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Real time observations of polylysine, dextran and polyethylene glycol induced mutual adhesion of erythrocytes held in suspension in an ultrasonic standing wave field

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Abstract. A technique which enables cells to be observed in suspension for times of the order of minutes (employing acoustic radiation forces in a 1 MHz ultrasonic standing wave field) is described. Video recordings of the mutual adhesion of human erythrocytes in suspension have been analysed. Concave-ended cell doublets and linear rouleaux developed in 0.5–1.5% w/v Dextran T500 by a gradual (2.5–17 s) increase in the area of cell contact over the cell cross-section. The concave-ended rouleaux form was not seen in polylysine or in polyethylene glycol. In 5–7% dextran and in 20 μg/ml polylysine mutual adhesion was a two stage process. Cells first form a strong local contact which persists (without apparently growing in area) for a number of seconds following which the cell surfaces move suddenly to form a spherical doublet. The average initial contact time and engulfment time for cells in 7% Dextran T500 are 18 and 2.7 s, respectively. The corresponding values for cells in 20 μg/ml, 14 kDa, polylysine are 2.7 and 0.3 s. There was no initial contact delay during spherical doublet formation in 1 mg/ml polylysine. Electron microscopy showed that the intercellular seam for spherical doublets formed with all three agglutinating molecules was bent in a wavy ($\lambda \approx 4$ μm) profile. The thickness of the intercellular space varied in a spatially periodic way ($\lambda \approx 0.8$ μm) for cells in polylysine. Examples of periodic intercellular spaces were seen by light microscopy in polyethylene glycol induced clumps. The role of interfacial instability in the adhesion processes is discussed.

Key words: Erythrocyte, cell adhesion, cell agglutination, acoustic radiation force, ultrasonic levitation, interfacial instability, polylysine, dextran, polyethylene glycol

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Introduction

The mutual adhesion of erythrocytes by polycations was first examined by Katchalsky et al. (1959). In a recent study (Coakley et al. 1985a) it was shown that the polylysine-induced intercellular contact points are spatially periodic and have an average separation of about 0.8 μm. The periodicity of the discrete contacts has been confirmed in freeze-fracture electron micrographs of unfixed cells (Hewison et al. unpublished). These authors have also shown that the periodicity was not striking in cells fixed in the high concentrations of osmium tetroxide used in the classic study (Katchalsky et al. 1959) thus accounting for the absence in that work of comment on the periodicity.

It has been pointed out that when two membranes approach each other mechanical disturbances of the membrane shape due to thermal or other fluctuations could spontaneously increase their amplitude under the action of net attractive inter-membrane forces (Dimitrov 1982; Steinchen et al. 1982; Wendel et al. 1982). Instability of an aqueous film can lead to growth of a spatially periodic surface wave (a squeezing wave) where wave crests move towards each other to give periodic membrane-membrane contact across the gap and/or the aqueous layer can develop a bending wave where the layer is deformed without thickness variation. Attention has been drawn to the fact that the seam of aggregation of polylysine-induced cell doublets as well as of the dextran-induced doublets examined by Skalak et al. (1981) have the appearance of a bending wave (Coakley et al. 1985b). In the case of polylysine a squeezing wave is superimposed on the bending wave (Coakley et al. 1985b).

The adhesion of an erythrocyte to a spherical erythrocyte fragment held in tips of microcapillaries in the presence of either dextran, plasma or of wheat germ agglutinin (Buxbaum et al. 1982; Evans and

Leung 1984) has been examined by video microscopy. Some observations on dynamic behaviour were made but the studies concentrated on the final equilibrium state. We report here on some real time observations of the dynamics of polymer-induced adhesion of erythrocytes in suspension. Such observations would be difficult to make using conventional light microscopy as cells in suspension could move out of the field of view or out of the plane of focus and would, in any case sediment rapidly on to the microscope slide. A technique, which allows cells levitated in a sound field to be observed for many seconds in suspension, is described below.

When erythrocytes in suspension are subjected to a standing wave sound field three sonic radiation forces will act upon them (Nyborg 1978). One radiation force operates to move the cells to planes which are at right angles to the direction of the acoustic particle velocity and are separated by a distance of half a wavelength. The production of cell bands in erythrocyte suspensions exposed to ultrasound has been observed (Dyson et al. 1972; Baker 1972; Gould and Coakley 1974; Vienken et al. 1985). A second force can result in mutual attraction between suspended cells. Finally, a radiation torque can align the cells in a preferred orientation in the sound field. The system described below takes advantage of the fact that when cells in polymer solutions are drawn into preferred regions of an ultrasonic standing wave field they can be retained there to await the arrival of a second cell with which a polymer-induced interaction occurs while both cells are still in suspension.

The real time observations of erythrocytes suspended in a sound field in solutions of polylysine, of dextran or of polyethylene glycol provide new information on the dynamics of erythrocyte mutual adhesion processes.

Materials and methods

a. Erythrocyte preparation

Human erythrocytes were obtained by finger puncture and collected into phosphate buffered saline (PBS) containing 145 m*M* NaCl, 5 m*M* phosphate at pH 7.32. The cells were washed and resuspended to a concentration of $1.2 \cdot 10^7$ cells/ml in fresh PBS, in solutions of poly-L-lysine (M.W. 14,000; Sigma Ltd.), of dextran T500 (M.W. 500,000; Pharmacia Fine Chemicals) or of polyethylene glycol (M.W. 8,000, Sigma Ltd.) in PBS.

In experiments where the effects of membrane stiffness on the adhesion process were investigated cells in saline were prefixed by addition of glutaraldehyde in saline to give the quoted concentration of glutaraldehyde in the cell suspension. The cells were maintained in glutaraldehyde for 5 min and then centrifuged. The supernatant was discarded and the cell pellet was resuspended in PBS.

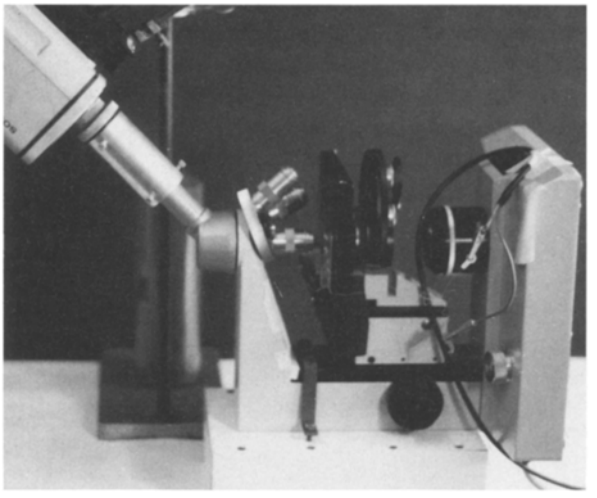

Fig. 1. Video-microscopy system for observation of cells in an ultrasonic standing wave field. A Vickers M15c microscope is turned through 90° to rest on its back. A video camera is attached to the microscope. A 1 MHz ultrasonic transducer is mounted on the microscope stage

b. Light microscope and video system

A Vickers M15c phase contrast microscope was fitted with a monocular viewing head, turned through 90° onto its back and bolted to a platform (Fig. 1). A Sony RSC-1110 (50 frames/s) rotary shutter television camera was connected to the microscope. The output from the camera was recorded on a Sony U-Matic VO-2360 video recorder. Monitoring of the field on view and subsequent analysis of the recordings were performed on a Sony-Videostrobe SVM-1110 motion analyser.

c. Generation of an ultrasonic standing wave field

A 2 cm × 1 cm rectangular section was machined from a 3 cm diameter lead zirconate PZT4 disc ultrasonic transducer (Vernitron Ltd.) and mounted on a perspex holder on the moveable microscope stage as shown in Fig. 2. The transducer was driven at its thickness resonant frequency of 1 MHz using a signal generator which could be continuously controlled from 0–50 V p-p. The voltage was routinely set at 25 V.

A 1 cm pathlength perspex light cuvette was shortened to a height of 1 cm. The base of the

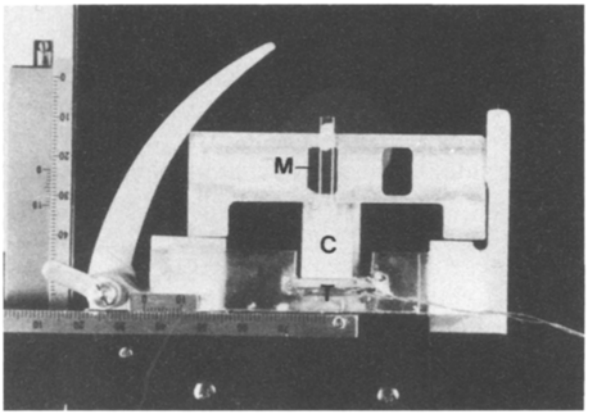

Fig. 2. The microscope stage. The 1 cm long perspex cuvette (*C*) of 1 cm × 1 cm cross-section, with a 12 µm thick plastic film base was filled with cell suspension and was acoustically coupled to the transducer *T* by a layer of silicone grease. Cells were drawn by capillarity from the cuvette into the microslide (*M*)

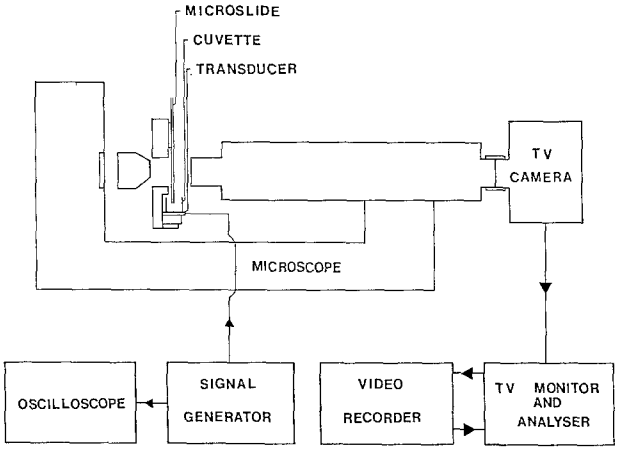

Fig. 3. A schematic diagram of the video-microscopy assembly

cuvette was cut off and was replaced by a 12 µm thick plastic film. A 2 mm recess was machined into the microscope stage to accommodate the cuvette on the transducer and to allow adjustment of the position of the cuvette. A thin layer of silicone grease on the ultrasonic transducer acted as an acoustic coupling agent between the cuvette and the transducer.

The cells were observed in 4 cm long rectangular microslides (Camlab Ltd.) which had been cleaned as described by Deeley and Coakley (1983). An empty microslide was attached with silicone grease to an aluminium slide which was then placed in the microscope's slide carrier (Fig. 2). The microslide was positioned so that the bottom end came halfway down the empty cuvette. The microscope was adjusted to obtain a focused (TV monitor) image of the inside surface of the part of the microslide which protruded from the cuvette. Figure 3 is a schematic diagram of the microscope system.

Equal volumes of stock erythrocyte suspension and of one of the polymer solutions (or of control polymer-free PBS) were mixed. The resulting cell suspensions had a concentration of $6 \cdot 10^6$ cells/ml either in PBS or in the concentration of polymer solution given in the Results. A 1.2 ml aliquot of the desired cell suspension was immediately introduced into the cuvette. The microslide filled with cell suspension, within seconds, through capillarity. Voltage was applied to the transducer and a field of view was selected to show an area of acoustic banding where a large number of cell-cell contacts could be observed as they occured in the bulk phase. Microslides of 0.1 mm and 0.3 mm pathlength were used with the X100 and X40 objectives respectively.

d. Viscosity measurements

The relative viscosity of dextran and of polyethylene glycol solutions was measured at 25 °C in a U-tube viscometer.

e. Erythrocyte adhesion by PEG in test tubes

In one series of experiments an erythrocyte pellet of 10^6 cells was resuspended in 1 ml of polyethylene glycol, whirl-mixed for one second and incubated for a known length of time at 37 °C. In other experiments washed erythrocytes resuspended in 1 ml of PBS were added to 1 ml of double strength PEG solution and incubated as before.

f. Electron microscopy

Cells were fixed by addition of glutaraldehyde solution to a final concentration of 0.7% w/v. The fixed cells were dehydrated using ethanol in a graded series (50%, 70%, 90%, 100%). The dehydrated cells were embedded in a 1:1 mixture of alcohol – L.R. white resin for 4 h at room temperature and then in pure L.R. white resin. The embedded cells were cured and 60 nm thick sections were cut. The sections were mounted on a copper grid coated with colloidin and carbon and were double stained with 5% uranyl acetate in 50% alcohol for 30 min (Reynolds 1963) before examination by electron microscopy.

Results

a. Observation on control cells in a standing wave field

Immediately on applying ultrasound (transducer voltage 27 V p-p) to suspensions of control erythro-

cytes in the microcapillaries cell movement was observed and, in about 5 s, regions (bands) of high cell concentration could be identified at locations separated by distances of 500–750 μm (the half-wavelength of 1 MHz sound in water is 750 μm) in the vertical plane as previously illustrated (Vienken et al. 1985). The cell bands were not continuous in the horizontal plane of the microslide cross-section. Rather, the cells tended to congregate near, but not on, the microslide walls. Individual cells retained the freedom to move relative to each other within the group. There was some bulk flow of the suspending phase liquid near the bands. Cells free in suspension in this bulk flow provided a reservoir from which (over several minutes) a succession of single cells could be observed microscopically as they came close to the cell band. These cells often interacted (because of an attractive intercellular radiation force; Nyborg 1978) with other single cells to form discrete pairs when they reached the boundaries of the cell bands, where bulk flow was slow. Such slowly moving discrete pairs could have a lifetime in excess of 30 s before the cells drifted away from each other. While close to each other both cells of the pair retained the capacity for independent movement i.e. the cell boundaries flexed (presumably because of thermal motion) independently and the cells could move slightly apart and make contact again at new regions of the membrane. This weak interaction between a pair of control cells will be referred to as acoustic interaction.

b. General observations of cells in polymer solutions

Extensive banding of cells suspended in either polylysine or dextran solutions was observed at ultrasonic transducer voltages which gave a clear banding effect in control cell suspensions. Single cells were seen to adhere to each other or to small cell agglutinates. These small agglutinates could come together to form larger agglutinates in the high cell concentration regions in the sound field. Unlike the case for sonically aggregated control cells in PBS there was no independent movement of cells within the agglutinates. At transducer voltages below those required for cell banding the incidence of cell-cell contact and subsequent adhesion was greatly reduced. This reduction occured because of the lower probability of cell-cell contact when the cells were not being concentrated by ultrasound.

c. Cell adhesion in dextran

Concave ended rouleaux and cell doublets were formed in the microcapillaries among cells in 0.5% w/v dextran. The cells approached tangentially and moved slowly over each other at an average rate of 0.4 μm/s. The fact that many rouleaux were seen in the microslides and yet that only a small number of cells were detected forming doublets in regions of the microslide where cells were comparatively still (little bulk flow) indicated that rouleaux were preferentially forming where there was fluid flow. It is therefore not sufficient just to bring cells close together in this low concentration of dextran in order to achieve adhesion. The experimental observation that in flowing suspensions rouleau size increases to a maximum with increasing shear rate has been attributed to enhanced collisions between cells (Samsel and Perelson 1984). The cells forming doublets in 0.5% dextran did not always fully overlap. The cells could be made to overlap by the mechanical disturbance of the doublets which resulted from sweeping the ultrasonic frequency thus changing the positions of the pressure nodes.

Cell adhesion in Dextran T500 concentrations of 1.0 and 1.5% w/v also occurred by rouleau formation where the end cells of the rouleaux retained a concave shape. An example of the buildup of a branched rouleau network (Samsel and Perelson 1984), in which the delay between the initial contact (Fig. 4b) of two established rouleaux to form a larger rouleau was about 9 s, is shown for cells in 1% w/v dextran in Fig. 4. The time for rotation of the smaller rouleau onto the network (Fig. 4d–h) was about 7 s. A very similar example of rouleau network formation was seen in cells in 1.5% w/v dextran. The time for rotation of the smaller rouleau in the latter case was 3 s. The average of 10 measured times for cells in 1.5% or in 2% dextran to complete contact over a cellular cross-section was 2.7 s.

At dextran concentrations of 5% and 7% the formation of a physical connection between cell rims could be resolved on the monitor as single cells came into close proximity. This connection will be referred to as 'a bridge'. A definite rapid local at-

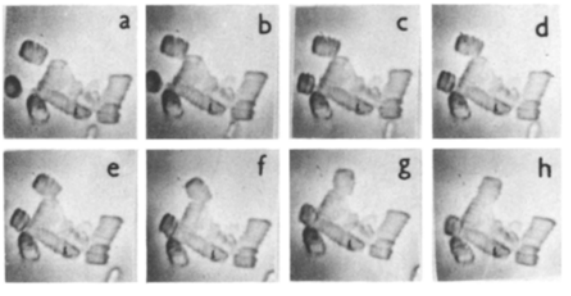

Fig. 4a–h. An example of the extension of a large concave-ended erythrocyte rouleau network in 1.5% dextran through adhesion of smaller rouleaux. Time; **a** zero, **b** 1.2 s, **c** 2.8 s, **d** 4.3 s, **e** 4.5 s, **f** 6.5 s, **g** 8.5 s, **h** 10.5 s

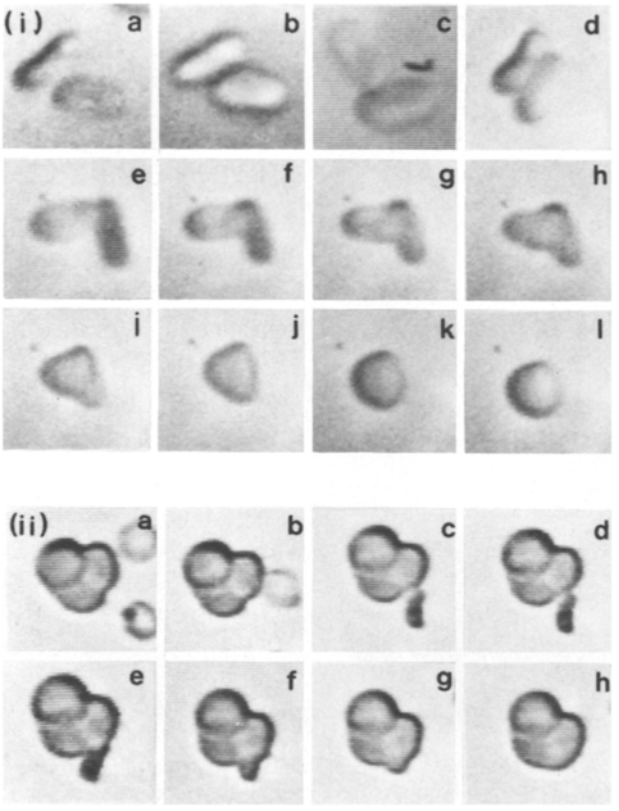

Fig. 5. **(i)** Video-micrographs of cells in 5% dextran. **a** Time zero, the cells are free in suspension 0.04 s before initial contact is made; **b, c, d** stable doublet forms at 4, 16 and 32 s respectively; **e** 40 s, the frame before the final adhesion stage begins; **f** 40.12 s; **g** 40.2 s; **h** 40.28 s; **i** 40.4 s; **j** 40.52 s; **k** 41.02 s; **l** 41.52 s; the final spherical doublet form has been reached. **(ii)** Video-micrograph of the addition of a single cell to a clump of adhered cells in 7% dextran. **a** Time zero, 2 s before contact is made, a cell flows close to the clump; **b** 5 s; **c** 10.7 s; **d** 11.5 s; **e** 14 s; **f** 15.8 s, 0.7 s after the final adhesion stage began; **g** 16.2 s; **h** 16.8 s

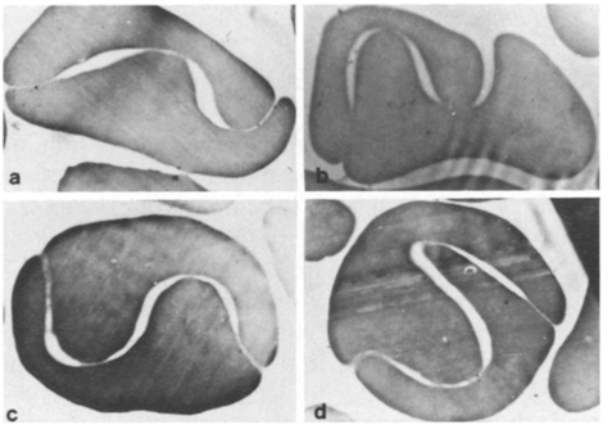

Fig. 6. Transmission electron micrographs of erythrocytes fixed after 45 s exposure to 7% dextran. Contact is made at the cell edges and at the regions of maximum membrane curvature

Table 1. Measured times for the formation of convex-ended agglutinates in polylysine or dextran following a period of stable cell-cell contact. Times are the averages from measurements on 15 adhesion events per polymer

Polymer	Concentration	Stable cell-cell contact time [s]	Agglutination time [s]	Viscosity [cP]
Dextran	7%	18	2.7	8.6
Polylysine	20 μg/ml	2.7	0.3	1.0

tractive movement of the cell membranes (lasting for times of the order of 50 ms) occurred as the bridge was formed. The apparent length of the bridge was about 3–4 μm when cells were in edge to edge contact. Figure 5 (i) shows that, for erythrocytes in 5% dextran a long period (40 s) of local cell-cell contact preceeded the sudden (1 s) movement to a convex doublet. Details (b, c, d) of Fig. 5 (i) illustrate different configurations of the doublet for which the contact area did not increase. The intercellular bridge was stable for a time before a sudden further rapid attractive movement of the cells occurred to give a larger clump in which the outer membrane was convex (Fig. 5 (i), e–l).

Bridge formation could also be identified when single cells came into close proximity with cell clumps. Video analysis showed that the complex of Fig. 5 (ii) was derived from the association of three spherical cell clumps which came together. The single cell which joins the complex in the figure is associated with the cell clump for 13 s before beginning the final 1.8 s adhesion phase. The lack of significant widening of the cell profile (implying an absence of cell deformation away from the contact point) as the cell advanced towards the clump in the final adhesion phase was also observed in other cases. Measured times for (i) the lifetime of the bridge before strong adhesion occurs and (ii) the cell movements leading to the strong adhesion of Fig. 5 are shown for cells in 7% dextran in Table 1.

Since, from Table 1, the average time for movement to spherical doublet formation was 2.7 s an experiment was performed to determine if cells fixed after 45 s exposure to 10% dextran would show intermediate stages of the doublet formation. The electron microscope thin sections contained many pairs of cells as in Fig. 6 a, c, d where contact was made at the cell rim and at regions of maximum curvature between the cells. Doublets with a contact profile as in Fig. 6 c, d but with contiguous membranes along all of the seam were also present in the thin sections. These doublets were assumed to have been formed before fixative was added to the cell suspensions.

d. Cell adhesion in polylysine

Video microscopy showed that the threshold concentration for adhesion of cells in 14 kDa polylysine lay between 8 and 10 μg/ml. Examination of a number of samples in this concentration range showed that no concave cell pairs or larger concave-ended rouleaux of the type shown in Fig. 4 were formed in the microcapillaries. When adhesion occurred it resulted in the formation of convex doublets or in larger convex complexes. 'Bridge' formation leading to doublet formation, as in the case of 5–7% dextran (Fig. 5), was the norm for cells in 20 μg/ml polylysine (Fig. 7). The bridge lifetime and the time to form a doublet are shown in Table 1. It can be seen from the Table 1 that the average times for doublet formation was greater in the more viscous dextran solution.

In addition to rim to rim cell contact as in Fig. 7 (i) parallel cells passing close to each other in 20 μg/ml polylysine can come together over their full parallel surfaces, without bridge formation, to give a convex doublet. This process could take as little as 80 ms.

A small number of examples were observed where cells which had formed a bridge in 20 μg/ml polylysine and were moving in slow bulk flow separated many seconds into the bridge lifetime. An indication of the mechanical strength of the bridge was provided by the observation that as a bridged doublet broke up in the flow the cells could become significantly distorted by elongation before separating. Marked distortion of stressed cells in plasma-induced rouleaux (Rowlands et al. 1983) and in stressed lectin-agglutinated erythrocytes (Evans and Leung 1984) has previously been reported.

In a number of cases in 20 μg/ml polylysine no measurable metastable bridge phase was formed during cell rim contact. The cell adhesion was completed rapidly without a pause. This form of delay-free convex doublet formation was the norm for cells in 1 mg/ml polylysine. It was never observed with cells in dextran.

The electron micrograph of Fig. 8 a shows a wavy (bending) seam profile (similar to those for dextran in Fig. 6) in a polylysine-induced doublet. The wavy fine structure (squeezing) of the contact seam of the polylysine treated complexes has been examined in more detail elsewhere (Coakley et al. 1985 a).

Cells fixed with gluturaldehyde at concentrations below 0.0012% w/v prior to exposure to 20 μg/ml 14 kDa polylysine showed bridge formation and strong adhesion as for unfixed cells. The bridge lifetime and the time for strong adhesion were similar to those for unfixed cells. In contrast the video microscopy showed that cells prefixed in glutaraldehyde concentrations greater than 0.0012% w/v became 'attached' to each other at a single point. Unlike the case for a pair of interacting unfixed control cells the attached cells were no longer capable of independent movement; the doublet moved as a single body. On the other hand (unlike the case for unfixed cells in the presence of polylysine) the sudden cell movement associated with bridge formation did not occur with 'attached' cells nor was it possible to optically resolve a bridge connection. These results showed that the loss, in glutaraldehyde prefixed cells, of the ability to form spherical doublets was associated with inhibition of the initial bridge formation process.

Conventional light microscopy of complexes of prefixed cells showed the regular intercellular spaces which are a feature of polylysine-induced complexes of control erythrocytes (Coakley et al. 1985 a).

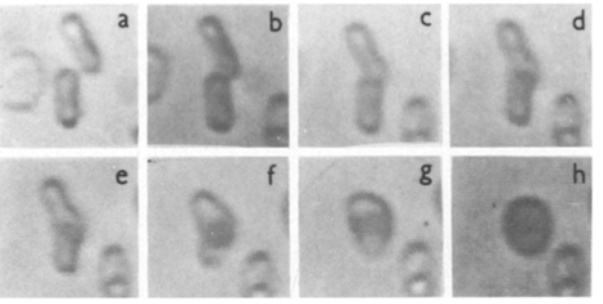

Fig. 7 a–h. Spherical doublet formation in 20 μg/ml polylysine. **a** Time zero; cells at the instant before contact is made; **b** 2.5 s cells during stable bridge period; **c** 3.3 s; the final adhesion stage begins; **d** 3.32 s; **e** 3.34 s; **f** 3.48 s; **g** 3.56 s; **h** 3.76 s

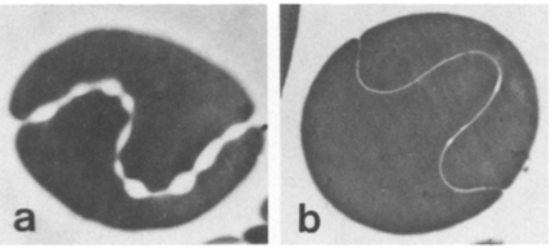

Fig. 8 a and b. Wavy seams of adhesion in cell doublets formed **(a)** in 20 μg/ml polylysine (bending and squeezing waves); **(b)** in 20% PEG (bending)

e. Cell adhesion in polyethylene glycol

Erythrocytes in single cell suspension in PBS were added to PEG in PBS. The resulting cell suspension was mixed in a Whirl-Mixer for 1 s. Conventional light microscopy of cells which were fixed in 4%

glutaraldehyde immediately or after standing in a test tube for 2.5 min showed small complexes, involving just two or three cells. These small complexes of adhered cells contrasted with the much larger complexes seen when a cell pellet was resuspended directly in PBS containing PEG. The latter observation is consistent with previous reports (Knutton 1979) where the formation of large complexes was described as being 'instantaneous'. The contrast with the situation where cells were in single cell suspension before being added to PEG suggest that pellets directly resuspended in PEG may never have separated into a single cell suspension. The results described below were obtained for cells which were in single cell suspension before addition to a PEG solution.

The threshold concentration for cell adhesion in a PBS solution of PEG 8000 was 18% w/v (final concentration). As for the case of cells in polylysine there was no evidence, either from electron or light microscopy, of concave-ended doublet formation in cell suspensions exposed to PEG. The electron micrograph of Fig. 8 b shows a wavy (bending) seam profile in a doublet formed in 20% w/v PEG. The electron micrographs of larger complexes showed regions of higher membrane curvature, implying tighter cell packing, than were seen in polylysine or dextran complexes.

Glutaraldehyde-fixed PEG complexes occasionally showed regular spatially periodic intercellular spaces as in Fig. 9. These spaces are of the same pattern as those seen in polylysine treated cells and they suggest that interfacial instability may be involved in cell adhesion by PEG. The freeze-fracture electron micrographs of erythrocytes exposed to PEG which have been presented by Knutton (1979) show localized cell-cell contact rather than a continuous intercellular seam.

Ultrasonic band formation in vertical microslides did not occur with cells in PEG concentrations greater than 4%. This unexplained absence of banding did not arise from increased viscosity of the cell suspending phase as the viscosity of cells in 7% dextran (7 cP) where banding occurred was greater than the viscosity (5 cP) of cells in 4% PEG. Neither may the result be explained by significant sound absorption in the PEG solutions as dextran has a higher attenuation coefficient than PEG (Hawley and Dunn 1969, 1970; Kessler et al. 1970). Despite the difficulty with cell banding in PEG a small number of adhesion events were detected with the video-microscopy system. Figure 10 shows doublet formation in a 25% PEG solution. The edge-on view of the cell bending involved in the doublet formation of Fig. 10 gives an impression of cell flexure consistent with that given, for cells in dextran, by the electron micrographs of Fig. 6.

f. Adhesion times in different solutions

Knowledge of the time required for different types of adhesion to occur makes it possible to estimate the force required to overcome the viscous drag on cells as they move towards each other to form a doublet. A typical doublet in 2% dextran developed from one cell sliding across the 7.5 μm diameter of the other in a time of about 2.5 s. The average velocity of one cell relative to the other was then 3 μm/s. Taking an equivalent spherical cell radius as 3 μm and the viscosity of the solution as 1.9 cP the retarding Stokes drag is $2.7 \cdot 10^{-8}$ dynes. It has been shown (Chien et al. 1977) that the shear flow stress neccessary to re-exposure 50% of the contact area of a doublet formed in 2% of 80 kDa dextran is 0.2 dynes/cm^2. For a doublet of cross-sectional area 44 μm^2 this stress exerts a tangential force of $9 \cdot 10^{-8}$ dynes on the cells. This maximum attractive force between the cells is similar in magnitude to the average force of $2.7 \cdot 10^{-8}$ dynes calculated from the rate of cell movement observed in the present work.

The energy dissipated in overcoming viscous drag has been estimated as the product of the Stokes drag and the diameter of a single cell for the situations described in Table 2.

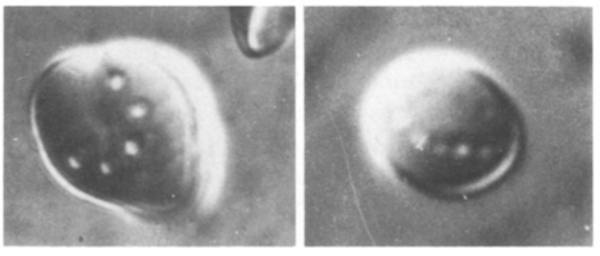

Fig. 9. Spatially periodic intercellular spaces in a complex formed in 20% PEG

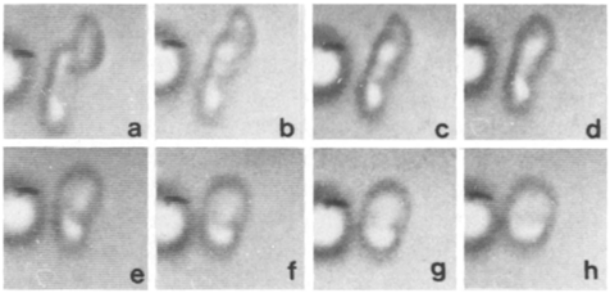

Fig. 10 a – h. Video-micrographs of cell doublet formation in 25% PEG. **a** Time zero; **b** 0.2 s; **c** 0.3 s; **d** 0.5 s; **e** 0.7 s; **f** 0.9 s; **g** 1.0 s; **h** 1.1 s

Table 2. Estimates of the energy dissipated in overcoming viscous drag during doublet formation in different suspending phases

Suspending phase	Doublet	Doublet time [s]	Viscosity [cP]	Viscous energy [10^{-10} ergs]
Dextran (1.5%)	Concave	2.7	1.4	0.15
Dextran (7%)	Spherical	2.7	8.6	0.89
Polylysine (20 µg/ml)	Spherical	0.3	1.0	0.94
PEG (25%)	Spherical	1.1	23	5.9

Discussion

The study of cell aggregation by conventional light microscopy suffers from the following limitations: (i) Cells sediment on to the glass coverslip (through a typical distance of 50 µm) in about 30 s thus limiting the time available for observation of cells in suspension. (ii) Since cells sediment at right angles to the focal plane of the objective they will rapidly pass through the shallow depth of focus of a high resolution objective. (iii) Since there is no mechanism for concentrating cells the probability of detecting two cells in close apposition, prior to agglutination, is low.

The combination of an ultrasonic field and the microslide cell holder described offer the following advantages over conventional microscopy. (i) Because of the accumulation of cells at particular regions in the microslide fields of view can be selected at which there is a high probability of detecting and recording a cell-cell interaction. (ii) The rate of movement of a cell in bulk flow close to a cell clump in the microslide is of the order of 2 µm/s so that typically a free cell can, even without movement of the microscope stage, be retained in the field of view for times of the order of 100 s. The fortuitous bulk flow provides a reservoir from which a continuous stream of single cells are drawn into the accumulation region for a long time after the initiation of the ultrasonic exposure. The 'useful life' (for observation of a cell-cell interaction) of a cell sample can therefore extend over many minutes. (iii) Few changes of focal plane are required because the cells accumulate near, but usually not on, the microslide wall. Since the direction of slow movement of cells in the bulk flow is parallel to the microslide wall many cells in the TV monitor field remain in focus for times of the order of 30 s.

The acoustic radiation force between cells in suspension in a sound field helps draw cells close together yet the interaction of cells which is induced by this force is, as described in the Results, qualitatively and quantitatively different from the polymer-induced adhesion. If the ultrasound is switched off when two cells in 20 µg/ml polylysine have been brought into close proximity by the ultrasonic field then the cells will progress through the bridge formation and strong adhesion stage. This observation indicates that the effects of acoustic inter-particle forces contribute little to the measured adhesion times. In any case the radiation force arguably carries out a requirement (that of bringing two cells close together for adhesion) which is achieved by bulk fluid flow when cells in polymer solution adhere in test tubes.

Since ultrasound exerts a torque on cells (Nyborg 1978) which often gave adjacent cells similar orientations it might be argued that the incidence of edge to edge contact observed in the present work has been exaggerated. We found that cells which approached each other in an edge-on way have bridge formation and adhesion times similar to those cases where cells approach at right angles or where single cells approach a clump at right angles. In addition when cells are clumped by polymers in shaken cell suspension (Katchalsky 1959) orientation of cells is, in any case, likely to occur in the resulting shear flow (Goldsmith 1968).

The observation here that for some concentrations of dextran and of polylysine the final rapid movement during spherical doublet formation is preceeded (see Evans 1980) by a metastable period of bridge contact (Figs. 5 (i), 7, Table 1) serves to emphasize the involvement of instability in the final rapid phase of adhesion. Electron microscopy (Figs. 6 and 8) shows that, for all three agglutinating molecules, the spherical doublet form is characterised by the presence of a wavy (bending) intercellular seam. An explanation of the development of the wavy seam should include the electron microscope observations (e.g. Fig. 6) that contact all round the cell rims preceeds both contact over the dimple region and the final development of the spherical form (Fig. 6 and the video micrograph of Fig. 10). Such an explanation would be that the fast phase of cell-cell attachment (Figs. 5 (i) and 7) involves the formation of a ring of contact on the cell rims and that the intercellular saline in the dimple regions (Fig. 6) becomes unstable through development of a bending wave. Skalak et al. (1981) calculated the surface energy of adhesion of axisymmetric red blood cells for the cases of bonding two cells in a rouleau and for the bonding of a typical cell in the centre of a long straight rouleau. It was assumed that the surface of adhesion common to two adjacent cells was a plane. They noted that the plane

assumption was not supported by their experimental observations at high adhesive energies but the complicated computational procedures were not undertaken for this non-axisymmetric curved-contact case. Skalak et al. (1981) suggested that the cylindrical rouleau form was probably unstable at sufficiently high adhesive energies.

The surface affinities for erythrocyte-erythrocyte adhesion in dextran has been examined quantitatively by measuring the interaction between a flaccid erythrocyte and 2–5 μm diameter spherical erythrocyte bodies held in the ends of micropipettes (Evans 1980; Buxbaum et al. 1982). It was considered that static deformation of the elastic red cell membrane stored energy conservatively and that the elastic energy opposed the reduction of free energy associated with formation of adhesive contact. The peak surface affinity of cells in 150 kDa dextran was calculated to be $2.2 \cdot 10^{-2}$ ergs/cm^2 (Buxbaum et al. 1982). For a 20 μm^2 engulfed area of a spherical fragment the elastic energy is $4.4 \cdot 10^{-9}$ ergs. The calculated elastic energy ($4.4 \cdot 10^{-9}$ ergs) is thus an order of magnitude greater than the estimate of viscous energy dissipated during the fast phase of spherical doublet formation in dextran and in polylysine (Table 2). Buxbaum et al. (1982) noted that, in general, solution viscosities affect the rate at which cell contact and adhesion take place.

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References

Baker NV (1972) Segregation and sedimentation of red blood cells in ultrasonic standing waves. Nature 239:398–399

Buxbaum K, Evans E, Brooks DE (1982) Quantification of surface affinities of red blood cells in dextran solutions and plasma. Biochemistry 21:3235–3239

Chien S, Sung AL, Kim S, Burke AM, Usami S (1977) Determination of aggregation force in rouleaux by fluid mechanical technique. Microvasc Res 13:327–333

Coakley WT, Hewison LA, Tilley D (1985a) Interfacial instability and the agglutination of erythrocytes by polylysine. Eur Biophys J 13:123–130

Coakley WT, Hewison LA, Tilley D (1985b) Interfacial instability and the agglutination of erythrocytes by polymers. Stud Biophys 110:89–94

Deeley JOT, Coakley WT (1983) Interfacial instability and membrane internalization in human erythrocytes heated in the presence of serum albumin. Biochim Biophys Acta 727:293–302

Dimitrov DS (1982) Instability of thin films between membranes. Colloid Polymer Sci 260:1137–1144

Dyson M, Woodward B, Pond JB (1972) Flow of red blood cells stopped by ultrasound. Nature 232:572–573

Evans EA (1980) Minimum energy analysis of membrane deformation applied to pipet aspiration and surface adhesion of red blood cells. Biophys J 30:265–284

Evans E, Leung A (1984) Adhesivity and rigidity of erythrocyte membrane in relation to wheat germ agglutinin binding. J Cell Biol 98:1201–1208

Goldsmith HL (1968) The microrheology of red blood cell suspensions. J Gen Physiol 52:5s–28s

Gould RK, Coakley WT (1974) The effects of acoustic forces on small particles in suspension. In: Proceedings of 1973 symposium on finite amplitude wave effects in fluids. Pergamon Press, London, p 241

Hawley SA, Dunn F (1969) Ultrasonic absorption in aqueous solutions of dextran. J Chem Phys 50:3523–3526

Hawley SA, Dunn F (1970) Ultrasonic relaxation spectra in aqueous solutions of dextran and polyethylene glycol. J Chem Phys 52:5497–5498

Katchalsky A, Danon D, Nevo A, Vries A de (1959) Interactions of basic polyelectrolytes with the red blood cell 2. Agglutination of red blood cells by polymeric bases. Biochim Biophys Acta 33:120–138

Kessler LW, O'Brien WD Jr, Dunn F (1970) Ultrasonic absorption in aqueous solutions of polyethylene glycol. J Phys Chem 74:4096–4102

Knutton S (1979) Studies of membrane fusion. III. Fusion of erythrocytes with polyethylene glycol. J Cell Sci 36:61–72

Nyborg WL (1978) Physical principles of ultrasound. In: Fry FJ (ed) Ultrasound: Its application in medicine and biology. Elsevier, New York, pp 1–75

Reynolds ES (1963) The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J Cell Biol 17:208–212

Rowlands S, Eisenberg CP, Sewchand LS (1983) Contractils: Quantum mechanical fibrils. J Biol Phys 11:1–4

Samsel RW, Perelson AS (1984) Kinetics of rouleau formation: II. Reversible reactions. Biophys J 45:805–824

Skalak R, Zarda PR, Jan K-M, Chien S (1981) Mechanics of rouleau formation. Biophys J 35:771–782

Steinchen A, Gallez D, Sanfeld A (1982) A viscoelastic approach to the hydrodynamic stability of membranes. J Colloid Interface Sci 85:5–15

Vienken J, Zimmermann U, Zenner HP, Coakley WT, Gould RK (1985) Electro-acoustic fusion of erythrocytes and of myeloma cells. Biochim Biophys Acta 820:259–264

Wendel H, Bisch PM, Gallez D (1982) Hydrodynamics of dielectric fluid films. Colloid Polymer Sci 260:425–434