CELL ADHESION PROCESSES IN A STANDING WAVE TRAP

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Abstract

A novel ultrasound trap is described to characterise and control the progression of cell to cell adhesion in suspension away from any deformable substratum. The full system consists of a single half-wavelength ultrasound chamber, epi-fluorescent microscope, video camera and image analysis routines. Mechanically strong and manipulable 2-D arrays of interacting mammalian cells can be produced in suspension. The acoustic interparticle forces do not affect the cell aggregate morphology and stability at surface separations of the order of 10 nm over which receptors operate. Temperature measurements showed that there was no measurable temperature increase in the trap ($\Delta T<0.5K$). The technique allows continuous monitoring of the progression of 2-D cell aggregate formation from initial cell-cell contact, offering the prospect to address fundamental biological questions with reference to tissue construction, intercellular communication and differentiation.

Introduction

Cell-cell interactions are ubiquitous throughout biology. All cells in multicellular organisms experience these interactions in a range of different contexts such as tissue construction, intercellular communication and information transfer. Cells can bind selectively to receptors present in the extracellular matrix (cell-matrix interaction) or to the plasma membrane of other cells (cell-cell interaction). Cell adhesion events can affect central aspects of the structure and function of individual cells, including cytoskeletal organisation, cell proliferation and differentiation.

Cell-cell adhesion and spreading have been studied as the ability of cells to adhere to a solid substratum, whereas less work has been done with cells in culture [1] or in cells of disaggregated tissues e.g. micromass culture of early chondrogenic cells [2]. Martin et al. [1] emphasised the need for fine quantification of the kinetics of cell adhesion as these could successfully represent complex biological reactions *in vivo*. However, even though valuable parameters such as aggregation percentage, average number of cells per aggregate and statistical distribution of aggregate size were extracted, the dynamics of cell adhesion - membrane spreading- remained unexplored, as there has been no simple method of maintaining aggregates in focus for a significant time.

Here, we apply a recently developed ultrasound trap that maintains cells in suspension away from any inorganic substratum and allows observation of cell-cell adhesion in a 2-dimensional aggregate developing in suspension.

The morphologies of 2-dimensional erythrocyte cell aggregates formed in the standing wave trap were examined. It was found that the aggregates were well packed with the cells arranged in hexagonal order as there are no strong net intercellular interactions on the macromolecular scale. In contrast, the morphology of aggregates formed by erythrocytes in the presence of the lectin wheat germ agglutinin (WGA) was dendritic. The perimeter of the final aggregates was quantified with fractal dimension measurements. The temperature dependence of plasma membrane spreading occurring on neighbouring neural cells was also investigated. At high temperatures (33^0C) neighbouring cells progressively spread over each other resulting in the loss of some of the individual cell boundaries. At temperatures as low as 12^0C, membrane spreading was repressed. Membrane spreading of erythrocytes in the presence of WGA was also observed.

The results suggest that the technique may aid expression of some aspects of the complex biological reactions occurring *in vivo* more effectively than do studies on solid substrata.

Methods

Ultrasound trap

The main features of an ultrasonic trap are: a transducer, a sample region and a quartz glass reflector, in a housing of radial symmetry (Fig.1). Two such traps were employed in the present work. The disc (12 mm diameter) transducers (Ferroperm, Kvistgart, Denmark) had a nominal thickness resonance frequency of 1.5 and 3 MHz. Their electrodes were etched to give 6 mm diameter circular back electrode. The quartz glass reflectors provided optical access to the sample region. The traps driven at around 1.57 and 2.45 MHz respectively, had an acoustic pathlength of $\lambda/2$ at the driving frequency.

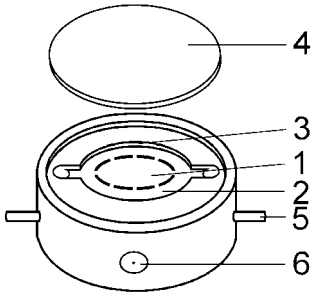


Figure 1: A radially symmetric ultrasound trap (Courtesy of L. Kutznetsova).
1) Active area, 2) Steel area, 3) Water layer,
4) Reflector, 5) Inlet/Outlet, 6) SMA connection

A cooling device or a water bath directly connected to a trap offer the ability to control sample temperatures *in situ* ranging from 10-40^{0}C. A 100 µm thermocouple bead located at the centre of the sample area allowed accurate temperature measurements without disturbing the sound field. The pressure amplitudes in the traps, calculated as described by Spengler et al. [3] were 0.27 MPa. The function generator, amplifier and control system providing the sinusoidal voltage applied across the transducer at the selected frequency have been already described [3].

Optical system and video recording

Observation in the direction of sound propagation, (z-) axis, was performed with an Olympus BX40 reflection epi-microscope. Video recording and sonication started shortly after the sample was pumped into the chamber. The aggregation process was observed under × 10 and × 50 magnifications and was video recorded onto a tape and PC by a PAL CCD camera (Fujitsu). The perimeter fractal dimension analysis was performed as described by Spengler and Coakley [4].

Erythrocytes and lectin

Human erythrocytes were collected by finger prick into 10 ml phosphate buffered saline (PBS), centrifuged and washed twice with PBS. Cells were resuspended in a volume of PBS supplemented with bovine serum albumin (BSA) to give a cell concentration of 10^6 or 2×10^5 cells/ml. A range of lectin solutions (0.05-10 µg/ml) was added to the cell suspension immediately before loading into the ultrasound trap.

Culture condition of 921202-6 (C6) neural cells

The 921202-6 (clone 6) cell line (isolated from the rat mesencephalon) was maintained as a replicative culture at 33^{0}C and 5% CO_2, in neuronal supplemented medium (NSM) and supplemented with 5 ng/ml FGF-2. The cells were attached onto gelatin/poly-L-lysine coated culture flasks. At the confluence phase cells were rinsed with Dulbecco's Phosphate Buffered Saline (DPBS) without Ca^{2+} and Mg^{2+}. Cells were then released from the plastic culture flask. 5 ml of NSM were then added. One sixth of the cells were plated to a fresh gelatin/poly-L-lysine coated flask. The remaining cells were centrifuged, resuspended in NSM and diluted to 10^5 cells/ml.

Results

Erythrocyte aggregate morphology

Control erythrocytes suspended in PBS/BSA moved into the focused node plane within 1.0 s. Single cells and miniclusters are in the central region of the node plane after 4.5 min (Fig.2a). New cells and clusters move into this region over the ensuing minutes (Figs. 2 b,c). Close packing of cells and the emergence of hexagonal order in the structure of the clusters is already clear in Fig. 2 (a, b). On contact with the main aggregate the cells of the cluster rearranged themselves to accommodate the aggregate's peripheral cells in the contact region so that the order in the aggregate structure was propagated (Figs. 2c, d). (This ability to rearrange order was also seen when 25µm latex particles in deionised water formed an aggregate in an ultrasonic trap [4]). The rearrangement of the control erythrocytes implies that the attractive interaction forces between the cells are weak and that the system is behaving as a reaction limited (RLCA) system. The fractal dimension of this aggregate was 1.16.

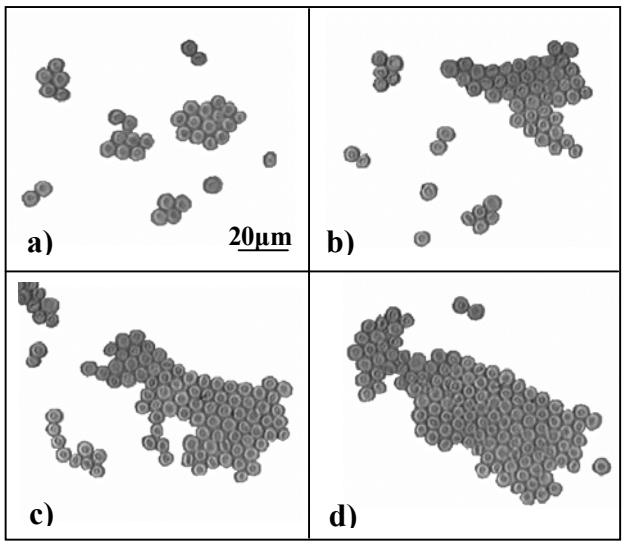


Figure 2: Growth of a typical closely-packed control erythrocyte aggregate

Erythrocyte aggregates formed in the presence of WGA (0.5 µg/ml) exhibited little evidence of the emergence of hexagonal order in the control situation either for clusters approaching the aggregation region (Fig. 3a) and for the final aggregates (Fig. 3d). The fractal dimension is this case was 1.49 (Fig.3d). Membrane spreading of erythrocytes suspended in PBS with WGA was observed.

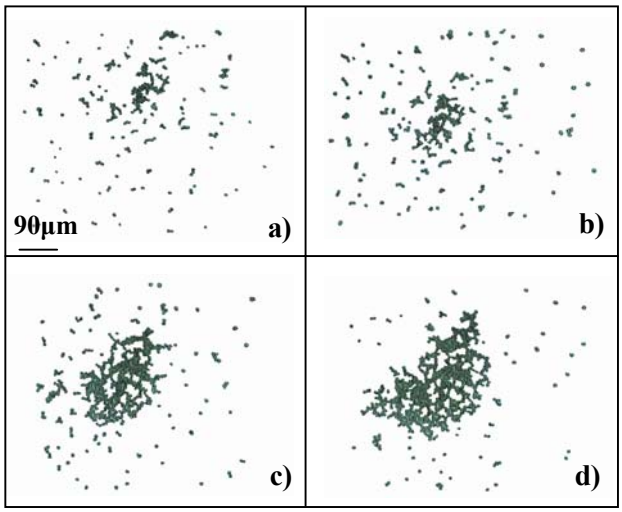
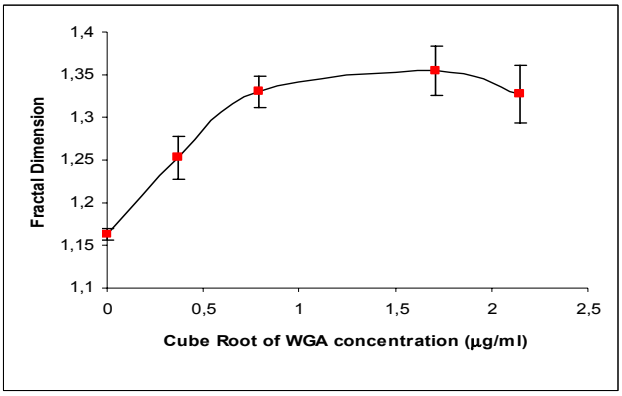


Figure 3: Growth of a dendritic erythrocyte aggregate in the presence of 0.5 µg/ml WGA

Erythrocyte aggregate fractal dimension dependence on WGA concentration

The fractal indices of the final erythrocyte aggregates formed in the trap are shown in Fig.4 for a range of [WGA]. The mean fractal indices were already slightly higher than control values at 0.05 µg/ml and had increased towards a limiting value at 0.5 µm/ml. Microscopic assessments of the aggregate morphology



showing progression from well packed to dendritic forms with increasing lectin concentration were consistent with the fractal measures.

Figure 4: Fractal dimensions of erythrocyte aggregates formed in WGA after 6 min, as a function of lectin concentration. The abscissa is presented as the cube root of [WGA] for data presentation purposes only.

Neural cells

Neighbouring neural cells suspended in DMEM at 33°C spread over one another in a time period of 20 min so that the ability to resolve some of the cell boundaries decreased (Fig. 5a-c). Eventually large bodies consisting of numerous cells were observed.

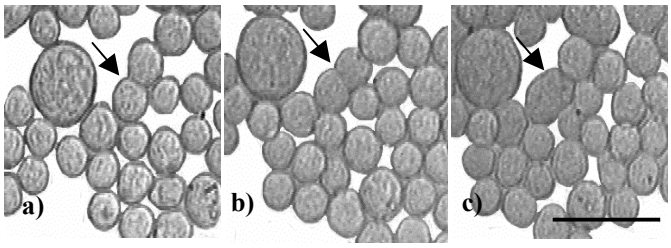


Figure 5: Development of membrane spreading (arrows) in a neural cell aggregate suspended in neuronal supplemented medium (33^0C) a) 0 min, b) 10 min and c) 20 min after ultrasound exposure (scale bar 25 µm)

On the other hand, neural cell membrane spreading did not occur in DMEM at 12°C for the first 15 min of ultrasound exposure; some signs of membrane spreading were evident after 20 min of sound initiation (Table 1).

Table 1: Temporal progression of the incidence of membrane spreading (as shown in Fig. 5a) of neural cells pre-suspended at different temperatures

Time (min)	12°C	33°C
5	0	10
10	0	23
15	0	28
20	3	38

Discussion

Closely packed aggregates (control erythrocytes) resulted when an incoming cell rolled about the edge of the main aggregate on collision or an incoming cluster rapidly reorganised its structure to accommodate the shape of the main aggregate. The collision efficiency, i.e. a measure of the occurrence of strong interactions on contact is low (approaching 0) for such closely packed aggregates so that the aggregation was reaction limited (RLA). Dendritic forms (erythrocytes with WGA) arose when incoming cells or cell clusters were

held without rolling or reorganisation on contacting the main aggregate. The collision efficiency approaches 1.0 for these aggregates so that aggregation is transport or diffusion limited (DLA). The aggregate morphologies observed in the trap reflect the known properties of the cell surfaces. Erythrocytes in buffered suspension in test tubes do not normally aggregate so that the collision efficiency is therefore low. Erythrocytes in suspension *in vitro* aggregate and agglutinate in the presence of WGA so that the collision efficiency for that case will tend towards 1.0. It has previously been shown, by light and scanning electron microscopy of samples taken at discrete sample points during a 1 h long incubation in a test tube, that erythrocyte membranes spread over each other as they agglutinate in the lectins WGA or concanvalin A [5]. The phenomenon has been confirmed here by real time observations of unfixed cells. Progressive spreading of neural cell membranes was temperature dependent; at 12^0C cells at first did not spread (a time lag of ~20min was required), a situation that reversed at 33^0C. Membrane spreading is the formation of motile cell surface protrusions i.e. lamellipodia, containing a meshwork of newly polymerised actin filaments. Gordon et al. [6] suggested that polymerisation of actin in solution proceeds more slowly at lower temperatures, whereas Howard and Oresajo [7], proposed that a low temperature *per se* might not be expected to start a signalling cascade event (that involves regulation by actin-binding proteins present in the cytoplasm, such as GTP-binding proteins) necessary for actin polymerisation. Video microscopy of the incidence of membrane spreading has confirmed the above suggestions.

Fractal analysis

The perimeter fractal index reported for control erythrocytes was similar to those reported for 25 μm diameter latex particles in deionised water (1.14). The fractal dimensions of the respective dendritic aggregates approached that (1.59) reported for a dendritic aggregate of 25 μm diameter latex particles in $CaCl_2$ solution in the ultrasonic trap (Spengler and Coakley, 2003). The fractal dimension for 0.05 μg/ml WGA was slightly but significantly higher than control values (Fig. 4) while that for 0.15 μg/ml WGA was already approaching the limiting value seen at higher concentrations. Studies of latex particles have shown that 'crossover' from low (RLA) to higher (DLA) fractal indices can occur when the short-range interactions between particles are modified e.g. by change in the suspending phase electrolyte concentration [8]. 'Crossover' occurred here at a WGA concentration of about 0.1 μg/ml.

Conclusion

The ultrasonic standing wave trap enables the aggregation process and the dynamics, i.e. membrane spreading, accompanying it to be continuously monitored. The morphology may be characterised by image analysis techniques such as perimeter fractal index determination. The influence of cell cross-bridging molecules on morphology can be clearly observed in the 2-D aggregates. The interacting cells are in suspension away from the influence of solid substrata. The technique is sensitive to concentration of cell-linking molecules and offers the potential to monitor the temporal progression of the expression of surface receptors on cultured cells in suspension.

References

[1] I. Martin, B. Dozin, R. Quastro, R. Cancedda, F. Beltrame. Computer based technique for cell aggregation analysis and cell aggregation in vitro chondrogenesis. Cytometry, vol.28, pp. 141-146, 1997.

[2] W. M. Kulyk, J. L. Franklin, L. M. Hoffman. Sox9 expression during chondrogenesis in micromass cultures of embryonic limb mesenchyme. Exp. Cell Res., Vol. 255, pp. 327-332, 2000

[3] J. F. Spengler, M. Jekel, K. T. Christensen, R. J. Adrian, J. J. Hawkes, W. T. Coakley. Observation of yeast cell movement and aggregation in a small-scale MHz-ultrasonic standing wave field. Bioseparation, vol.9, pp. 329-341, 2001.

[4] J. F. Spengler, W. T. Coakley, An ultrasonic trap to monitor morphology and stability of developing microparticle aggregates. Langmuir, vol.19, pp. 3635-3642, 2003.

[5] H. Darmani, W. T. Coakley, A. C. Hann, A. Brain. Spreading of wheat germ agglutinin-induced erythrocyte contact by formation of spatially discrete contacts. Cell Biophys. vol. 16, pp. 105-126, 1990..

[6] D. J. Gordon, Y. Z. Yang, E. D. Korn. Polymerisation of Acanthamoeba actin. Kinetics thermodynamics co-polymerisation with muscle actin. J. Biol. Chem., vol. 251, pp. 7474-7479, 1976.

[7] T. H. Howard, C. O. Oresajo. The kinetics of chemotactic peptide-induced change in F-actin content F-actin distribution and the shape of neutrophils. J. Cell Biol., vol. 101, pp. 1078-1085, 1985.

[8] D. M. Asnaghi, M. Carpinetti, M. Giglio, M. Sozzi. Coagulation kinetics and aggregate morphology in the intermediate regimes between diffusion-limited and reaction-limited cluster aggregation. Phys. Rev., vol. A45, pp. 1018-1023, 1992.