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Interfacial instability and the agglutination of erythrocytes by polylysine

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Abstract. Human erythrocytes have been exposed to poylysine of molecular weight range 4 to 220 kDa and concentration range 0.5 to 2,000 μg/ml at 37 °C. Threshold concentrations for cell agglutination by the polycation have been determined for the samples of different molecular weight. Light and electron micrographs show that, in the erythrocyte agglutinates, cell-cell contact is generally made only at discrete, spatially periodic, regions which are distributed over a significant part of the cell surface. The average spacing between contact regions is 0.83 μm. The cell membrane has a wavy profile between contact regions. Agglutination occurs only in cell samples whose electrophoretic mobility is significantly altered by polylysine and, in agreement with a previous report, occurs even when the electrophoretic mobility reaches high positive values. The electrophoretic mobility data implies that agglutination requires some protrusion of polylysine from the cell glycocalyx. We discuss how a resulting net attractive intercellular force could act to destabilize the aqueous layer between two cells, allowing surface wave growth which results in spatially periodic contact regions. Examples of situations where cell and membrane contact might be explained by the general concept of interfacial instability are discussed.

Key words: Erythrocyte, interfacial instability, surface wave, polylysine, cell agglutination, cell adhesion, acrosome reaction, membrane fusion

Introduction

Many of the features of the agglutination of human erythrocytes by polycations were established in experiments carried out about thirty years ago (Nevo et al. 1955; Katchalsky et al. 1959). It was concluded that the common property causing agglutination was the high charge density of the polycations. Erythrocyte agglutination continued to occur under conditions where the polycations had changed the cells' electrophoretic mobility to positive values. The polycation-erythrocyte membrane interaction was reversible; however agglutinates formed by polycations were not dispersed by washing the cells. Katchalsky et al. (1959) and Danon (1963) studied the cell-cell contact of agglutinated cells by electron microscopy. Their micrographs showed regions of close cell-cell contact interrupted by areas where there were spaces between the cells. The authors did not comment on any spatial periodicity of the contact interruption regions.

Following our recent suggestion (Coakley et al. 1984) that erythrocyte agglutination by polylysine at 37 °C results from an interfacial instability (Prevost and Gallez 1984; Wendel and Bisch 1984) we present the evidence for the occurrence of regularly spaced cell-cell contact regions in cell agglutinates. In contrast to our previous experimental studies of surface wave growth on erythrocytes as they were heated through the denaturation temperature (49.5 °C) of the cytoskeleton protein, spectrin (Coakley et al. 1978; Coakley and Deeley 1980; Deeley and Coakley 1983; Doulah et al. 1984), the wavy profiles were observed in the present study in cells at 37 °C, where the cytoskeleton was intact.

The discussion draws on the increase in the understanding of the structure and charge distribution of the erythrocyte glycocalyx (Levine et al. 1983) and the interpretation of electrophoretic mobility data (Donath and Pastushenko 1980) which has occurred since the early agglutination studies (Katchalsky et al. 1959) were carried out.

Materials and methods

a. Preparation of cell suspensions

Human blood was collected by finger prick into 145 m*M* NaCl, 5 m*M* N-2-hydroxyethylpiperazine-

N'-2 ethanesulphonic acid (HEPES) (Sigma Ltd.). The pH was adjusted to 7.32 by dropwise addition of 1% NaOH. The cells were usually washed three times by centrifugation at 3,000 *g* for 3 min and were finally resuspended in buffered saline (control samples) or in poly-L-lysine (Sigma Ltd.) solutions. The final cell concentration was $7 \cdot 10^6$ cells/ml for agglutination studies and $3 \cdot 10^6$ cells/ml for electrophoretic measurements. Blood from some donors became echinocytic on the second wash. In sample preparation blood from these donors was washed once because echinocytic cells showed little aggregation.

b. Microscopy of cells which had been exposed to polylysine

1.0 ml samples of cell suspension were incubated in polylysine solutions, with occasional shaking, for 2 min at 37 °C.

(i) Unfixed cells. Erythrocytes exposed to high molecular weight polylysine tended to flatten on the substratum on coming into contact with glass. It is known from electrophoretic mobility studies (Nevo et al. 1955) that the polylysine interacting with the cell glycocalyx is in equilibrium with the polylysine in solution. We found that cells exposed to the lower concentrations of polylysine which were capable of agglutinating cells remained agglutinated but did not flatten on coming into contact with glass following a wash in buffered saline. The washed cells were loaded into a 100 µm pathlength microslide (Camlab. Ltd.) which had been cleaned as described by Deeley and Coakley (1983) and were examined by light microscopy using a Nachet NS 400 Universal microscope, usually in the differential interference contrast mode.

(ii) Fixed cells. Polylysine treated cells were fixed by addition of 1.0 ml of 8% glutaraldehyde (GA) in buffered saline. After 15 min fixation the cells were examined by light microscopy, as above, or were prepared for electron microscopy. Cells fixed in 0.8% GA gave identical results to those fixed in 8% GA. Some samples were fixed with 1% OsO_4, the fixative employed by Katchalsky et al. (1959), rather than with GA.

For scanning electron microscopy fixed cells were dehydrated in a graded series (50, 70, 90, 100%) of ethanol, transferred to a polycarbonate millipore filter and critical point dried for 30 min. The cells were mounted on a specimen stub, gold coated and examined in a Philips 400 S(T)EM attachment using a secondary electron detector.

For transmission electron microscopy dehydrated fixed cells were embedded in a 1:1 mixture of alcohol-L.R. white resin for 4 h at room temperature and then in pure LR white resin. The embedded cells were cured in a capsule for 24 h at 60 °C. 60 nm thick sections were cut using an Ultracut microtome (Reichert UMH). The sections were mounted on a copper grid coated with colloidin and carbon and double stained with 5% uranyl acetate in 50% alcohol for 30 min (Reynolds 1963). The sections were examined in a Philips 400 transmission electron microscope.

c. Cell electrophoresis

Erythrocyte electrophoretic mobility was measured in a glass cell of rectangular cross-section at 37 °C in a modified (Sutherland and Pritchard 1979) Mark II cytophorimeter (Rank Bros., Botisham, England) as previously described (Coakley and Deeley 1980). An average electrophoretic mobility (Jones 1975) was calculated from measurements on 20 cells.

Following measurements on each polylysine treated sample the system was rinsed thoroughly with 1 *M* NaOH, distilled water and a sample of the next cell suspension to be examined (Nevo et al. 1955).

Results

Figure 1 shows differential interference contrast light micrographs of erythrocytes which have been exposed to 20 µg/ml polylysine of 14,000 molecular weight. There are many small beadlike shapes within the aggregates of cells. Single cells which are not part of aggregates are similar in appearance to control cells i.e. they have not undergone any of the common erythrocyte shape changes (e.g. stomatocyte or echinocyte) which occur in the presence of surface active agents (Fujii et al. 1979) or when extracellular pH and monovalent ion concentration are both changed (Glaser 1982). Arrays of beads as in Fig. 1 were also seen when 5 m*M* phosphate replaced HEPES as the buffer in the cell suspension and fixative solutions, showing that the buffer did not influence the pattern formation. The arrays of beadlike shapes as in Fig. 1 were also clearly detectable in bright field and in phase contrast microscopy. Unless stated otherwise the micrographs presented here show cells which were exposed to 20 µg/ml of 14 kDa polylysine.

In Fig. 2a the shallow depth of focus of the ×100 oil immersion objective facilitates the detection of a mosaic pattern within a small aggregate of erythrocytes. Figure 2b shows that a regular spatial pattern arises only at the region where both cells overlap. Figure 2c shows beading patterns in cells which were fixed only in OSO_4, as described by Kat-

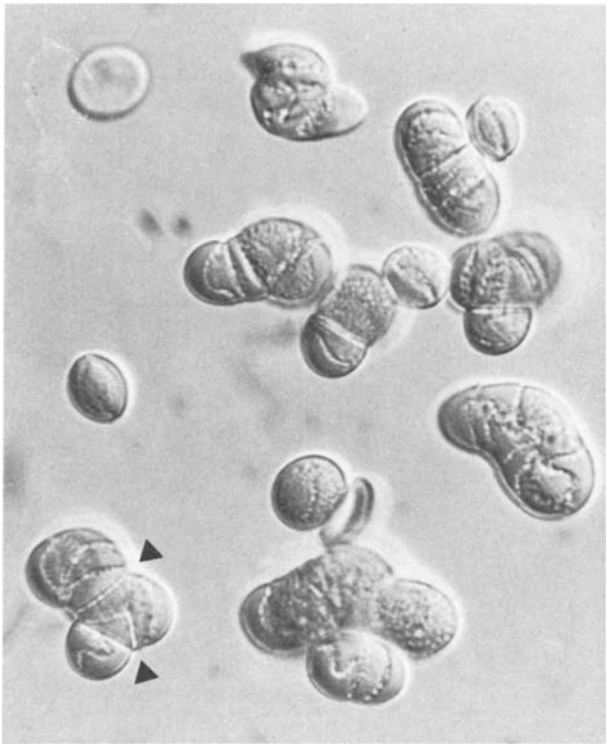

Fig. 1. Differential interference contrast micrograph (×40 objective) of cells which were agglutinated by exposure to 20 μg/ml polylysine of 14 kDa molecular weight for 2 min and were then fixed glutaraldehyde; some linear arrays of cell-cell contact regions are shown (arrowheads)

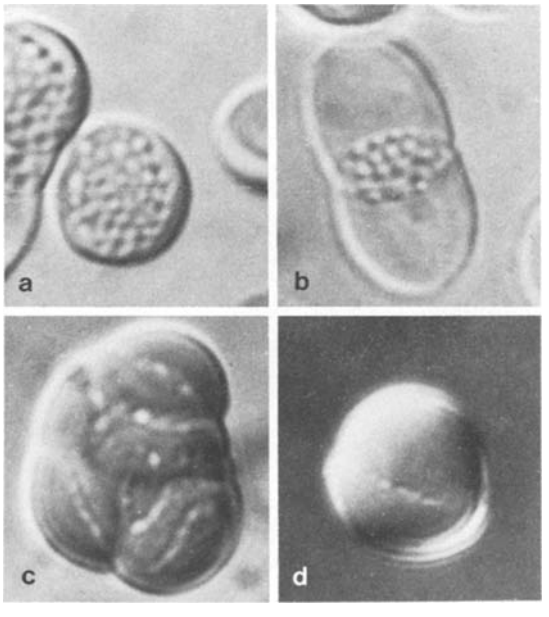

Fig. 2 a – d. Objective oil immersion light micrographs of cells which had been exposed to 14 kDa polylysine; (**a**) a small clump of cells fixed with glutaraldehyde; (**b**) two glutaraldehyde fixed cells showing agglutination over a small region of cell overlap; (**c**) agglutinated cells fixed with OsO_4; (**d**) unfixed cells washed once after exposure. Polylysine concentration (**a – c**) 20 μg/ml; (**d**) 10 μg/ml. ×100

chalsky et al. (1959). When cells which had been exposed to 20 μg/ml polylysine were loaded, without fixing, into microslides the cell clumps flattened onto the glass. Beaded patterns could be discerned on regions of the larger cell clumps which were not in direct contact with glass. When cells were exposed to 10 μg/ml polylysine and washed once before loading into the microslide interaction of cells with glass was much reduced. Figure 2d shows a linear array of beads at a contact region in such unfixed, washed cells. Figure 2d and other similar observations show that a regularity of contact points is not an experimental artifact arising from cell fixation in the presence of polylysine.

Transmission electron micrographs (*a*) of control cells and (*b*) of a general field of polylysine treated cells fixed with GA, are shown in Fig. 3. Regular arrays of contact regions can be seen in the cell aggregates. Some higher magnification examples of cell-cell contact are shown in Fig. 4. Sections through the aggregates show regular wave-like profiles between adjacent cells. The arrowed cell in Fig. 4a has a wavy profile as it interacts with four adjacent cells. A further example of regular contact points is shown in Fig. 4b. In Fig. 4c the elliptical pattern of intercellular spaces surrounds two circles which are more lightly stained than the background. We interpret these circles as being the parts of intercellular beads which fall within a section across a cupped contact region. The ellipse, together with the small circles, provide an impression of how regularly waved cell profiles develop over the three dimensional contact region between two cells.

Aggregates of polylysine treated cells are shown in the scanning electron micrographs of Fig. 5. The aggregates often take the form of linear stacks of cells fitting into the saucer like depression in the two extreme cells (Fig. 5b). High magnification details of aggregated cells are shown in Fig. 5c, d. Figure 6 shows the rare occurrence of an agglutinate containing two cells with pitted regions. Because the pits are restricted to a local circular region rather than covering the whole cell surface we suggest that they have become exposed because of the breakdown of an agglutinate on handling during, or after, fixation.

The average separation of intercellular spaces was measured during light microscopy of cells fixed after exposure to polylysine of molecular weight 4 kDa (1 & 2 mg/ml), 14 kDa (0.02 & 1.0 mg/ml), 60 kDa (4 & 40 μg/ml) and 220 kDa (2 & 20 μg/ml). Measurements of the spacing interval were made with a calibrated micrometer eyepiece and a ×100 oil immersion objective. In order to correct for any contraction of membrane due to fixation the diameters of some unagglutinated cells in each field were

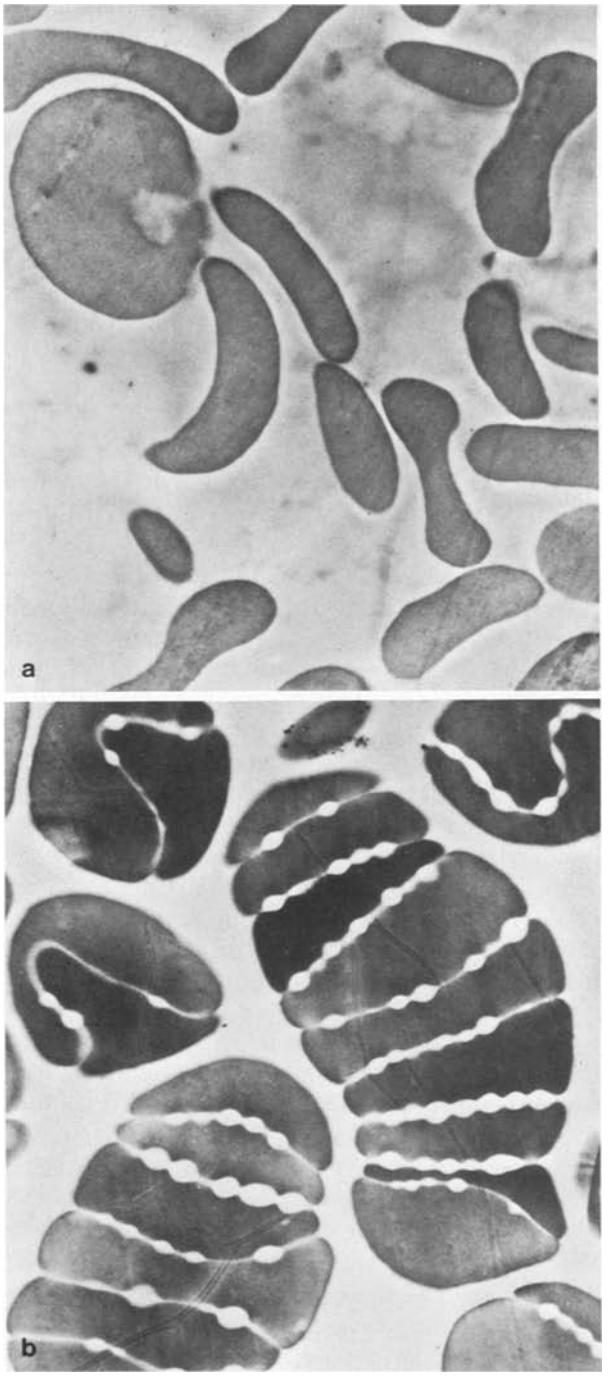

Fig. 3 a and b. Transmission electron micrographs of (**a**) control cells (**b**) polylysine agglutinated cells fixed with glutaraldehyde. Final print magnification (**a**) ×42,000, (**b**) ×45,000

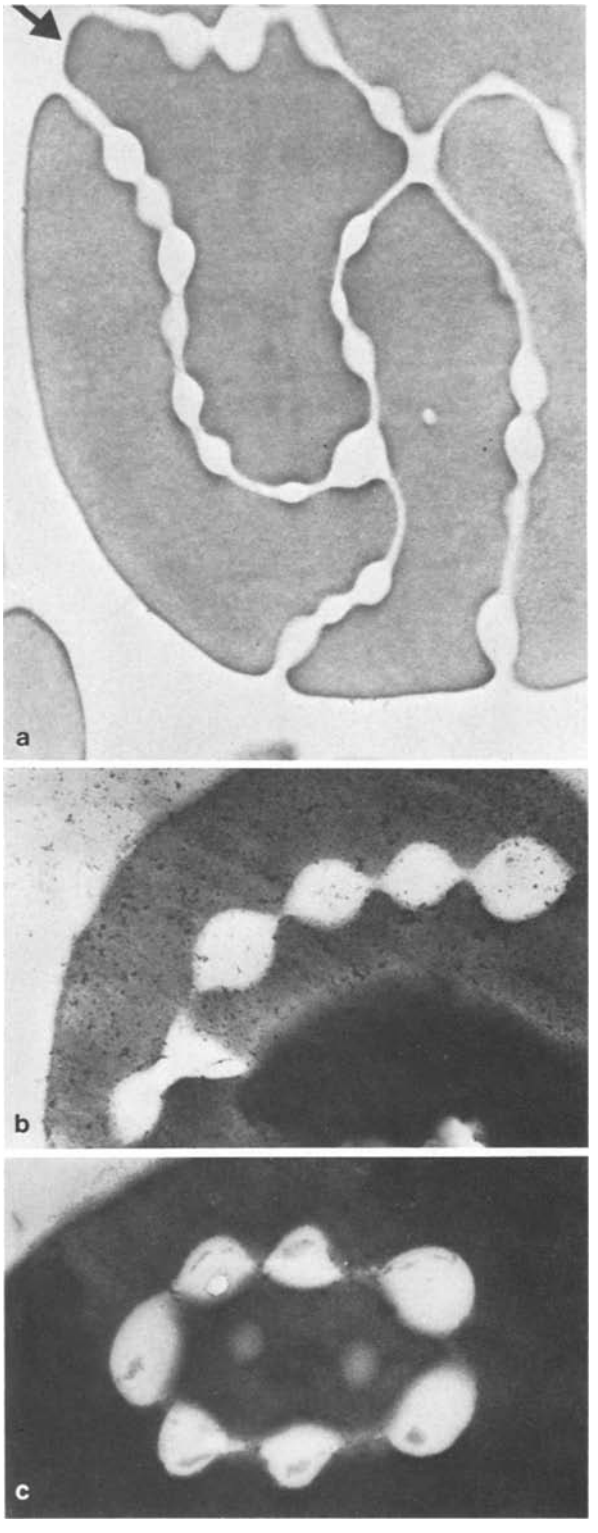

Fig. 4 a – c. Transmission electron micrographs of details of GA fixed cell agglutinates. The arrowed cell in (**a**) shows a wavy profile where it interacts with four adjacent cells. Final print magnification (**a**) ×12,600; (**b**) ×16,000; (**c**) ×19,500

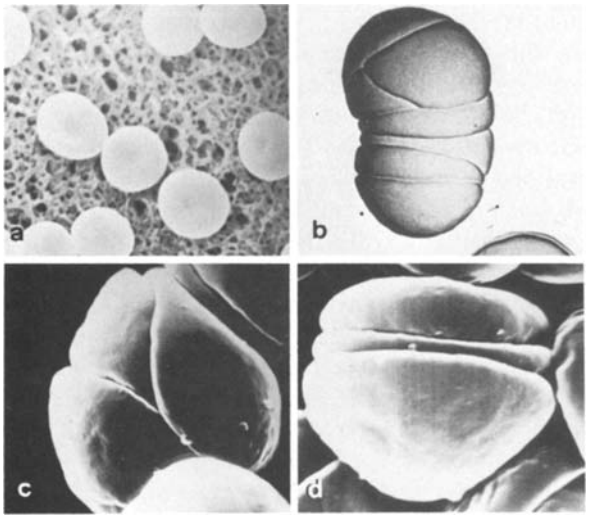

Fig. 5 a – d. Scanning electron micrographs of (**a**) control cells and (**b – d**) cell agglutinates. Final print magnification; (**a**) ×1,000; (**b**) ×2,400; (**c**) ×4,400; (**d**) ×4,800

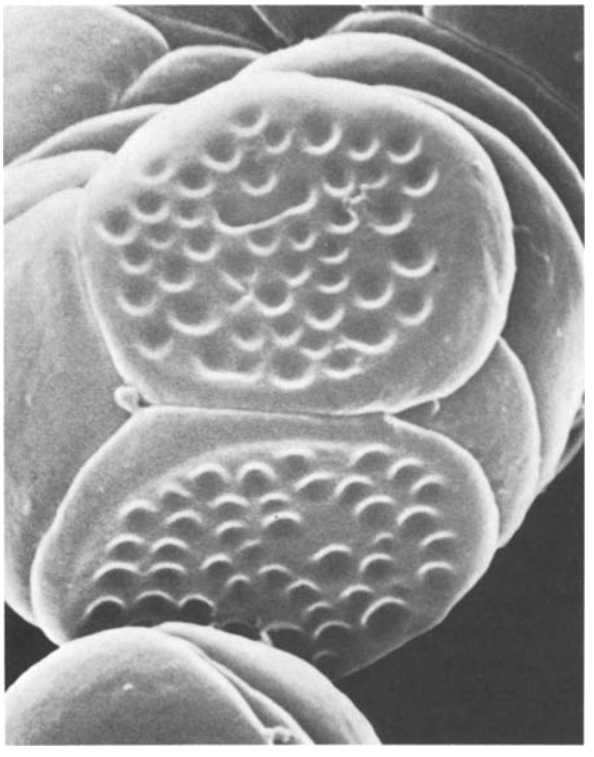

Fig. 6. Scanning electron micrograph of a cell clump showing two cells with a regular beading pattern over a discrete region of each cell (×7,000)

also measured. The spacing intervals were expressed in microns by assuming that the measured diameters of fixed cells represented the 7.65 μm diameter (Fung et al. 1981) of unfixed cells. The average separations for the different samples ranged from 0.7 to 1.0 μm. The mean spacing, with 95% confidence limits, was 0.83 ± 0.12 μm. There was no detectable pattern of dependence of spacing on molecular weight or polycation concentration. Because factors like difference in cell aggregate size, the limits of resolution of the microscope and the relative ease of measuring the linear arrays of contact regions (arrowed in Fig. 1) led to some selection of the spatial separations measured no further effort was made to refine the spacing measurements. We conclude that, providing agglutination occurred, any dependence of separation distance on the size and concentration of the polycation was small.

Table 1. Threshold concentrations, c, for the agglutination of erythrocytes ($7 \cdot 10^6$ cells/ml) by polylysine of different molecular weights, M. The threshold data for dextran (10^8 cells/ml) is taken from Jan and Chien (1973)

Polymer	M (kDa)	c (μg/ml)
Polylysine	4	1,000
Polylysine	14	8
Polylysine	60	0.25–0.5
Polylysine	210	0.25–0.5
Dextran	20	120,000
Dextran	42	30,000
Dextran	74	5,000

The critical polymer concentration for cell agglutination is shown, for the polylysines of different molecular weight, in Table 1. The reported concentrations (Jan and Chien 1973) required for erythrocyte agglutination by the neutral polymer dextran are also shown. These latter concentrations were about 10,000 times greater than the concentration of polylysine (of similar molecular weight) required to achieve agglutination. Transmission electron micrographs of dextran agglutinated cells (Jan and Chien 1973) show parallel cell-cell contact without any sign of the wavy profiles seen in Figs. 3 and 4. These features suggest that the pathways to agglutination by charged or uncharged polymers involve fundamentally different mechanisms.

The electrophoretic mobilities of cells exposed to different concentrations of polylysine are shown in Table 2.

Discussion

Very little agglutination and only a small decrease in electrophoretic mobility occurs when cells are exposed to 1,000 μg/ml of 4 kDa polylysine while marked aggregation and a significant decrease in electrophoretic mobility occurs on exposure to 20 μg/ml of 14 kDa polylysine (Table 2). In saline of

Table 2. The electrophoretic mobility, u (measured at 37 °C), of erythrocytes which agglutinate when exposed to concentrations, c, of polylysine of different molecular weights, M

M (kDa)	c (μg/ml)	u (nm/s/V/m)
0	0	− 1.3
4	1,000	− 1.1
14	20	+ 1.1
14	1,000	+ 2.1
60	4	+ 1.1
220	2	+ 1.6
220	4	+ 2.8

high ionic strength (e.g. 145 m*M*) charges at the outer surface of a glycocalyx contribute much more to the cell's electrophoretic mobility than do charges deeper in the glycocalyx (Donath and Pastushenko 1980). The observation in the present work that increased agglutination accompanies significant changes in electrophoretic mobility (Table 2) indicates that the smaller poylysine was buried deep in the glycocalyx while the larger molecules reached and probably protruded from the outer edge of the glycocalyx. In agreement with Katchalsky et al. (1959) strong agglutination was observed in the present work under conditions where the electrophoretic mobility reached positive values comparable in size to the negative values measured for control cells. Cationic polylysine molecules of high localized charge density protruding from opposite cells would tend to avoid each other and be attracted towards localised negatively charged areas of the opposite cell. A nonuniform (in magnitude and sign for distances of the order of polymer dimensions (nms)) distribution of charge over the surface of polylysine-treated cells could thus explain the observed net attractive force under conditions where the electrophoretic mobility is positive and high.

Fricke and Sackmann (1984) have attributed the rapid spontaneous fluctuations of the thickness of local regions of erythrocytes in suspension (cell flickering) to the development of overdamped surface waves on the erythrocyte. Brochard and Lennon (1975) showed that the root mean square amplitude of the thickness fluctuation was 80 nm and calculated a fundamental wavelength value of 0.59 μm at 10 Hz. Fricke and Sackman (1984) took 1 μm as the wavelength in their calculations to assess the contributions of different membrane properties to cell flicker. These wavelengths are similar to the average spatial separation distance of 0.83 μm measured in the present work.

In the following discussion, we will assume that distribution of polylysine *molecules* is uniform on the cell surface (over wavelength distances) and will argue that the observed periodic contact points arise from the growth of an interfacial instability. An interface is stable if, following a small disturbance, it returns to its initial form. Perturbations can originate outside the system as with various types of vibration, or inside, when random thermal motion leads to small fluctuations in interfacial composition or shape (Miller 1978). The deformation of a plane interface which arises from a small disturbance can be expressed as a Fourier integral so that it is sufficient, for stability analysis, to determine the response of the system to simple periodic sine disturbances. If all of the simple disturbances die out so will the general perturbation and the initial state of the system is stable. If a sine disturbance of any wavelength grows then the system is unstable. If the system is unstable over a range of wavelengths the wavelength with the fastest growth rate will dominate the instability growth. The unstable system may reach a new steady state with the interface deformed but intact or the interface may break up to form droplets or particles (Miller 1978).

Attractive (e.g. van der Waal) forces which increase in magnitude with decreasing distance tend to destabilize a water layer between two lipid phases while repulsive forces (e.g. electrostatic and hydration forces (Israelachvili 1984) tend to stabilize the system (Gallez et al. 1984). The aqueous layer can become unstable either *(i)* by growth of a bending wave, in which the transverse movements of both interfaces are in phase and there is no thickness variation in the layer of *(ii)* by growth of a squeezing wave in which a periodic variation in layer thickness develops (Steinchen et al. 1982; Wendel et al. 1982). While it is not possible (because, among other reasons, of complexities associated with the presence of a glycocalyx) to apply the above treatments quantitatively to our situation the emergence of a squeezing wave as one of the two fundamental solutions to a thin layer instability problem suggests a mechanism through which points on two adjacent cells can move towards each other in a spatially ordered pattern.

Figure 7 illustrates how polylysine could allow a disturbance to grow between two cells which approach each other. In the control cells of Fig. 7A, the intercellular water layer is stabilized by the repulsive, non-specific, steric (Bell et al. 1984) and electrostatic forces associated with the glycocalyx. Any small disturbance will die out and the cells will eventually move apart. In polylysine-trated cells the additional attractive force due to glycocalyx cross-linking by the polycation acts with the van der Waal force to destabilise the water layer so that a squeezing wave grows on the layer and the wave crests come into contact.

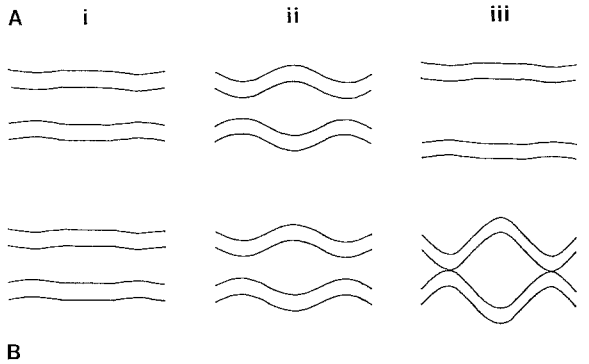

Fig. 7. Schematic representation of how (**A**) two control cells (i) approach each other; (ii) suffer a disturbance which initiates surface wave growth on the intercellular water layer. Further growth is inhibited by repulsive forces at the cell surface and in (iii) the cells drift apart. In (**B**) polylysine treated cells approach each other in (i) and are disturbed in (ii). In (iii) polylysine protruding from each glycocalyx penetrates the glycocalyx of the opposite cell; the resulting attractive force acts with the destabilizing van der Waal force to overcome the stabilizing mutual cell repulsive forces and the disturbance grows

Situations where the instability principle might influence biological processes include the following. *(i)* Membrane "undulation" (Petzoldt 1982) occurs during the acrosomal reaction in sperm. Periodic contacts between the plasma membrane and the outer acrosomal membrane lead, through membrane fusion, to the production of hybrid vesicles containing equal amounts of membrane from both parent membranes (Russell et al. 1979; Meizel 1984). The involvement of phospholipase A_2 in the acrosome reaction (Meizel 1984) would act to destabilize the system by reducing the hydration repulsion force and leaving a fusogenic (lysophosphatidylcholine) residue in the membranes. It has already been suggested by Prevost and Gallez (1984) that a squeezing wave may precede fusion of liposomal membranes. *(ii)* The similarity between the spatial periodicity of circular pores in the flat cisternae of the Golgi apparatus (Krstic 1979) and the periodicity of the recesses in the discoid cells of Fig. 6 suggest that the pores in the Golgi apparatus contribute to its stability. *(iii)* An instability growing between deformable membrane and a substrate may account for the uniform distribution (Baranowski et al. 1977) of cell-substrate contact points during amoeba locomotion (localized regions of deformable plasma membrane occur in amoeba during processes such as induced pinocytosis where wavy plasma membrane becomes detached from the dense filamentous region which normally underlies it (Klein and Stockem 1979)).

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