A technique for encapsulation of high cell density aggregates

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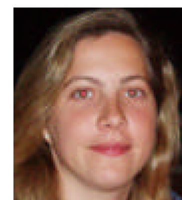

Introduction

The alginate encapsulation of cells to confine biomass within microcapsules for cell grafts and its recent combination with biosil capsule coating is an active area of research (Carturan et al., 2004). The microcapsules produced by droplet formation of a cell suspension in alginate into a $CaCl_2$ solution are spherical in form. The cells are dispersed within the capsule and capsule size is restricted by the need to maintain cells of the propagating cell mass within the limits set by nutrient supply and gas exchange. We report proof of principle here of a gel encapsulation technique that departs from the minimum surface area to volume restriction of spherical microcapsules and allows gelation of preformed cell aggregates reducing the need for pre-graft incubation. The process involves forming a discoid cell aggregate in an ultrasound standing wave resonator and then introducing an alginate/$CaCl_2$ pre-gel into the ultrasound trap where it preferentially sets about the cell aggregate. An ultrasound standing wave field is generated by a resonant system applied to an ultrasound trap and is characterized by a spatially periodic distribution of acoustic forces with peaks of high pressure and areas of minimal acoustic pressure amplitude. When ultrasound is applied to a suspension of cells, these tend to move towards areas of minimal pressure, thus creating a spatial arrangement within the suspension. Gherardini et al. (2005) formed aggregates of *Saccharomyces cerevisiae* in agar gel and then triggered gel formation across the whole cell suspension. Subsequent sectioning and staining of the gel showed that both propagation and viability were retained in the treated cells. The approach is modified here in a manner that restricts gel formation to the region of the cell aggregate so that it may be withdrawn for subsequent application. Once the cell suspension is inserted to the trap and ultrasound is applied in a specific frequency value -called resonance- and due to the lateral radiation force, cells begin to migrate to the center of the trap thus forming a discoid aggregate, usually within seconds (Fig. 1a).

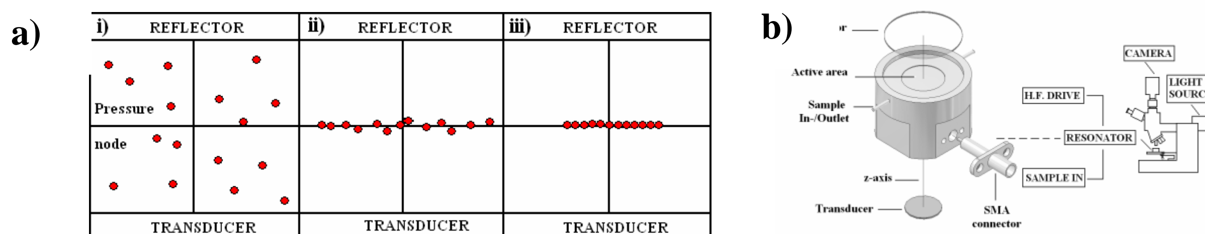

Fig. 1: a) Schematic diagram of the temporal progression (from time zero (i) to less than one second (ii) to tens of seconds (iii)) of aggregation of suspended cells in a single pressure node half-wavelength ultrasound trap; b) Schematic diagram of the cylindrical steel trap assembly, epi-microscope, sample loading and ultrasound generation. Its main components were a 3 MHz disc transducer attached to a steel acoustic coupling layer, a sample volume and a glass acoustic reflector.

Materials and Methods

Alginate Hydrogel Preparation and estimation of solution viscosities

The alginate pre-gel was prepared as appropriate mixtures of low viscosity alginate (BDH, Poole, England) and $CaCl_2$ solutions. Alginate/$CaCl_2$ solution viscosity was estimated from video-determined sedimentation times for spherical Ballotini glass beads (average radius 220 µm and material density is 5,245 kg/m^3) over a measured distance in a test tube.

Test suspensions

Human erythrocytes were obtained by finger prick. A drop of blood was diluted in 10 ml of phosphate-buffered saline (PBS) and the suspension was centrifuged at 1200 rpm, washed twice and resuspended in 10 ml of PBS containing 0.5 mg/ml bovine serum albumin (BSA). The erythrocyte concentration was then adjusted**,** using PBS/BSA solution, to $1x10^5$ cells/ml.

Ultrasound standing wave trap

The ultrasound trap (Fig. 1b) employed in the present work had four layers: a transducer (Ferroperm, Kvistgard, Denmark) nominally resonant in the thickness mode at 3 MHz and mounted in a radially symmetric housing, a steel layer coupling the ultrasound to a one half wavelength ($\lambda/2$ or 0.25 mm depth, where λ is the wavelength of sound in water at 3 MHz) aqueous layer and a reflector that provided optical access from above (Fig. 1b). The outer diameter of the cylindrical steel body was 35 mm. The disc transducer (12 mm diameter) was driven at 2.2 MHz. Its back electrode was etched to a 6 mm diameter circle so as to give a single central aggregate in a single half-wavelength trap. The quartz glass reflector had a thickness of 0.5 mm ($\lambda/4$ in glass) so as to locate the single pressure node plane half way through the sample volume. The transducer drive voltage came from a function generator (HM 8138, HAMEG, Germany). The sound pressure amplitude (P_o) was estimated as described by Khanna et al. (2003), as 0.85 MPa. Observation in the direction of sound propagation (negative z-axis) was performed as described by Bazou et al. (2005). The trap was placed on a stage of a microscope (Olympus BX40) fitted with a Fujitsu video-camera. The microscope was pre-focused on the trap's pressure nodal plane. Samples were fed to the trap with a Minipuls peristaltic pump (Gilson). Sonication and video recording started shortly after the sample was introduced into the trap. Video records were converted into 'tiff.' files by MiroVideo capture DC30 software and imported to Adobe Premiere. Selected frames were then imported to Adobe Photoshop 5.5 for further analysis.

Production of localized gels in the ultrasound trap

Once cell aggregates were formed in the ultrasound trap, the alginate pre-gel was slowly drawn into the trap with the peristaltic pump. The pump flow was set on a very low speed so as not to disturb the aggregate. On continuous application of ultrasound the pre-gel sets around the cell aggregate over a period of 10 min. Encapsulated cell aggregates were recovered from the trap with the peristaltic pump and placed on histobond slides (RA Lamb) to assess the mechanical stability of the removal process.

Results and Discussion

Erythrocyte Aggregate Formation in an Ultrasound Standing Wave

Upon ultrasound initiation erythrocytes began migrating to the pressure node plane at the center of the trap. Cell suspensions of 10^6 cells/ml produced multiple-layered while 1×10^5 cells/ml produced single-layered aggregates. The lower concentration results are presented here for clarity of microscopic presentation (Fig. 2a, b).

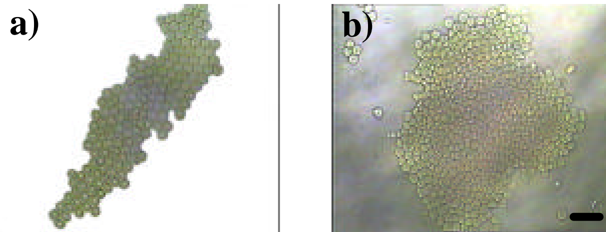

Fig. 2: a) Erythrocyte aggregate formation in an ultrasound standing wave trap after 30 sec; b) gelation of an erythrocyte aggregate. Scale bar is 25 µm.

Hydrogel Construction and Rheological Tests

The selected gelation time was 10 min, in order to avoid gelation occurring instantly after mixing the alginate with the $CaCl_2$ solution in bulk or in the inlet tube. The measured viscosity values are given below (Table 1):

Solution	Viscosity value
Distilled H_2O	1.0 mPa s
3% low viscosity alginate in distilled H_2O	275 mPa s
3% low viscosity alginate & 5mM $CaCl_2$ in distilled H_2O	355 mPa s

Table 1: Rheological test results of the solution examined in the present study

Therefore, a solution consisting of distilled water with 3% alginate and 5mM $CaCl_2$ satisfies the previously mentioned requirements and forms a mechanically stable and uniform gel.

Cell Encapsulation

With the progress of time and as gelation was occurring it was observed that single cells coming into the aggregate would hit the gel boundary and stop migrating. This observation constitutes a strong

indication that the alginate/$CaCl_2$ system was forming an alginate hydrogel about the cell aggregate (Fig. 4a-c).

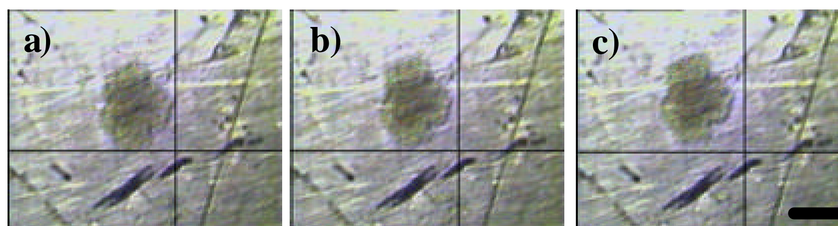

Fig. 4: Sequence of images (a-c) with horizontal and vertical axis set (fixed cross-wire) illustrates that the alginate hydrogel and the cell aggregate move lateral as a system, rather than independently, on slightly changing the drive frequency, as the distance between the cell aggregate and the fixed cross-wire increases. Scale bar is 100 µm.

The mechanism through which solidification of the alginate pre-gel about the cell aggregate was initiated has not been established. One possible mechanism is that ultrasound induced microstreaming (Spengler et al., 2003) ensuring good mixing of the alginate and radiation force attracting the growing sol particles into the region of cell aggregation Preliminary work has also shown that 2 and 3-D aggregates of HepG2 cells formed in the ultrasound trap and subsequently recovered from it, exhibit strong F-actin accumulation at sites of cell-cell contact after 3 days in culture; the viability of these aggregates remained essentially unchanged at 88 %.

Conclusions

In the present study, the principle of gel encapsulation of cell aggregates in an ultrasound trap is established. The alginate/$CaCl_2$ pre-gel preferentially sets about the cell aggregate. The discrete encapsulated cell aggregates (discrete capsules) are discoid in shape and have a thickness that allows accessibility to nutrient and gas exchange. The technique described here departs from the minimum surface area to volume restriction of spherical microcapsules, thus providing an alternative method of alginate encapsulation of cells.

References

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