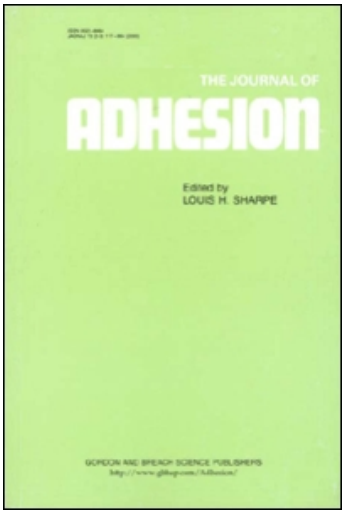

The Journal of Adhesion
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713453635

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To cite this Article McLver, Donald J. L. and Schürch, Samuel(1987) 'Polymer Mixing and the Thermodynamics of Cell Adhesion at Fluid Interfaces', The Journal of Adhesion, 22: 4, 253 — 289
To link to this Article: DOI: 10.1080/00218468708071247
URL: http://dx.doi.org/10.1080/00218468708071247

J. Adhesion, 1987, Vol. 22, pp. 253–289
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Printed in the United Kingdom

Polymer Mixing and the Thermodynamics of Cell Adhesion at Fluid Interfaces†

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(Received December 22, 1986)

The mechanism of adhesion of cells to other cells or to non-cellular surfaces is a central problem in cell biology and biotechnology. The present studies were carried out to investigate the relationships between cell surface thermodynamics, the kinetics of cell adhesive behaviour and the molecular and morphological structure of the cell surface, when cell adhesion is elicited by defined physiological stimuli. The surface thermodynamic studies are applications of classical capillarity in which we measure the wetting of cell surfaces (the surface affinity or work of adhesion) by aqueous phase separated polymer solutions of dextran and poly (ethylene glycol), before and after exposure of the cells to the adhesive stimuli. For the phase system used here {4%/4% w/w dextran mol. wt. 2×10^6/poly (ethylene glycol) mol. wt. 2×10^4 in HEPES buffered physiological saline}, the measured interfacial free energy was 4.02×10^{-6} J m^{-2}. Isolated leukocytes were exposed to adhesion-promoting chemoattractant stimuli (for example, the tripeptide N-formyl methionyl leucyl phenylalanine or serum complement activated yeast particles). We observed increases in the work of cell adhesion to the dextran phase which were proportional to the stimulus dose; at the maximally effective doses, the cell–liquid–liquid contact angles changed by about 50°, corresponding to 2×10^{-6} J m^{-2} changes in the work of adhesion. In parallel with the thermodynamic measurements we measured the kinetics of cell activation to provide the time scale of cell behaviour which is missing from the equilibrium surface energy measurements. Infrared photometry (optical density and perpendicular light scattering) provided information on the kinetics of cell adhesion and morphological responses to stimuli; simultaneous measurements of oxygen free radical-dependent chemiluminescence and spectrophotometric measurements of

† Presented at the Tenth Annual Meeting of The Adhesion Society, Inc., Williamsburg, Virginia, U.S.A., February 22–27, 1987.

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enzyme activity defined the kinetics of biochemical activation. The thermodynamic studies were carried out during the plateau of the time-response curves; under these conditions both the kinetic and thermodynamic measurements have similar stimulus dose-response curves, suggesting that both types of process are manifestations of the same cell surface events. The exact relation of the surface affinity changes to the molecular mechanism(s) of cell adhesion is not yet clear, however. Synthetic structural studies (model membrane reconstitution) indicate that the cell surface/polymer phase affinity is a function of the concentration, molecular weight and conformational state of the cell surface glycopolymers (the "glycocalyx"). Analytic studies have shown correlations between surface affinity changes and the molecular weight/concentration profiles of isolated cell membrane glycoproteins. Modelistic considerations suggest that the cell surface glycoproteins which determine the phase wetting behaviour have concentrations in the micromolar range. If these aqueous phase reactive glycocalyx proteins are the molecules which mediate cell adhesion elicited by small specifically recognised ligands, then it may be necessary to modify the current model of cell adhesion which proposes that adhesion results from a balance between "specific bonding and non-specific repulsion." For example, in stimulus induced leukocyte adhesion a "specific induction, non-specific execution" model may be more useful.

KEY WORDS Bioadhesion; cell adhesion; fluid interfaces; polymer mixing; properties of cell surfaces; thermodynamics.

1 INTRODUCTION: MEASURING AND MODELLING CELL ADHESION

"*Until our assays of adhesion are improved considerably we will remain ignorant of the relative contributions of strength and permanence in adhesion interactions.*"[1]

The mechanism of adhesion of cells to other cells or to non-cellular surfaces is a central problem in cell biology and biotechnology. Any detailed understanding of these processes must account for

1) the physical strength (thermodynamics) of cell adhesion and its relation to the nature and range of the forces between the adhering molecules;

2) the permanence (kinetics) of cell adhesion, and

3) the way that chemical selectivity (structure) operates in different cellular adhesive events.

The term "cell adhesion" encompasses a wide variety of adhering cells and surfaces, ranging from such processes as the aggregation of

cells in flowing blood to the adherence of cells to plastic tissue culture dishes or implanted biomaterials. The molecular details of these events remain only partially characterised, but it seems unlikely from the outset that any single method of measurement or model of adhesion would suffice to describe the diversity of these adhesive processes.

In this communication, we will focus on the ways in which surface thermodynamic studies highlight the similarities rather than the differences in cell adhesion. Historically, this has been the role of thermodynamics: to provide a framework for describing the magnitude and direction of diverse natural processes, without requiring or using molecular information. Eventually, of course, a full understanding of adhesion requires that the thermodynamic measurements be related to the structure of the adhering surfaces.[2]

Measuring cell adhesion

The variety of methods that has been employed to measure cell adhesion seems only to emphasise this diversity. The experimental approaches that have been used to quantify cell adhesion include adherent particle counting,[3] force-displacement measurements,[4] particle size distribution,[5] hydrodynamic shearing of cells in suspension[6,7] or attached to surfaces,[8,9] light scattering[10,11] and surface thermodynamics.[12] Each of these approaches has features to commend it, but all have their limitations. So far none has permitted the detailed, simultaneous correlation of adhesive energies and kinetics with the biochemical structure of the cell surface that is necessary to formulate and test critical theories of the mechanism(s) of cell adhesion.

The traditional biological approach to quantifying cell adhesion is the direct counting of the "adhering" cells, using morphological, chemical or radiochemical detection methods.[13] The biological advantage of this approach is that it necessarily provides a functional assessment of adhesion. The major difficulty is that what is observed (the quantity of adherent cells) depends on the distraction procedure, *i.e.*, on the method used to separate "bound" and "free" cells in these kinds of measurements. The cell responses are quantized (*i.e.*, cells are either "stuck" or "not stuck"), so it is difficult to make estimates of graded adhesion energies. The forces

required to remove adherent cells do not necessarily provide information about adhesive energies; the large hysteresis between the energies of cell attachment and detachment probably arises because detachment includes contributions from time-dependent (irreversible) work as well as from the reversible work of adhesion.[32]

The traditional physical approach to the energies of surface interaction is to measure the force required to produce a measured surface displacement. Provided reversibility can be guaranteed (no easy task when dealing with phenomena that are usually time- and path-dependent), this will probably always be the most informative method of studying adhesion. In aqueous media—the predominant biological milieu—this approach has been applied with increasing refinement over the past decade: micromechanical[14] or osmotic force[15] measurements, coupled with multiple beam optical interference[14] or X-ray diffraction[15] measurements of surface separation have revealed a wealth of information about the range and magnitude of surface forces. However, the technical requirements of these measurements—molecular smoothness or structural repetition which yields an X-ray diffraction pattern—have so far limited their application to non-cellular systems. When micro-mechanical force-displacement measurements are carried out on living cells the fine details of the range of the surface forces are lost, and an integral of work and distance is recorded.[4]

The distribution of particle sizes in either microscopic optical measurements[16] or electronic sizing channelizers[5] has been a useful way of following the growth of cell clumps due to aggregation. These procedures provide evidence of cell-cell adhesion, but once again estimates of adhesion forces are not readily obtainable.

Light scattering measurements have also been widely employed to study cell adhesion. When a suspension of particles is illuminated with a beam of collimated light, some of the light may be absorbed, some transmitted and some scattered. Measuring the luminous intensities of each of these processes is a powerful tool for obtaining information on the morphological properties of the components of the suspension—the size, shape, state of aggregation, etc. Born[10] introduced transmittance photometry to the study of blood platelet aggregation, and showed that microscopic aggregation was time correlated with increased light transmittance. Since then, measure-

ments of light transmittance and scattering by cell suspensions have been widely used to study cell responses to stimuli.[17] The light scattering approach has two major virtues. First, it has good kinetic sensitivity (providing a millisecond time scale for cell behaviour). Second, it has good statistical reliability, being based on measurements of large numbers of cells (typically 10^5–10^7). In addition, other spectral data may be obtained at the same time as scattering is used to investigate morphology. Information on interaction forces is not readily obtainable, however, and the results of scattering experiments have usually been interpreted empirically, mostly in terms of light or electron microscopy.

Surface thermodynamics has been used as a tool for investigating cell adhesion for more than 50 years.[18] While this is also a potentially powerful approach, it has suffered from the limitation of identifying the cell surface thermodynamic potential functions which are *relevant* to studying cell adhesion. The problem is essentially one of the macrocosm and the microcosm: given the microheterogeneity of cell surfaces, it is not clear what a macroscopically averaged "surface energy" measurement on a multicomponent cell surface really means. Nevertheless, measurements of cell surface thermodynamics have shown a number of correlations with cell behaviours such as phagocytosis and adhesion to synthetic polymeric surfaces.[19] A related approach which has been widely investigated as a tool in cell surface analysis is the partition of cells between liquid phases.[20] Surface thermodynamics contributes to the partition behaviour of cells, via the difference in interfacial energy of the cells in each phase but there are other factors at work as well: external forces in addition to interfacial work (such as hydrodynamic flow and gravity) also influence cell partition.[21]

Modelling cell adhesion

Based on these kinds of measurements, the current model of cell adhesion is that of a "competition between non-specific repulsion and specific bonding."[22] According to this model, cell adhesion occurs when a sufficient number of cross-bridging molecules bind to the surfaces of adjacent cells and oppose the distracting forces which tend to keep the cells apart. Lectins,[23] antibodies[24] and "Cell

Adhesion Molecules" (or CAM's[25]) are examples of molecules which cross-bridge specific binding sites; non-specific cell cross-linking (the so-called rouleau formation) may also be mediated by binding of molecules such as dextran or fibrinogen.[26]

The mechanics and thermodynamics of the "non-specific repulsion, specific bonding" model (which we shall subsequently call "model 1" for short) have been developed in detail, both for the cases where the bridging molecules are continuously[27] or discretely[28] distributed on the cell surface; direct micromechanical force measurements of cell cross-bridging under equilibrium conditions have confirmed the predictions of this model. Model 1 explicitly assumes that the same molecular mechanisms are responsible for recognising and executing cell adhesion. For both the specific and non-specific macromolecular cross-bridging (agglutination) reactions described above, this is a reasonable assumption.

For many types of cell adhesion, however, we suggest that model 1 is not adequate; the agglutination described by model 1 is a sufficient but not necessary condition for cell adhesion. There are many cellular adhesive events where the specificity of adhesion arises from a low molecular weight adhesion-inducing ligand which is of itself too small to provide the specific cross-bridging required by model 1. An important example of such a process is the leukocyte aggregation induced by the chemoattractant tripeptide N-formyl methionyl leucyl phenylalanine;[16] the stimulation of cell adhesion to foreign surfaces by activated complement[29] may well be a similar phenomenon. A considerable amount is known about the recognition of these specific adhesion-inducing ligands, but much less is known about the sticky molecules—the glue—which actually mediate the cell adhesion. Given the fact that activated leukocytes, for example, may stick quite indiscriminately to a variety of surfaces, we suggest that small-ligand-induced adhesion may be better described by the reverse of model 1 or a "specific induction, non-specific execution" model, which we call model 2 for short. The idea behind model 2 is not new (for example, see Refs. 30, 31). What is new is the availability of methods for testing it.

These methods, which are predominantly applications of classical thermodynamics to cells in liquid polymer solution phases, are based on measuring the static (equilibrium) affinity of cell surfaces

for fluid interfaces under conditions where the cell adhesiveness is varied by environmental manipulation, and where parallel studies of the kinetics, morphology and biochemistry of the adhering cells allow these events to be correlated. From a knowledge of the composition of the liquids used for the affinity measurements, it is possible to make some testable inferences about changes in the cell surface under defined conditions of adhesion, and to relate these inferences to the energies between the adhering molecules.

2 SURFACE THERMODYNAMIC APPROACHES TO CELL ADHESION

In principle, an approach which could yield information about the time- and path-independent (*i.e.*, equilibrium) adhesive forces between cell surfaces is one based on surface thermodynamics. This idea is not new either: attempts to describe cell adhesion in terms of the formalisms of classical thermodynamics had already been made by the second decade of this century.[18] These early approaches regarded cell adhesion as arising from a "a pure surface tension · · · and the fluidity of the protoplasm";[18] in more modern terminology this balance of opposing forces would be described as the resultant of surface affinity (the surface free energy reduction on contact, or work of adhesion) and cell deformability.[32] However, after this seminal early work (and for no apparently decisive reasons), enthusiasm waned for using equilibrium thermodynamics as a tool in analysing cell adhesion. At least two influences were likely responsible. On the one hand, the growing appreciation of the biochemical complexity of cells may have pointed to the improbability of usefully describing microscopically heterogeneous cell surfaces in terms of macroscopically averaged potential functions. For example, the fact that cell receptors for drugs and hormones only covered a small fraction of the cell surface was already widely appreciated by 1933.[33] In addition, the growing recognition of irreversibility in biology[34] may have made equilibrium approaches to what is manifestly a non-equilibrium phenomenon, seem unrealistic. The strengths and limitations of the equilibrium approach to cell surface interactions have recently been

discussed.[22] In Section 7 we will briefly discuss the relations between microscopic receptor distribution and equilibrium macorscopic surface thermodynamics in relation to the question of cell adhesion induced by low molecular weight, structurally specific ligands which do not *per se* have the capacity to stick cells together.

In the early 1960's thermodynamic approaches to cell adhesion were taken up again, this time from the viewpoint that whole cells could be regarded as miscible or immiscible liquids.[35] Despite its limitations, we believe that elements of this theory remain useful for modelling some important aspects of cell adhesion, in particular, those aspects which primarily involve the isotropic polymer solution-like behaviour of the cell surface glycoproteins (the glycocalyx) rather than the thixotropic or viscoelastic behaviour of membrane or cytoplasmic deformation. Subsequently, the thermodynamic analysis of cell adhesion phenomena received a considerable impetus from advances in the study of contact angles and their application to a variety of cell-substrate and cell-particle interactions.[12]

The latter studies take as their starting point the equation developed by Thomas Young in 1805[36] to describe the equilibrium relationship at the three-phase line between two fluids and an ideal, non-deformable solid substrate (Figure 1):

$$\gamma_{23} = \gamma_{13} + \gamma_{12} \cos \theta \tag{1}$$

where γ_{12}, γ_{13} and γ_{23} are the interfacial free energies between the three phases (denoted 1, 2 and 3) and θ is the contact angle at the three-phase line. Deviations of real contact angles from Young's equation are usually attributed to metastable wetting states arising from chemical heterogeneity or surface roughness.[37] The scale of microscopic irregularities which result in macroscopic contact angle changes is a current area of active research concern; as far as heterogeneity is concerned, modelistic considerations indicate that dimensions $<0{\cdot}1\ \mu$m will not give rise to macroscopic contact angle hysteresis;[38] since most cell surface chemical heterogeneities are expected to be much smaller than this, macroscopic contact angles on living cells should reflect an equilibrium average of the effects of the chemical determinants of the surface. The exact influence of the second cause of contact angle hysteresis—microscopic surface roughness—still remains unsettled.

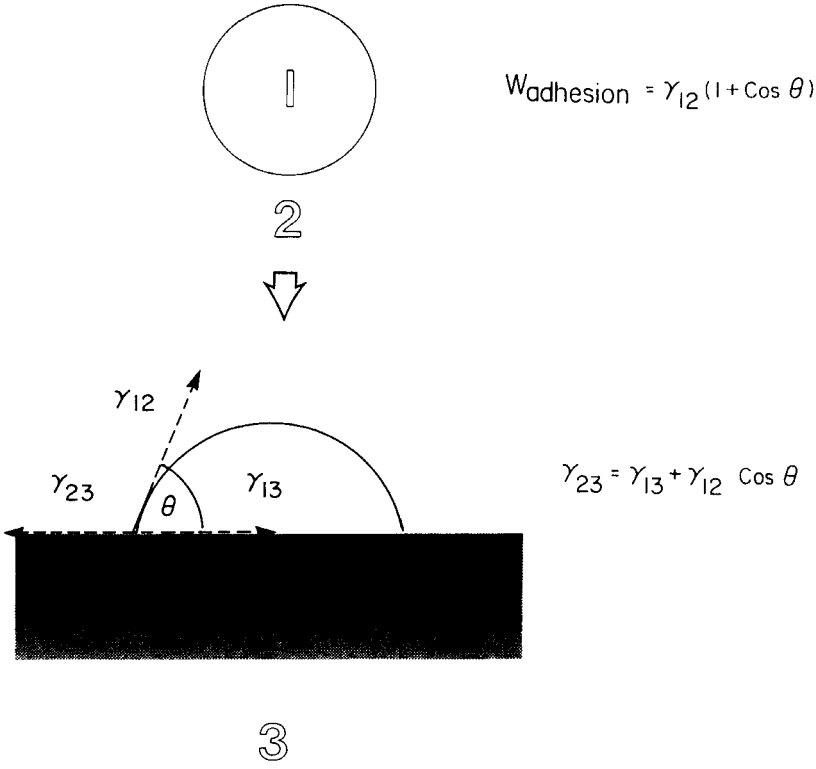


FIGURE 1 Relationships between the interfacial free energies, γ, and the contact angle θ, at a three-phase boundary.

The last decade has seen a great increase in the application of surface thermodynamics to the investigation of a variety of cell surface phenomena (for a recent review, see Ref. 19), as well as the appreciation of some of the problems associated with it.

Like the methods described above, our own approach to cell adhesion has been based on the measurement of contact angles at cell surfaces, and the estimation of the work of cell adhesion under various biological conditions. Our approach differs from those described above in that it uses aqueous solutions, rather than the vapour phase, as the reference state for calculating cell surface thermodynamic potentials.

3 CELL SURFACE CONTACT ANGLES IN LIQUID-LIQUID SYSTEMS

In our early studies of cell surface contact angles in liquid/liquid systems (*e.g.*, Ref. 39) we used droplets of polar hydrocarbon oils immersed in saline solutions to measure angles at the oil/saline/cell interface. Figure 2A illustrates the application of this approach to

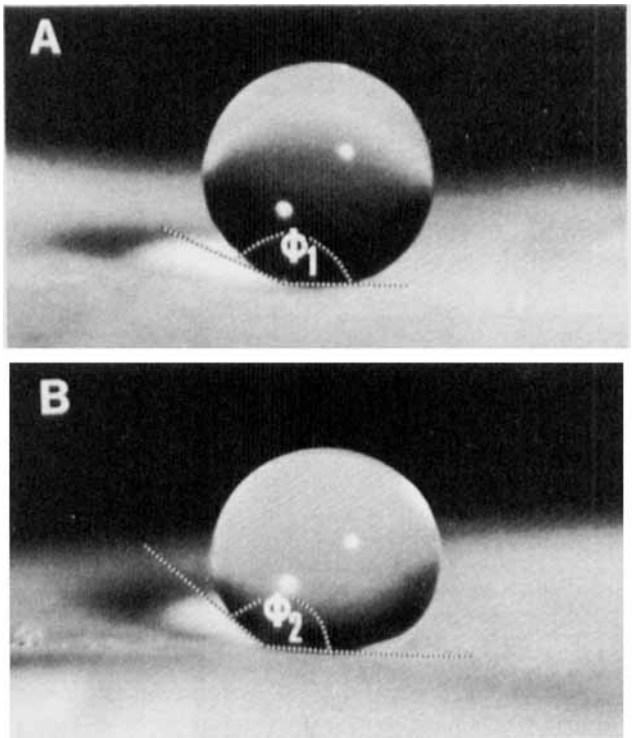


FIGURE 2 Adhesion of endothelial cells at oil-water interfaces. A A droplet of isopropyl salicylate ($\gamma_{12} = 13.3\,\text{mJ m}^{-2}$) resting on the endothelial surface of the aorta from a healthy rabbit. The contact angle is 150°. B A droplet of isopropyl salicylate resting on the surface of an endothelial lesion from an animal with advanced diet-induced atherosclerosis. The contact angle is 137°. Both droplets are approximately 1 mm in diameter. C Scanning electron micrograph of specimen A, stained with silver to highlight the endothelial cell boundaries, and showing the intact endothelial cell monolayer. The endothelial cells range in length from 30 to 50 μm; the dotted line shows the approximate boundary of the three phase line which would be formed by droplet A at a contact angle of 90°. Adapted and reproduced from Ref. 39.

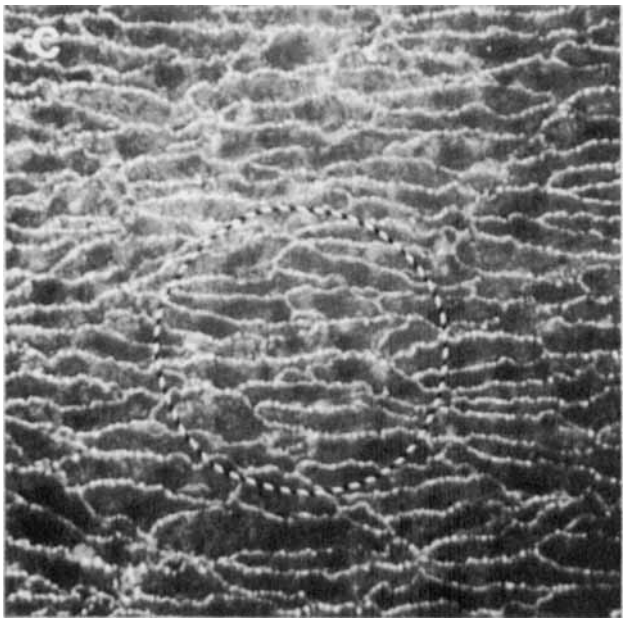


FIGURE 2 (*continued*)

the endothelial cell surface of isolated arteries. The measured contact angles and liquid-liquid interfacial free energies, and a semi-empirical equation of state (see equation [2][40]) indicated that the cell-saline interfacial free energy was very low, $\sim 10^{-2}$ mJ m^{-2} or less. This value is one order of magnitude lower than direct measurements of bilayer interfacial free energies,[41] and two orders of magnitude lower than interfacial free energies calculated from measurements of contact angles on lipid monolayers at oil-water interfaces,[42] indicating that the contact angles at the cell/saline interface were a result of non-lipid surface active materials in the cell membrane (*i.e.* membrane proteins), an idea originally proposed by Davson and Danielli.[43] Interestingly, these cell/oil/water adhesion studies also showed that pathological processes (in this

case, advanced diet-induced atherosclerosis, which resulted in focal changes in the endothelial surface) resulted in changes in the interfacial free energies of the cells which could be distinguished from healthy control animals or from non-affected cells in the diseased animals (Figure 2B). Morphological studies (Figure 2C) showed that the cell layer remained intact through the experimental procedure. On the basis of these liquid/liquid adhesion measurements, we suggested that this kind of study might be a useful approach to assessing one of the factors thought to be involved in the initiation of atherosclerosis: an altered adhesion potential of the blood vessel wall for blood elements such as platelets or leukocytes.[39]

However, the use of oil-water interfaces in these studies created a problem of sensitivity: because of the high values of the cell/oil/water contact angles (a consequence of the high oil/water interfacial free energy, and the low cell/water interfacial free energy) it proved impossible to obtain contact angles of less than 150° on normal cells; the difficulty of accurately measuring such high angles introduced a substantial uncertainty into the cell/saline free energy estimates. In addition, since cells do not usually live in a hydrocarbon environment, the biological effects of this procedure may not be innocuous.

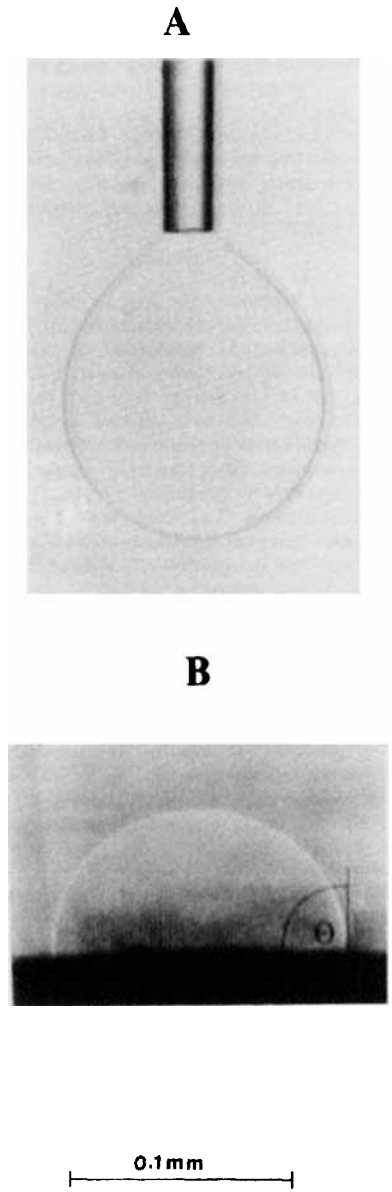
Accordingly, most of our subsequent studies of cell surface thermodynamics have been carried out using a procedure which minimises these problems: aqueous phase separated polymer systems. These systems have been extensively studied by Albertsson and co-workers,[20] and have both of the characteristics required for studies of the surface thermodynamics of living cells: ultralow interfacial free energies ($\sim 10^{-3}$ mJ m^{-2}) and excellent biocompatibility. These polymer systems resemble blood plasma in their physiochemical properties (indeed, dextran solutions have been in clinical use as plasma expanders for many years), and they may be buffered, sterilised, rendered isotonic and provided with many of the factors required for normal cell function. They have been used extensively for dynamic studies of cell separation by phase partition.[44]

Measurements of static contact angles of polymer phase drops with living cells were carried out independently in our laboratory[45] and that of Fisher.[46] Since then we have studied the effects of

environmental factors on the thermodynamic properties of the cell surface revealed by this method, seeking to correlate biochemical and biophysical information about the cell surface, its relation to the mechanisms of cell adhesion, and its alteration by pathological processes.

The method is illustrated in Figures 3A and 3B. A droplet of the denser polymer phase (4% dextran, ave. mol. wt. 2×10^6) is formed in the lighter phase (4% polyethylene glycol, ave. mol. wt. 2×10^4, PEG) and the interfacial free energy is determined from the deformation of the drop profile by gravity. Tables of drop profile parameters[46] may be used for this calculation. The drop is allowed to fall onto the surface of interest, and it spreads until a final advancing contact angle is achieved. If suitable digitising and computing facilities are available, a more precise analytic procedure is provided by the computer programme of Rotenburg, Boruvka and Neumann,[68] which uses a least squares algorithm to fit a LaPlacian curve to the drop profile, and which calculates the interfacial free energy, the drop volume and surface area, and the contact angle formed by the drop. Depending on the polymer concentration, for drops of the size illustrated in Figure 3B (0.1–1 mm), spreading takes 1–4 minutes.

Figure 3B shows the drop from Figure 3A, after it has reached its stationary state on the cell surfaces. In this case, the cells were a purified (~99%) population of lung alveolar macorphages cultured on a cellulose acetate filter for 24 hours before the contact angle measurements were made. At this magnification (60×) the individual cells (12–15 μm in diameter) can just be resolved. Each drop samples about 7×10^3 cells. The contact angle (calculated either by direct measurement with a protractor eyepiece, or by the computer curve fitting procedure),[68] is 95°, corresponding to an affinity (work of adhesion) of the cell surface for the dextran phase in the PEG phase of 4×10^{-3} mJ m^{-2}. Subsequent studies, described below, have shown that the polymer phase contact angle is quite sensitive to changes in the cell surface induced by a variety of different means, and that the chemical basis of these changes probably lies in alterations in the amount, molecular weight or conformation of cell surface glycoproteins. The method has also been used to investigate the effects of mechanical removal of endothelial cells from the arterial wall.[50]



4 EQUIVALENT SOLUTION PROPERTIES OF THE CELL SURFACE

Abandoning the vapour phase as the reference state for cell surface thermodynamics may arguably have some benefits in terms of sensitivity and biocompatibility (*e.g.*, Ref. 69), but it definitely carries a price: in the absence of an independent relation between the contact angle and the interfacial free energies Young's equation cannot be solved for solid substrates. Hence, contact angle measurements on solid surfaces yield only the difference in interfacial free energies of the solid surface between the two fluid phases. An empirical functional relation (an equation of state) between the free energies in three-phase systems has been proposed[40] having the form,

$$F(\gamma_{13}, \gamma_{23}, \gamma_{12}) = 0 \tag{12}$$

but so far this relation applies only to the type of two-component systems for which it was derived, where γ_{12} (using the terminology of Figure 1) is of the magnitude of the energy of the hydrocarbon-vapour interface, *i.e.*, in the mJ m^{-2} range.[42] For systems of μJ m^{-2} interfacial free energies (such as the aqueous polymer phases of interest here), the empirical equation of state does not hold.[49]

In order to stay with thermodynamics, therefore, we are restricted to expressing contact angle data in terms of the difference in free energy of the substrate with respect to the two fluid phases. Quantitatively, this difference is expressed as the work of adhesion, W_{adh}, where

$$W_{adh} = \gamma_{12}(1 + \cos\theta) \tag{13}$$

Equivalently, and for a constant γ_{12}, the difference in interfacial

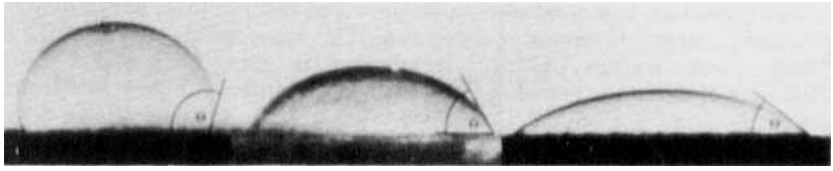
FIGURE 3 Adhesion of isolated cells in phase-separated aqueous polymer systems. A A pendant drop of the denser (dextran-rich) phase hanging in the less dense (polyethylene glycol-rich) phase of a 4%/4% dextran (ave. mol. wt. 2×10^6)/polyethylene glycol (ave. mol. wt. 2×10^{-4}) phase system. The aqueous phase is Hank's balanced salt solution, pH 7.4. The interfacial free energy of this system is 4.02×10^{-3} mJ m^{-2}. The maximum drop diameter is approximately 100 μm. Differential interference (Nomarski) contrast. B The droplet shown in A resting on a layer of pig alveolar macrophases sedimented onto a cellulose acetate filter. The final advancing contact angle is 95°. Individual cells can just be distinguished at this magnification (60×). Polarization contrast. Reproduced from Ref. 45.

free energy between two situations can be expressed as the change in the cosine of the contact angle, $\Delta \cos \theta$ (*e.g.*, see Figure 10). In general, we express the liquid-liquid adhesion measurements in terms of the work of adhesion of the cells to a drop of the dextran phase immersed in the polyethylene glycol phase.

Here, however, we want to explore briefly an extra-thermodynamic approach to interpreting the polymer phase contact angle measurements. The strategy is based on an analogy with Zisman's operational parameter, the so-called "critical surface tension for spreading" or "γ_c").[51] For low energy solid/liquid/vapour surfaces and negligible equilibrium spreading pressure, Zisman defined γ_c as the limiting value of a cosine plot of the contact angle *versus* the liquid surface tension. As Zisman was careful to point out, γ_c is not a thermodynamic property of the surface, and it therefore cannot be used in a thermodynamic analysis. However, along with molecular considerations, a critical spreading type of approach does offer some useful insights into the interaction of polymer phases with cell surfaces.

The approach is illustrated in Figure 4. In 4A, droplets from the dextran-rich phase of three different two-phase systems are shown on sedimented layers of isolated human erythrocytes. As the polymer concentration is reduced, the contact angle is progressively lowered. In the Zisman approach, a "γ_c"-like parameter would be defined as the limiting liquid surface tension when θ approaches zero. We have also used a "γ_c" type of parameter to express the properties of the cell surface;[45] it is not clear, however, what a "γ_c" determined under these conditions really means. More informative perhaps is the limiting value of the polymer *concentration* when θ approaches zero. Assuming that the interfacial free energy between the cell and the test droplet in this case is very low (certainly a minimum, and possibly zero: Ref. 52), at this point the polymer phase in the test drop is equivalent in composition to a polymer phase comprised of the molecules making up the cell surface. We call this limiting composition of the test droplet phase, determined as the contact angle approaches zero, an "equivalent solution" to the cell surface. For equivalent molecular weights and conformations (*i.e.*, polymer-solvent interactions), the time- and space-averaged polymer compositions of the two systems (the cell surface and the test drop) should be identical.

A



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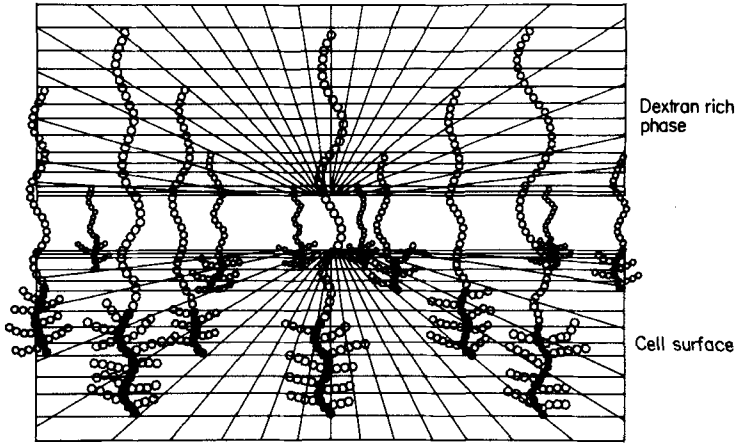


FIGURE 4 "Critical spreading" of polymer phases on isolated cells. A Variation of contact angle with polymer concentration of dextran/polyethylene glycol droplets on layers of human erythrocytes for dextran (ave. mol. wt. 5×10^5) polyethylene glycol (ave. mol. wt. 2×10^4) phase systems. The concentrations (w/w) of the polymers and the contact angles are: 5%, 106° (left); 3·7%, 61° (middle) and 3.3%, 38° (right). The droplet diameters are approximately 1 mm. Polarization contrast. Reproduced from Ref. 45. B Schematic quasi-lattice model of polymer-polymer interactions at an idealised cell surface as the contact angle and the cell/dextran phase interfacial free energy approach zero. Reproduced from Ref. 48.

This hypothesis is illustrated schematically in Figure 4B, a quasi-lattice model of polymer–polymer interactions between the polymer segments at the cell surface and in the aqueous phase.[48] In this model, lowered contact angles (lowered interfacial free energies or increased work of adhesion of the cell surface to the dextran phase) correspond to increased mixing between the two phases, while raised contact angles (increased mixing between the two phases, while raised contact angles (increased interfacial free energies or increased adhesion to the PEG phase) correspond to phase de-mixing. From a consideration run on to next page (15) of the phase diagrams of these phase systems (*e.g.*, Refs. 20, 45) these changes could be mediated by changes in the molecular weight or the concentration of cell surface polymers. In addition, polymer solution theory (*e.g.*, Ref. 53) predicts that alterations in polymer-solvent interactions (conformation) at the cell surface will be expected to influence such mixing behaviour.

We have not yet obtained any direct evidence that the latter factor (cell surface polymer conformation) exerts an influence on cell surface/polymer phase interactions, but several experiments are consistent with the hypothesis that changes in the molecular weight and amount of cell surface polymer influences cell-medium contact angles.

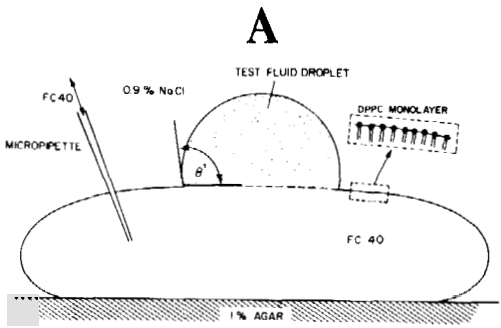
5 CELL SURFACE COMPOSITION AND SURFACE THERMODYNAMICS

Our approaches to the experimental investigation of the relationships between cell surface composition and surface thermodynamics have taken two routes: synthesis and analysis. First, we carried out some simple recombination experiments in which the surface thermodynamic behaviour of model (synthetic) surfaces made of defined chemical components was compared with the behaviour of intact cells. The other route was correlating contact angle measurements with biochemical analysis of the membrane composition of cells cultured under conditions which produce well-defined permanent changes in membrane composition.

The experimental basis of the synthetic approach is the ability to measure contact angles on defined molecular monolayers at defor-

mable interfaces. The experimental procedure is chosen to facilitate both contact angle measurements and analysis of interface shape, while at the same time circumventing the problems of surface film leakage which attend traditional film balances of the Langmuir–Wilhelmy type.[54] This is achieved in a "mini surface balance," illustrated schematically in Figure 5A. A five-phase system is used: a hydrophilic substrate (*e.g.*, 1% agar gel) provides a leakproof base for a lipid monolayer spread at an oil/water interface. By adding or removing oil the area of the monolayer can be controlled just as in a Langmuir trough. The choice of a dense hydrophobic and oleophobic liquid fluorocarbon ("FC40," perfluoromethyldecalin, 3M Company) allows the creation of a fluid oil/water interface which is deformed by gravity, but not by the hydrocarbon test drop, whose density (1.02 g/ml) is chosen to be slightly greater than that of the aqueous phase (1.01 g/ml), but much less than that of the fluorocarbon (1.85 g/ml). We call this system a pseudo-solid, since with respect to hydrocarbon/water contact angles the fluorocarbon oil behaves as a solid. That is to say, because of the density differences the usual "Neumann triangle" relationship associated with deformation at fluid three-phase lines[54] does not occur. As a "solid" this system is rather unusual: since the surface monolayer is both smooth and homogeneous, it is free from contact angle hysteresis, and the contact angles measured on this surface are unambiguously equilibrium angles. With respect to profile analysis, however, the fluorocarbon behaves as a true fluid. Figures 5B illustrates this experimental arrangement when a phospholipid monolayer is spread at the oil-water interface: the contact angle of 105° corresponds to a measured monolayer-water interfacial free energy of 3.6 mJ m^{-2} on this partially compressed monolayer.

When isolated membrane proteins are added, the wetting behaviour of intact cells can be reproduced. Figure 6 shows the effects of progressive addition of membrane components on the wetting behaviour of synthetic and native erythrocyte surfaces. In 6A, the left-hand photograph shows a hydrocarbon test droplet resting on top of a fluorocarbon drop (diameter 1 cm); the hydrocarbon has spread to a contact angle of about 5°, since in so doing the high energy (50 mJ m^{-2}) fluorocarbon/water interface is replaced by the lower energy hydrocarbon/water interface (in this case, 22.5 mJ m^{-2}). In the adjacent photograph a phospholipid



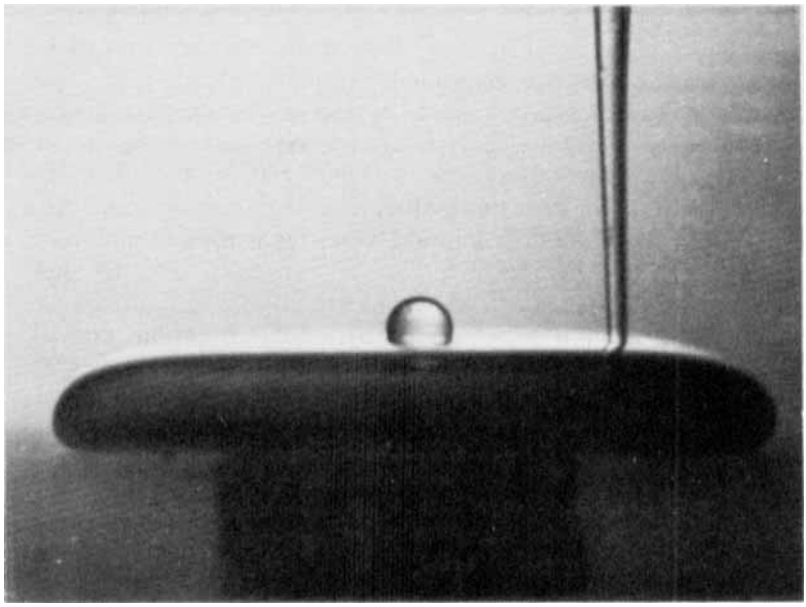


FIGURE 5 Simultaneous equilibrium contact angle measurement and shape analysis at fluid interfaces. A Schematic diagram of the mini-surface balance with a dense "pseudo-solid" (fluorocarbon) subphase. B Hydrocarbon droplet contact angle (the test drop is a 1:1 mixture of dibutyl/dioctyl phthalate, density 1.02 g/ml, interfacial free energy 22.5 mJ m^{-2}) and interface profile of a dipalmitoyl phosphatidylcholine monolayer at a fluorocarbon oil-water interface. The contact angle is 105° and the monolayer-water interfacial free energy is 3.6 mJ m^{-2}. Reproduced from Ref. 42.

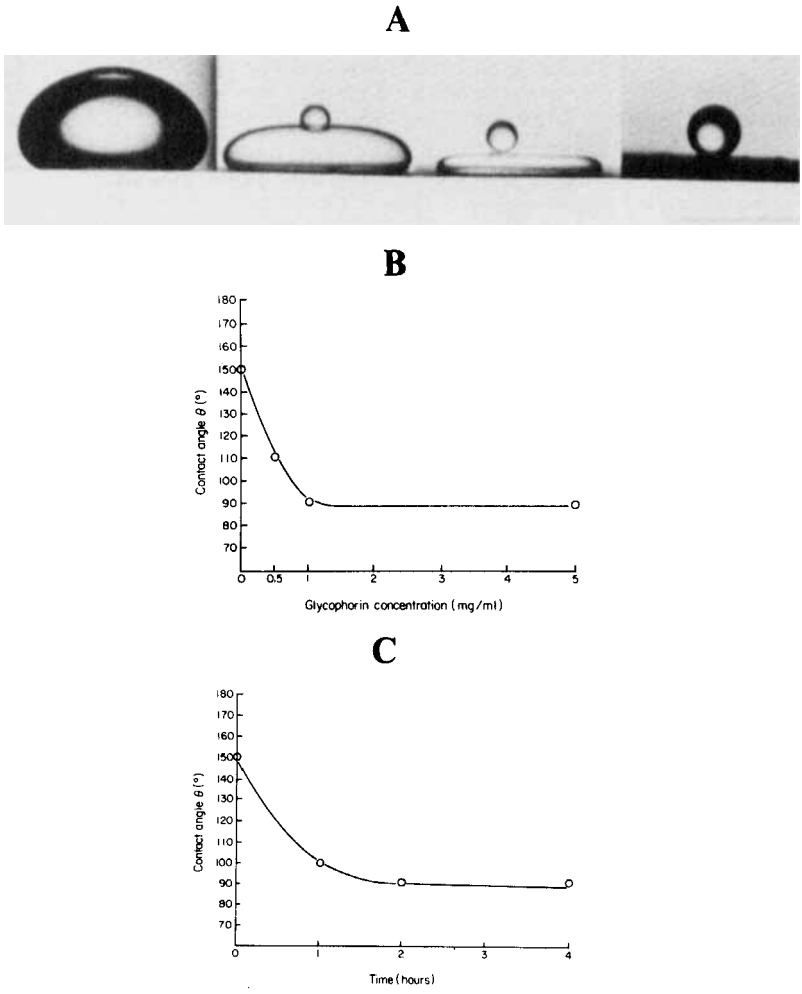


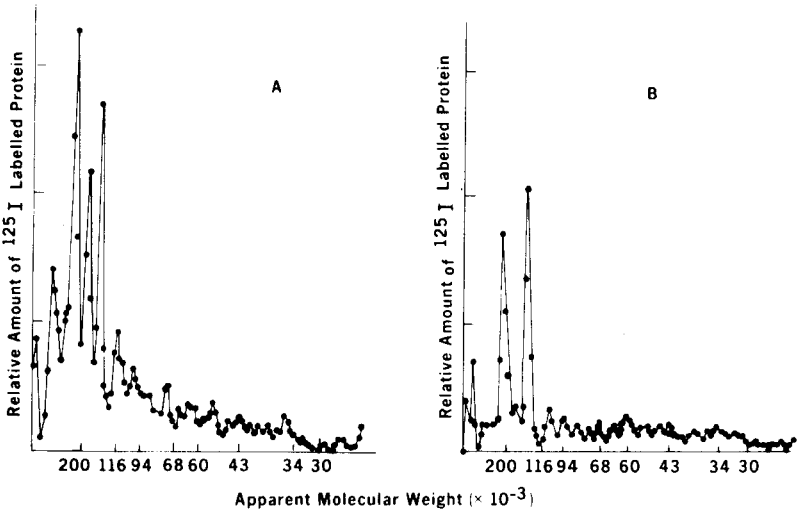
FIGURE 6 Interfacial free energies of native and model (reconstituted) cell surfaces. A Surface wetting by oil droplets (1:1 dibutyl phthalate/dioctyl phthalate). a: fluorocarbon/water interface, $\theta = 5°$; b: phospholipid/water interface, $\theta = 90°$; c: phospholipid/glycophorin/water interface, $\theta = 175°$; d: intact erythrocytes, $\theta = 175°$. B Effect of bulk phase glycophorin concentration on dextran/polyethylene glycol contact angles at the oil/phospholipid/water interface. 4%/4% phase system. C Kinetics of glycophorin effects on dextran/polyethylene glycol contact angle at the oil/phospholipid/water interface. 4%/4% phase system. Reproduced from Ref. 49.

monolayer (dipalmitoyl phosphatidylcholine) has been spread at the fluorocarbon/water interface: the equilibrium balance between interfacial free energy and the gravitational force arising from the density difference between the FC40 and aqueous phases now occurs with substantial deformation and flattening of the FC40 drop, and an increase in the contact angle to 90°, at a monolayer-water interfacial free energy of 5 mJ m^{-2}. Next, addition of the major erythrocyte membrane glycoprotein glycophorin to the bulk phase results in the same wetting behaviour as observed in intact erythrocytes (right-hand photograph): contact angles of 175° are observed in both cases. When polymer phase systems are used instead of oil droplets, the glycophorin addition is associated with similar wetting behaviour to that observed in intact cells, except that the wetting changes are in the opposite direction: glycophorin causes the dexaran contact angles to decline. This decline shows saturation with respect to bulk phase protein concentration (6B) and constancy with respect to time (6C). Of course, the correlations between the wetting behaviour of these model membranes and real cells are only semi-quantitative, since the monolayer glycophorin concentration is not known in these experiments. However, the experiments do suggest that the contact angles are indeed a function of the cell surface protein concentration. This conclusion was corroborated by experiments in which the amount of radiolabelled protein adsorbed at the interface was directly correlated with the reduction in dextran contact angle.[55,56]

In complementary experiments, the protein composition of cell membranes was varied *in vivo* by manipulating the growth conditions of cells in culture, and the measured contact angles compared with the measured glycoprotein composition of the isolated membranes.[57] V79 fibroblasts were exposed to varying oxygen concentrations, a procedure known to induce long-lasting

FIGURE 7 Environmental effects on membrane composition and surface thermodynamics of cultured V 79 fibroblasts. A Radiodensitometric profile of membrane glycoproteins from normal aerobic (A) and hypoxic (B) fibroblasts. I^{125}-concanavalin A was used to label the membrane glycoproteins separated by polyacrylamide gel electrophoresis. B Comparison of contact angles formed by dextran droplets from a 4%/4% phase system on layers of V79 fibroblasts grown under aerobic (a) and hypoxic (b) culture conditions. Reproduced from Ref. 57.

A



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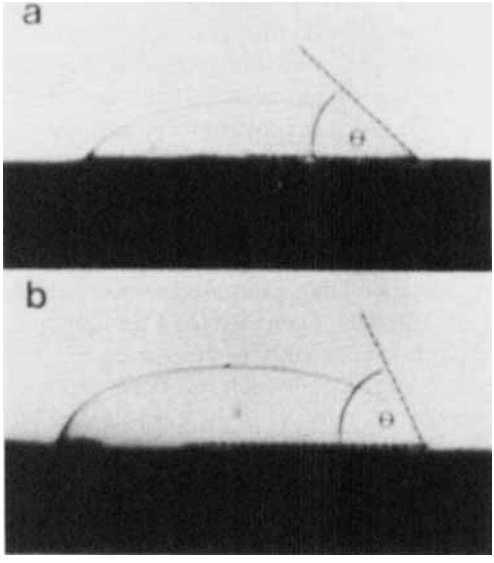


changes in membrane protein composition and in substrate adhesion. The isolated cell membrane proteins were separated by gel electrophoresis, and labelled with a radioactive ligand, the mannose binding lectin concanavalin A. The densitometric profiles of the autoradiograms of the isolated proteins are shown in Figure 7A: hypoxia induces a reduction in both amount and molecular weight of the membrane proteins. As expected on the basis of the discussion in section 3 above, these changes are associated with increased dextran contact angles (B). Since both concentration and molecular weight changed, these experiments cannot clearly assign the contact angle changes to either factor and more quantitative work in this area is needed. About the relationship of surface wetting to the third factor—macromolecule conformation—we can as yet say nothing, except to propose that polymer phase wetting studies like these reported here may turn out to be a useful probe of the configuration of macromolecules at surfaces.

6 STIMULUS-RESPONSE COUPLING AND CELL SURFACE WETTING

In the experiments just described, altering the cell culture conditions was associated with morphological evidence of reduced cell-substrate adhesion,[57] and the increased dextran contact angles observed on the less-adherent cells support the hypothesis that adhesion and cell surface/polymer phase thermodynamics are somehow related. However, the hypoxia experiments represent rather drastic changes in cell behaviour and are not a very sensitive test of the hypothesis. We have, therefore, examined several cellular responses which are related to more physiological adhesive events *in vitro*, and we have examined in some detail the time- and dose-relationships between cell surface thermodynamic changes and the stimuli which elicit the cellular responses.

One cell adhesion process which has been studied in considerable depth by a number of workers is the response of blood neutrophils to the chemotactic peptide N-formyl methionyl leucyl phenylalanine (fMLP). This low molecular weight ligand elicits several behavioural responses from the neutrophil, including both increased cell-substrate and cell-cell adherence (aggregation), and a variety of

biochemical responses including the production of oxygen-free-radicals.[58] Figure 8A shows the dose-response relationships for three photometric indices of these responses: perpendicular light scattering, light transmission and oxygen-radical-dependent chemiluminescence.[59,72] The light scattering changes predominantly reflect polarisation of the cells, the light transmission changes are

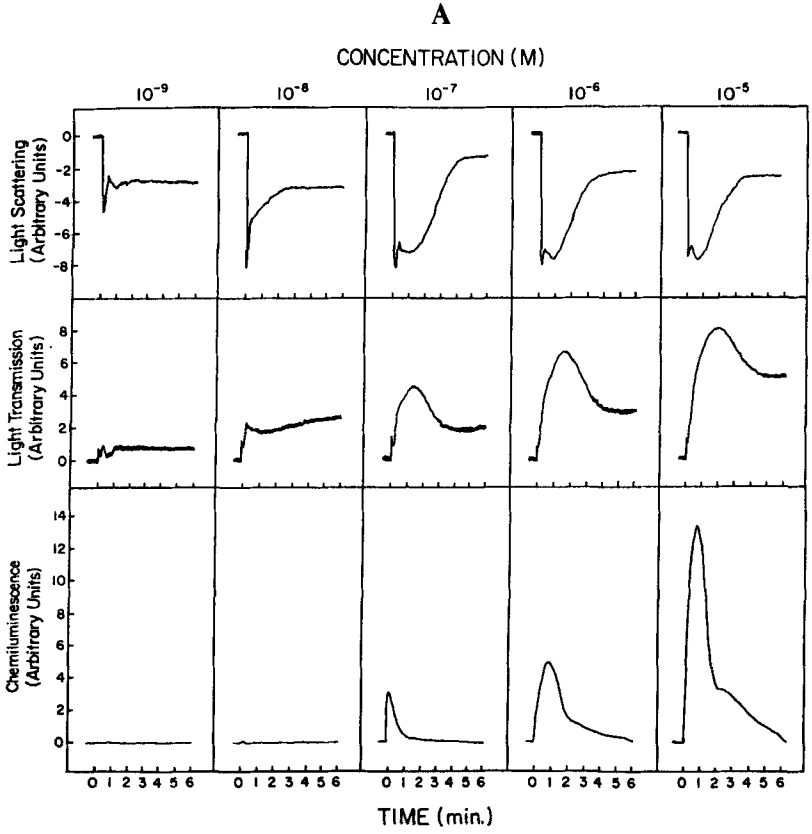


FIGURE 8 Effects of the soluble chemoattractant tripeptide N-formyl methionyl leucyl phenylalanine (fMLP) on human blood neutrophil behaviour and surface thermodynamics in polymer phases.[59] A Dose-response curves for photometric measurements of the responses of human neutrophil suspensions to fMLP. B Dose-response relations of neutrophil contact angle changes associated with exposure to fMLP. Left: 10^{-6} M ($\theta = 60°$); centre 10^{-7} M ($\theta = 73°$); right: control ($\theta = 95°$).

B

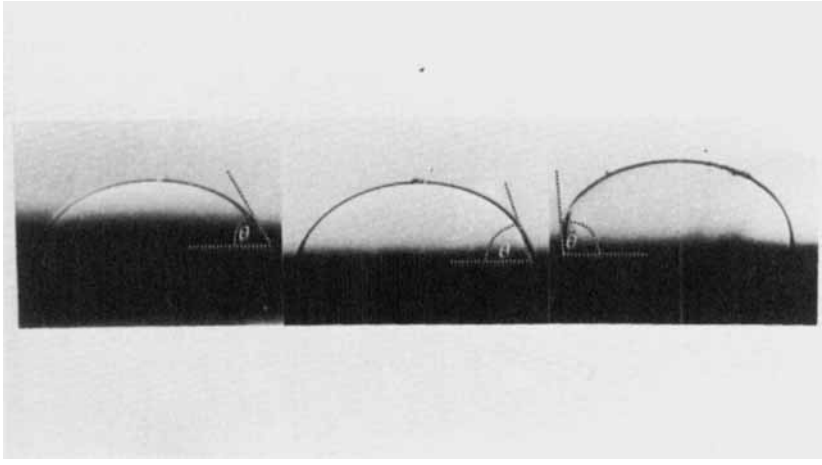


FIGURE 8 (*continued*)

associated with cell adhesion (aggregation), and the chemiluminescence reflects the biochemical activation of the cells. All of these responses show a dose-dependence on the fMLP concentration. So do the changes in the cell/dextran phase contact angles illustrated in Figure 8(B), which show a 50% increase in the work of adhesion to the dextran phase between the control and maximally fMLP-stimulated cells, *i.e.*, a ΔW_{adh} of 2.0 μJ m^{-2}.

Macrophages are another type of cell which show dose-response relations for stimulus-induced adhesion in polymer phases. When macrophages are exposed to particulate stimuli (Figure 9) they show dose relations between measurements of biochemical activation (the production of oxygen radicals and the secretion of lysosomal enzymes) and changes in the adhesion of the cells to the polymer phases.[60] Figure 10A is a comparison of the potency of a number of stimuli in eliciting changes in macrophage surface thermodynamics: serum-treated yeast particles (opsonized zymosan) are the most potent stimulators of changes in adhesion to the polymer phases, followed by the protein kinase C activator phorbol myristate acetate

(PMA) and the arachidonic acid metabolite leukotriene D (LTD_4).[61] As with the effects of fMLP on neutrophils, the contact angle dose-response curve for leukotriene D_4 (Figure 10B) indicates a sensitive and specific relationship between the surface thermodynamic measurements and cell stimulation by the ligand.[61]

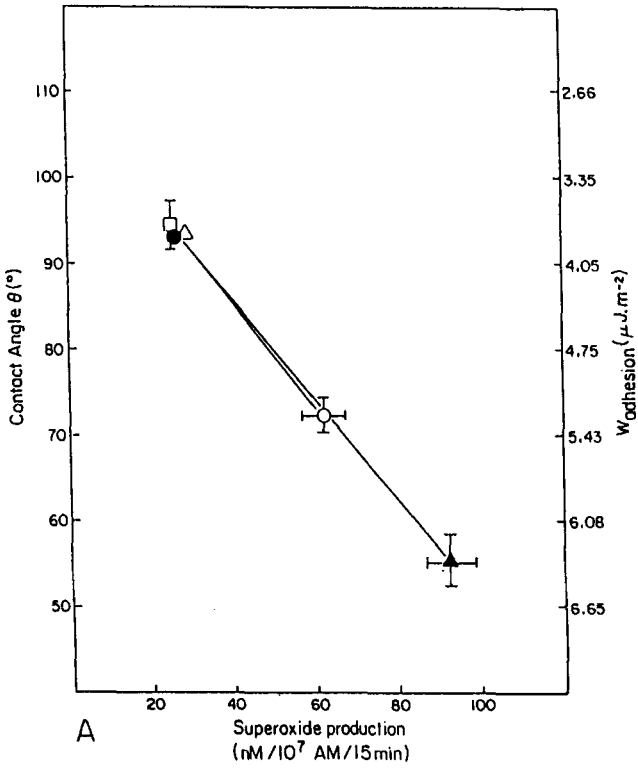


FIGURE 9 Effects of the particulate stimuli opsonized zymosan and non-opsonized silica on porcine alveolar macrophage surface thermodynamics and enzyme activity.[60] A Relation between contact angles, work of adhesion and superoxide anion production by macrophages exposed to zymosan (circles) or silica (triangles). Opsonized particles are indicated by filled symbols, non-opsonized particles by open symbols. Control cells are indicated by the square. 4%/4% phase system. B Relation between contact angles, work of adhesion and lysosomal enzyme release by alveolar macrophages exposed to zymosan or silica particles. Symbols as denoted in A. Dotted line indicates release of N-acetyl beta glucosaminidase, solid line release of arylsulfatase. 4%/4% phase system. Each point is the mean ± S.E. mean of 6 separate enzyme determinations or 30–40 separate contact angle measurements.

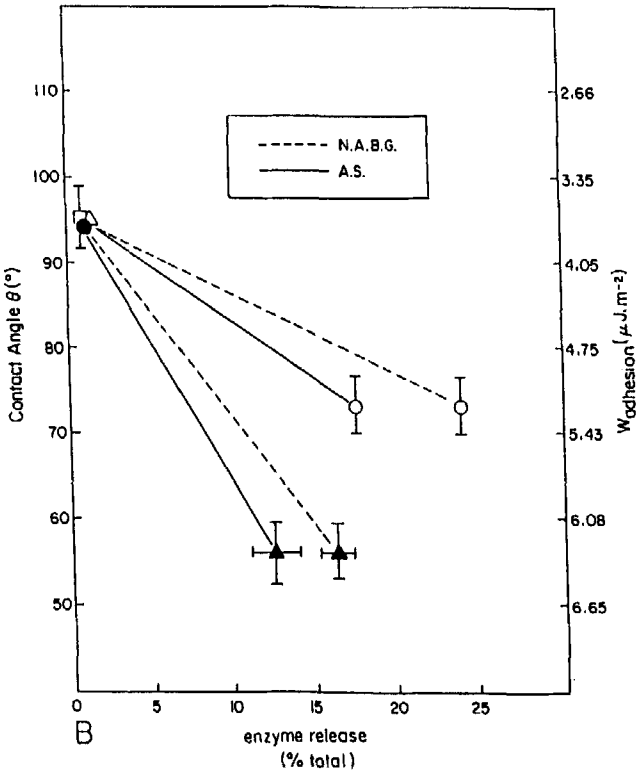
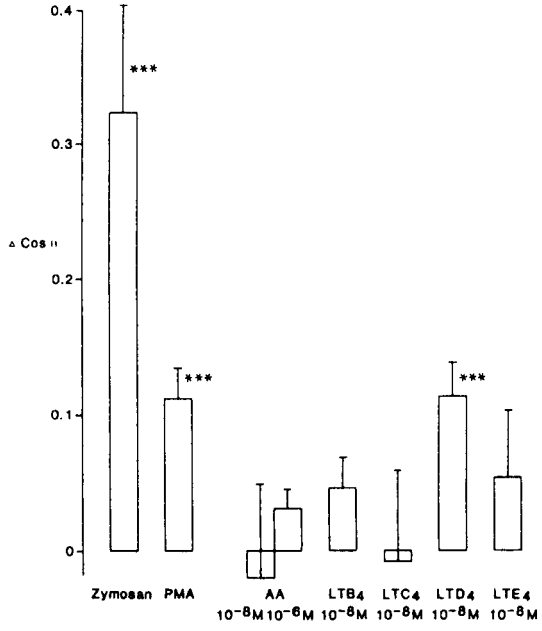


FIGURE 9 (*continued*)

FIGURE 10 Effects of particulate and soluble stimuli on surface thermodynamics of alveolar macrophages in the 4%/4% phase system.[61,62] A Comparison of the surface thermodynamic effects of the particulate stimulus opsonised zymosan with the effects of various soluble ligands. Differences in surface free energy are expressed as the change in the cosine of the contact angle between control and treated cells. Asterisks indicate differences in $\cos\theta$ which are significant at the $p < 0.001$ level. PMA, phorbol myristate acetate; AA, arachidonic acid; $LTB_4 - C_4 - D_4$ and $-E_4$, leukotrienes B to E.[61] B Dose-response curve for the effects of LTD_4 on alveolar macrophage surface free energy in the dose range 10^{-9} M to 10^{-7} M. Each point is the mean ± S.E. mean of 20–30 separate contact angle measurements in 6 different experiments.[62]



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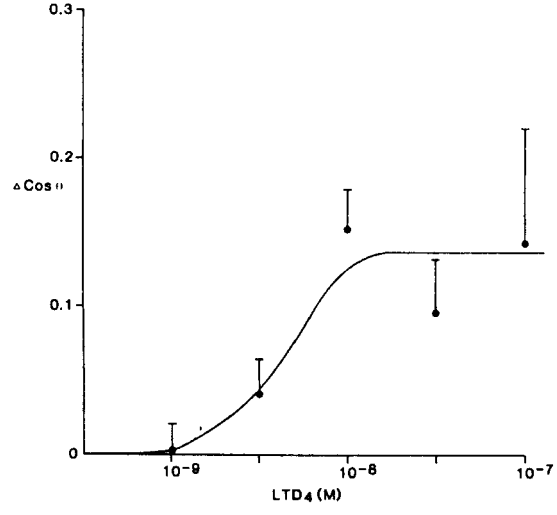
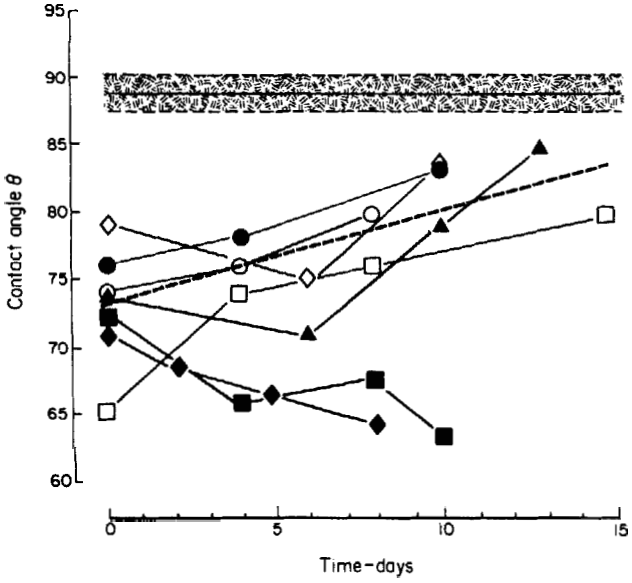


B

A



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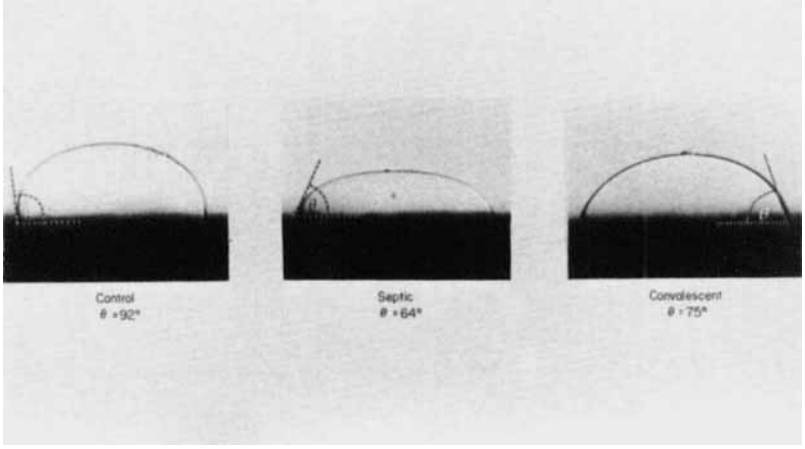

Finally, Figure 11 shows the results of a study of human neutrophil surface thermodynamics in a clinical disorder which is associated with massive intravascular neutrophil adhesion, the Adult Respiratory Distress Syndrone, or "A.R.D.S."[70,71] In Figure 11(A) the contact angles on isolated neutrophils from 7 patients with A.R.D.S. are compared with normal age- and sex-matched controls. In this small sample, two trends are apparent: first, there is a significant lowering of the neutrophil contact angles in all patients when compared with the control group and, second, there is a correlation with clinical course. The two patients who died showed continuously lowered contact angles, while the convalescing patients showed a gradual return towards control values. The contact angle changes shown in Figure 11(B) are reminiscent of the *in vitro* response of isolated neutrophils to fMLP, suggesting that the changes may be due to *in vivo* activation of the cells by chemoattractants.

These studies collectively point to a general association between stimulus-specific cell activation and increased cell adherence to the dextran phase of the polymer phase systems; these associations do not however, prove that there is necessarily any causal relationship between the changes in the cell surface which produce the contact changes, and the molecular events which actually mediate cell-cell, cell-particle or cell-substrate interactions. That is to say, it may not be the adhesive events *per se* which produce the contact angle changes, but some other, rather ubiquitous process associated with cell surface activation. In the absence of information about the molecules actually responsible for mediating any particular adhesive events, this conjecture cannot be directly tested. The only proof of a causal relationship between cell adhesion and the surface thermo-

FIGURE 11 Neutrophil surface thermodynamic changes in a clinical disorder of neutrophil adhesion, the Adult Respiratory Distress Syndrome (A.R.D.S.).[71] A Contact angles and clinical courses of 7 patients with A.R.D.S. and 4 normal controls (hatched area). The standard errors of the mean (not shown) are around 3%. The two downward sloping curves (black diamonds and squares) were for non-survivors; the remainder of the patients survived. Each point is the mean of 30–40 separate contact angle measurements. B Contact angles on neutrophils isolated from a patient with A.R.D.S. associated with systemic sepsis (the patient represented by the open squares in A.), compared with those from a healthy age- and sex-matched control subject.

dynamic measurements would be the demonstration that the isolated molecules which actually do mediate the ligand-induced adhesive interaction(s) described above could reproduce the wetting changes in recombination experiments such as those described in section 5. In the meantime, we propose a working hypothesis which we believe is consistent with the data so far, and which suggests a somewhat different way of looking at the thermodynamics of ligand-specific cell adhesion from the sequence of events described by model 1.

7 POLYMER MIXING AND CELL ADHESION

The hypothesis is that the binding of structurally specific, adhesion promoting stimuli (such as fMLP) to cell surface receptors induces changes in cell surface molecules which are responsible for adhesion of the cells both to polymer phases and to other cells or substrates.

The experimental results discussed above indicate that specific ligand-induced cell adhesion to foreign particulate surfaces (phagocytosis) or to other cells (aggregation), and such biochemical evidence of cell activation as enzyme activity or secretion can all be correlated in a ligand dose-dependent fashion with changes in the surface affinity of the cells for polymer phase solutions. In general, when cells become stickier to substrates or to each other they also become stickier to the dextran phase of the dextran/PEG phase system. Some evidence also suggests the opposite behaviour: inhibition of adhesion is associated with an increased affinity for the PEG phase.[57,70] These general trends are similar to the surface tension studies reported by Neumann and co-workers[19] for a variety of cell-particle and cell-substrate adhesive processes, where lower surface tension (more hydrophobic) cells are generally less adhesive than higher surface tension (more hydrophilic) cells.

None of these correlations proves that any averaged thermodynamic properties of the cell surface are causally related to any of the adhesive events occurring in these experiments. The correlations could be merely with some effect of cell activation on cell surface chemistry which happens to occur under the same conditions as adhesion; such coincidences, while they might be interesting in their own right, would not be expected to contribute much insight into the mechanisms of adhesion.

On the other hand, it is possible that the correlations actually are causally related to the adhesion occurring in these experiments, and that the changes in cell/polymer phase affinity do reflect the intermolecular forces which drive cell adhesion under these conditions. If this is the case, then it is necessary to modify the "specific bonding, non-specific repulsion" model for cell adhesion elicited by small ligands.

We suggest that adhesion elicited by structurally specific low molecular weight, non-cross linking ligands may be better described by a "specific induction, non-specific execution" model, *i.e.*, model 2. In circumstances where large bridging molecules such as lectins or multivalent antibodies provide both recognition and adhesion functions, model 1 serves well. But where the recognition and adhesion are separate molecular processes, and particularly in those kinds of rapid, reversible adhesion events such as the ones studied here (typically the kind of adhesion encountered in the blood and in host defence mechanisms), model 2 suggests that it may be useful to analyse cell adhesion as a problem in polymer phase mixing.

The simplest form of such a model is based on a proposal like that of Steinberg,[35] that cell adhesion occurs when the free energy of the adherent state is lower than that of the non-adherent state. An equilibrium approach to cell adhesion requires that the adhesion is measured on a time scale that is slower than biochemical transients such as those shown in Figure 8. In other words, that the cell surface is considered constant with respect to irreversible metabolic processes which may move the cell from one metastable state to another.[22] The approach describes simply what the adhesion molecules prefer to do and where they prefer to go after they have been synthesised, and before they have been degraded. Such an approach has definite limitations: it will not, for example, account for processes such as cell "grip" which depend on the expenditure of contractile energy.[63] However, to the extent that the model will not include irreversible sources of cell adhesive behaviour, it may in fact allow an experimental separation of those processes which depend on pure adhesion ("stick") and those which depend on "grip." That is to say, the failures of the model may be as useful as its successes.

Consider two cell types, a and b: equilibrium adhesion between the two cells can only take place if the free energy of adhesion,

(W_{a-b}) is negative

$$i.e.,\ W_{a-b} < 0 \tag{4}$$

the condition

$$W_{a-b} = 0 \tag{5}$$

corresponds to a non-adherent dispersion of the cells; the condition

$$W_{a-b} > 0 \tag{6}$$

(*i.e.*, a positive free energy of adhesion) corresponds to de-mixing or repulsion of the cells.

For the three types of adhesion $a-a$ (homotypic aggregates of a), $b-b$ (homotypic aggregates of b) and $a-b$ (heterotypic aggregates of a and b), and the three conditions $W<0$ and $W>0$, ten different combinations can be distinguished.[34] In the blood, for example, Eq. (4) could correspond to the margination of leukocytes, Eq. (5) to the free mixing of non-adhering cells in the plasma and Eq. (6) to the repulsion of the normal endothelium for blood components.

Let us now take one of the simplest possible models of a cell—a non-deformable particle coated with a completely deformable (liquid-like) polymer solution. The free energies of adhesion in Eq. [4–6] may be replaced with a polymer interaction parameter, such as the Flory–Huggins χ.[53] Equivalent treatments (*e.g.*, Refs 64, 65) attribute positive (repulsion) or negative (attraction) interaction energies to an effective polymer "excluded volume." In terms of surface thermodynamics in polymer phases, adhesion ($W<0$) corresponds to phase mixing (minimum interfacial free energy or a contact angle of 0° as in Figure 4B), while repulsion (maximum interfacial free energy or a contact angle of 180°) corresponds to phase separation. To the extent that cells are actually deformed on contact, deformation energies will also enter into the balance of forces between polymer affinity and the work of cell deformation.[17,31]

If this cell surface phase mixing and demixing behaviour does in fact mediate the kind of cell adhesion events described by model 2, the contact angle changes are clearly stating that the adhesion molecules must be quite widely distributed on the cell surface. The equivalent solution of an activated neutrophil is a 3·2% phase of

dextran 2×10^6. Assuming, for example, that the adhesion molecules have an average molecular weight of 200 k daltons (*e.g.*, see Figure 7A), then the surface concentration of the adhesion molecules could well be in the 0.3% or micromolar range. This is many orders of magnitude higher than the concentrations at which most ligands activate their specific receptors in cell membranes;[64] it may be noteworthy however that the fibrinogen binding site in blood platelets (the so-called glycoprotein IIB–III complex) actually constitutes 18% of the platelet membrane protein.[67]

Model 1 describes the situation where the specificity of both recognition and adhesion lies in the agglutinating molecules. By contrast, model 2 describes cell adhesion as a problem in polymer phase mixing, and attributes adhesion (either positive or negative) to stimulus-induced changes in the concentration, molcular weight or conformation of glycocalyx polymers, and consequent changes in their free energies of mixing with polymers on the surfaces of other cells or substrates.

Supported by Grants from the Medical Research Council of Canada (D.M., S.S., E.T. and N.P.) and the Heart and Stroke Foundation of Ontario (D.M.).

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