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Electro-acoustic fusion of erythrocytes and of myeloma cells

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Mammalian cells can be concentrated in a sound field. A method is introduced, which combines the reversible aggregation of cells in a sound field with the electrical breakdown of cell membranes to fuse cells, which are in contact. Human red blood cells and mouse myeloma cells are fused by means of that procedure.

Introduction

It has been known since at least 1874 that particles can be aggregated by suspension in a sound field [1]. Radiation forces of non-zero-time average arise due to non-linear effects when sound is propagated in a fluid [2]. The attractive forces responsible for particle agglutination, which can lead to erythrocyte chain formation [3,4], have been attributed to these radiation forces [2–5]. The agglutination forces will operate both in a travelling-wave or a standing-wave field. However, in a standing-wave field radiation pressure also operates to move suspended particles to planes which are separate by half a wavelength and are at right angles to the direction of propagation of the sound field [5,6]. The concentration of suspended erythrocytes at positions separated by half a wavelength in a standing-wave field has been observed experimentally [6,7].

In recent years Zimmermann and co-workers [8–11] have developed methods of fusing biological cells by the application of extremely short (μs range) electric-field pulses. The only requirement is that the cells must first be brought into close membrane contact. This can be achieved by high suspension densities [8,9], dielectrophoresis [9], by inhomogeneous magnetic fields if the cells are first coated with small magnetic particles [12] or by means of an antigen/antibody reaction of cells pretreated with avidin/biotin [13]. A further possibility is to fuse cells, which have grown together as a monolayer, by means of an electric-field pulse [14].

In this communication we introduce a simple method of cell fusion which combines ultrasonic aggregation of cells with electric-field pulses and provides a large yield of fused cells.

Materials and Methods

(1) Experimental arrangement. Ultrasound was generated by driving a 30 mm diameter lead zirconate PZT5 disc at its thickness resonant frequency of 1.0 MHz using a Toellner signal generator (Toellner GmbH, Frankfurt/Main, F.R.G.) or the GCA fusion equipment (GCA Corporation, Chicago, IL, U.S.A.).

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The bottom of a perspex cuvette (1 × 1 cm) was cut off and the cuvette was released with a very thin plastic film, then filled with a solution, consisting of phosphate-buffered saline (PBS = solution F). Sound waves from the transducer were thus able to penetrate into the solution F in the cuvette without significant absorption of the sound at the base of the container (Fig. 1). The output from the loaded signal generator could be continuously controlled from 0 to 50 V p-p. It was not possible to measure the pressure distribution within the container but in separate experiments it was found that the peak sound intensity at the last maximum in the near field of the transducer was 1.0 W/cm when the transducer was coupled to a large tank of degassed water under travelling-wave field conditions [15] and was driven at 50 V p-p.

The fusion chamber consists of two stainless-steel cylinders arranged one inside the other. The cylinders are maintained in a concentric configuration by a plastic spacer which also serves to insulate the cylinders from each other. The gap between the cylinders was 200 μm. The two metal cylinders, which are the electrodes of the fusion chamber, are connected to a pulse generator (GCA Cell Fusion System, GCA, Chicago, IL, U.S.A.). The cells to be fused are sucked into the gap between the electrodes by means of a pipette. The fusion chamber is then immersed in the solution F filled container (Fig. 1).

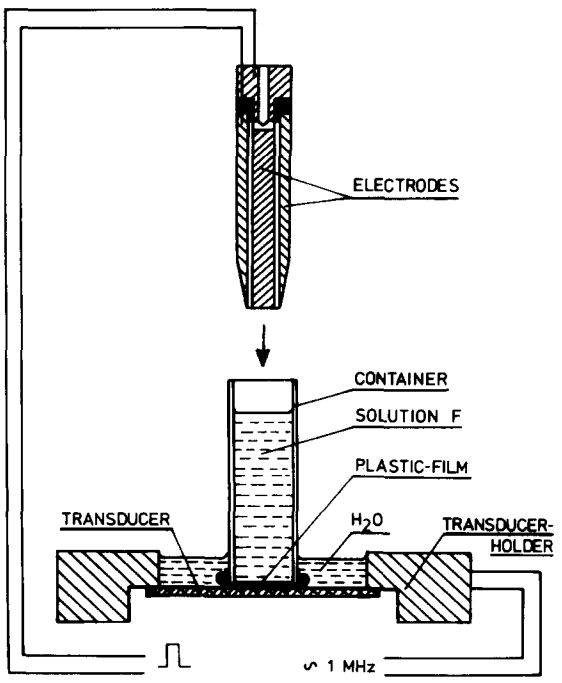


Fig. 1. Set-up for electro-acoustic fusion of erythrocytes and myeloma cells. For details, see text.

(2) Preparation of the cells. Freshly drawn human erythrocytes or SP 2/0 mouse myeloma cells are washed in an isotonic sodium chloride solution and then incubated for 5 min in a solution of 1 mg/ml pronase (SERVA GmbH, Heidelberg, F.R.G.) in phosphate-buffered saline. The cells are then washed once in solution L which has the following composition: 0.28 M inositol, 0.25 mM magnesium acetate, 0.1 mM calcium acetate, 0.36 mM KH_2PO_4 and 0.86 mM K_2HPO_4.

A 20 μl aliquot of cells suspended in solution L is transferred to the fusion chamber which had been rinsed with an aqueous solution of 0.1 mg albumin/ml prior to the experiment.

(3) Electro-acoustic fusion. Following the immersion of the fusion chamber in the container the cells are exposed to a sound field for a period of 60 s. After 10 s three 10 μs pulses of 80 V and 60 V amplitude are applied to the erythrocyte or myeloma cell suspensions, respectively. The optimum interval between the pulses has been found experimentally to be 1 s. Following the third pulse ultrasound is applied to the chamber system for a further 50 s.

The temperature of a 3.5 ml L-solution was measured after irradiation, at 50 V p-p transducer voltage, in a perspex container for various times by means of a thermometer. The temperature increase was only 1 Cdeg in the first minute of irradiation. The total ultrasonic exposure time for cells was therefore limited to 1 min so that temperature effects in the radiation assembly would not be significant.

Results

Extensive cell fusion occurred when a suspension of erythrocytes was exposed to the combinations of ultrasound (Fig. 2) and electric-field pulses described in Materials and Methods (Fig. 3a). Fusion of myeloma cells which had been exposed

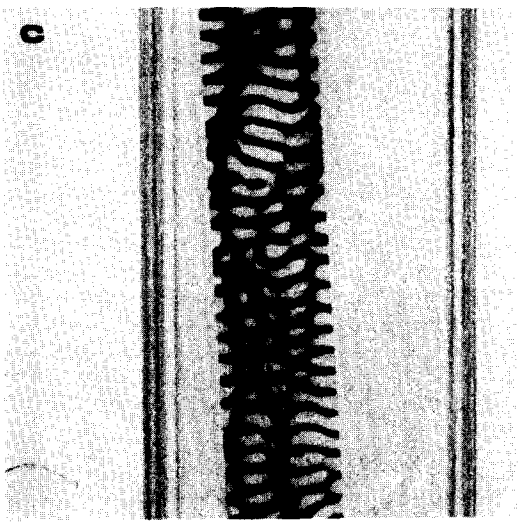


Fig. 2. To illustrate the banding of red blood cells following sound irradiation a Camlab-Microslide is filled with a suspension of erythrocytes and inserted into the container of the electro-acoustic-fusion set-up (a). Two seconds after the beginning of irradiation cells are not any more suspended homogeneously (Fig. 2a) but show a banding over the whole length of the microslide (b), due to the sound aggregation forces. At higher magnification (c) the concentration of red blood cells at planes separated by half a wavelength and at right angles to the direction of sound propagation is nice to be seen.

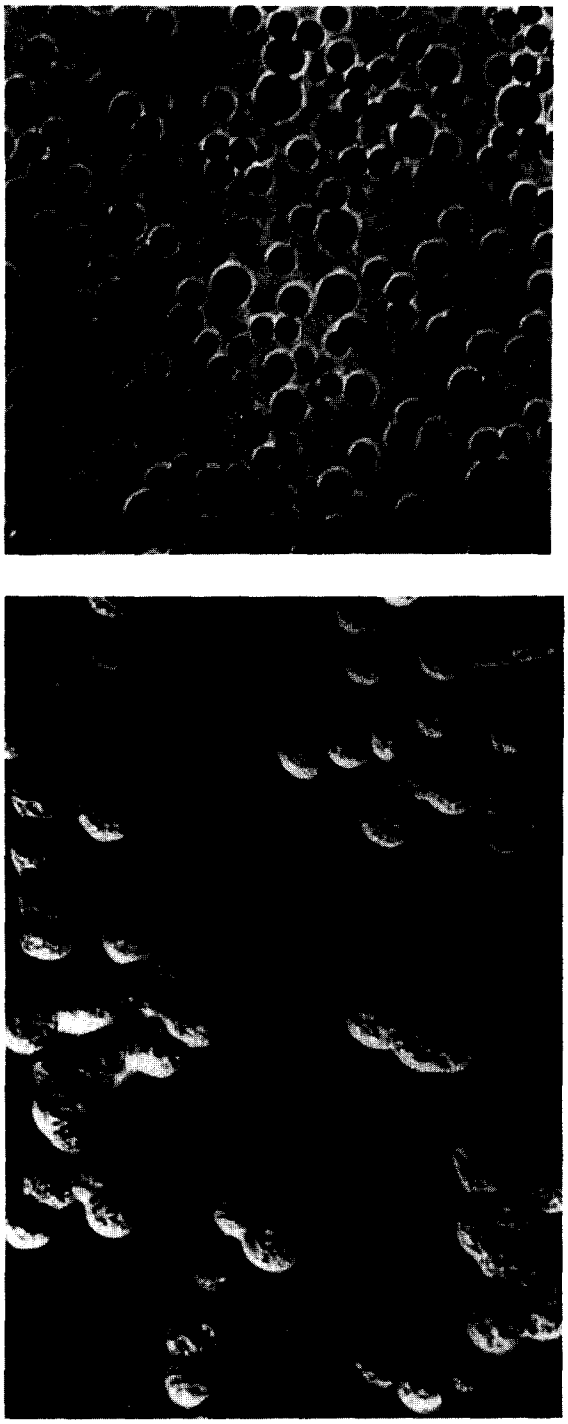
to the electrofusion procedure are shown in Fig. 3b. No fusion was observed in cell suspensions exposed to ultrasound alone in the absence of the electric-field pulses.

Some fused erythrocytes were observed when cells, at a concentration of approx. $5 \cdot 10^8$ cells/ml were exposed to the electric-field pulses in the absence of ultrasound but the number was small compared with that observed when ultrasound and electric-field pulses were combined. This result confirmed an earlier report by Zimmermann and Pilwat [8] that erythrocytes could be fused by application of electric-field pulses alone provided that the suspension density was sufficiently high or that the pulse was applied to a cell pellet. No fused cells were observed when erythrocytes, at a concentration of 10^5–10^6 cells/ml were exposed to electric field pulses in the absence of ultrasound.

At cell concentrations of 10^4–10^5 cells/ml fusion preferentially took place between two cells whereas at higher concentrations aggregates of four or more cells were also observed. This can be easily demonstrate by fusing SP 2/0 mouse myeloma cells. Using an initial concentration of 10^6 cells/ml doublets as well as aggregates of more than two cells are observed. Fig. 3b shows a suspension of myeloma cells two minutes after irradiation and field-pulse application, taken from the fusion chamber and suspended on a microslide. The elongated cells will become completely spherical like the cells in Fig. 3a, if phosphate-buffered saline is added to the suspension 10 minutes thereafter.

The occurrence of fused doublets at low cell concentrations could be particularly significant from the point of view of fusing myeloma cells with lymphocytes for the production of hybridoma cells. Preliminary experiments showed that considerable amounts of mouse hybridoma cells could be

obtained when lymphocytes and myeloma cells were exposed to ultrasound and field pulses. The number of clones obtained in this way seemed to be higher compared to the yield when using chemicals (poly(ethylene glycol), PEG). The yield of hybridomas could apparently be increased if more conductive solutions were used.



A number of experiments were carried out to establish if a standing-wave field, in which cells would accumulate at regions separated by half an acoustic wavelength, could be set up in a transparent cell container of a width similar to the 200 μm gap of the fusion chamber. A 5 cm long, rectangular cross-section, glass microslide (Camlab, Cambridge, U.K.) of 2.0 mm width and 0.2 mm pathlength was filled with an erythrocyte suspension.

The microslide was placed in a cuvette, containing phosphate-buffered saline, which was mounted on the ultrasonic transducer (Fig. 2a). The formation of cell bands could be discerned after 1 s when the transducer voltage was 50 V p-p. The development of bands was complete within 5 s (Fig. 2b). The high magnification micrograph of Fig. 2b shows that the separation of cell concentration regions corresponded to approximately half the wavelength of sound in water.

In a further preliminary experiment it was shown, that cell banding could occur in a gap such as that between the two metal cylinders of the fusion chamber. For this purpose two glass cylinders with slightly different diameters, made of Pasteur pipettes were placed one inside the other. The gap of approx. 200 μm width was filled with a suspension of red blood cells. Irradiation with ultrasound led to cell banding in this system.

Fig. 3. (a) Fused erythrocytes after electro-acoustic treatment of a suspension of human red blood cells. 20 μl of the cell suspension are sucked into the fusion chamber and the chamber is immersed into the container of the ultrasound set-up. After a 10 s period of sound irradiation three electric-field pulses of 10 μs duration and 80 V amplitude are applied. Fusing cells are now maintained in contact by a further sound irradiation of 50 s and then ejected from the fusion chamber to a microslide. After ten minutes small amounts of phosphate-buffered saline are added and the cells become spherical. (bar: 25 μm). (b) Fusing myeloma cells (SP2/0) two minutes after electro-acoustic treatment. Following the same protocoll as in (a) with the exception of a pulse amplitude of 60 V, the cells are pipetted on a microslide. The elongated cell aggregates will become spherical after the addition of phosphate-buffered saline approx. 10 min later. (Interference phase micrograph, bar: 20 μm).

Discussion

When a cell suspension is exposed to ultrasound, radiation forces of non-zero-time average [2] arising from two different mechanisms can operate to bring the cells close together. One such radiation force (F_1) operates to bring cells into contact in such a way that the line connecting their centres will become aligned in a plane at right angles to the direction of the velocity amplitude in the absence of the particle [5]. An expression has been derived for the magnitude of F_1 [2] and a criterion suggested for determining the minimum value of the velocity amplitude required to overcome the tendency, due to thermal motion, for two particles to drift apart [16]. In a standing-wave situation a second force F_2 operates to move cells close to the velocity maxima in the sound field [5,6]. F_1 has its maximum value at velocity maxima. Consequently, the combination of the effects of both forces in a standing-wave field will move particles to preferred areas in the sound field and at these positions the aggregation forces will be at maximum. It is not known how closely two cells can approach each other in ultrasonic fields such as those generated in the present work. It is generally accepted [9] that fusion can occur when the membranes of at least two cells come within 2 nm of each other. It may be that the cells do not come this close to each other in the ultrasonic fields used here but that the required proximity for fusion is achieved during application of the breakdown pulse. As described in detail elsewhere [9] high local suspension densities can lead to dielectrophoresis during the application of the electrical breakdown pulse because the electric-field distribution is strongly inhomogeneous under these conditions. Thus electrofusion may occur in ultrasonically aggregated cells because high local concentrations can be achieved in a cell suspension of low initial concentration. The ultrasonic technique has the advantage over some other cell concentration techniques that the initial homogeneous cell suspension is concentrated into many small locally concentrated regions rather than into a single pellet.

At the present time it is difficult to decide which technique, electro-acoustic or conventional electrofusion, would be most appropriate for the fusion of particular cell types. In general, the electro-acoustic method enables cells to be fused in conducting solutions i.e. in partly physiological electrolyte solution. It shares this advantage with the magneto-electrofusion method [12], while the purely electrical fusion technique [9–11] – if no sophisticated equipment is used – has to be carried out in weakly conducting solutions because of the associated slight increase in temperature. Fusion in physiological solutions could lead to a considerable increase in the viability of the fused cells (see above). On the other hand, we cannot rule out the possibility that the opposite effect might occur with fusion in conducting saline. As discussed elsewhere [11] the use of non-electrolyte solutions might serve to protect the cell interior, if the membrane has been rendered permeable, from the electric field to which it is exposed for a short period of time. The possibility of comparing electrofusion in conducting and weakly conducting solutions will enable the better method to be selected for any cell type.

Compared to magneto-electrofusion [12] the electro-acoustic method has the advantage that the properties of the cells do not have to be changed artificially (e.g. by labelling the outer membrane with small magnetic particles).

The electro-acoustic method could have another advantage when fusing cell species which exhibit different complex dielectric constants at those frequencies of the alternating electric fields (dielectrophoresis) used to establish membrane contact. This is because the existence of an aggregation force F_1 in the electro-acoustic method requires only that the density of the cells be different from the density of the suspending fluid. The standing-wave force F_2 depends on the compressibilities of the cells as well as their densities. These cell properties are, to a first approximation, similar for all mammalian cells. This fundamental difference between the cell properties used to aggregate cells with ultrasound rather than with an alternating electric field justifies further studies in this area and promises interesting results in the future, particularly in the area of production of hybridoma cells.

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