stable. Preliminary measurements of interfacial tension at pH 9 indicate a very low interfacial tension.
Figs. 6–10. Cells on an oil/water interface. × 200.

Fig. 6. Cells on the oil/water interface at pH 8.25 about 30 s after inversion. In this and the following photographs only a portion of the whole visible field is reproduced.

Fig. 7. Cells on the oil/water interface at pH 8.25 about 1 min after inversion.

Fig. 8. Cells on the oil/water interface at pH 8.25 about 5 min after inversion.

Fig. 9. Cells on the oil/water interface at pH 7.0 about 30 s after inversion.

Fig. 10. Cells on the oil/water interface at pH 7.0 about 5 min after inversion.

Fig. 11. Single red cell adherent edge-on at an oil/water interface at pH 6.4. Oil uppermost. × 1000.
Red cell adhesion to oil/water interface