A new ultrasound-based cell immobilisation technique

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ABSTRACT

Ultrasonic standing waves fields can act on particles in suspension and can concentrate them in nodal (or antinodal) planes of acoustic pressure. This principle of ultrasonic cell trapping is applied in numerous biotechnological applications, such as mammalian cell filtering and red blood cells sedimentation. We developed a new ultrasound-based cell immobilisation technique, which allows the manipulation of cells/particles in different gel matrices. This creates a defined distribution of particles entrapped within the gel. The challenge now is to develop novel biotechnological applications of this new technique. The paper discusses the potentials of ultrasonic cell manipulation as a new cell-immobilisation/encapsulation technology.

INTRODUCTION

Bio-encapsulation of living cells into a polymer gel matrix is one of the most common techniques used for manipulating microbial cells (McLoughlin, 1994). Essentially, this technique creates a spatial arrangement of the cells within the semi-rigid matrix, and results in a defined microenvironment where colonies can develop. A new immobilisation technique based on the use of ultrasound has been investigated (Radel et al, 2000). It is based on the principles that an ultrasonic standing wave field of appropriate frequency and intensity applied to a homogeneous cell suspension caused cells to quickly move into areas of lower ultrasonic pressure, called pressure nodal planes (Gröschl et al., 1998). Based on these principles, an acoustic filter for mammalian cells initially designed for experimental purposes (Trampler et al., 1994) and further developed for industrial applications ("Bio Sep", Applicon, The Netherlands), is routinely used in cell perfusion systems. A number of studies indicated that the acoustic field caused no variation in particle integrity and cell viability in mammalian cells and erythrocytes (Doblhoff-Dier et al., 1994; Gröschl et al., 1998).

Direct investigation of those phenomena that determine the spatial arrangement of the cells is therefore fundamental to understand acoustic filtration principles. Analysis is limited by the fact that spatial arrangement only exists for as long the acoustic field is applied. Recently we have developed a gel-immobilisation technique that allows the particle to be retained in the spatial arrangement, which results from the application of the acoustic forces and lasts even if the force field is removed (Gherardini et.al 2001). This allows a direct investigation on the effect of ultrasonic waves on cell location and intracellular interaction. In this paper, we demonstrate the versatility of this technique, with the use of several different ultrasonic devices, biological systems and supporting matrices. This technique, which combines expertise in classic gel

immobilisation and ultrasound technology, will benefit new biotechnological application such as cell encapsulation.

MATERIALS AND METHODS

The different devices employed in this investigation are designed according to the general model of enhanced cell sedimentation by ultrasound standing waves (Gröschl, 1998).

The acoustic devices

The plane resonator. The Paar USSD-05 resonator with a chamber containing 60ml of suspension was employed in batch mode. The system consists of a piezoelectric ceramic transducer, a cooling chamber and an active volume chamber. Superposition of the incoming waves and reflected waves formed the quasi standing wave. The experiment was carried out generating a plane wave with a frequency of approximately 2.2MHz and 14W of electrical true power input on a lead zirconate titanate (PZT) transducer area of 12.5 cm^2. The chamber was filled with cell-gel mixture through the top aperture. When the gel is formed, the chamber can be dismantled and the gel collected to use for further analysis.

The cylindrical resonator. A sine wave of the required frequency was generated by a Philips PM5192 programmable synthesiser. The synthesiser output went to a broadband power amplifier (Model A150, ENI Rochester, N.Y.) controlled by a PC programme. The ceramic transducer, (22mm i.d., 37.6mm length, 1.6mm wall thickness) was made of PC4D PZT (Morgan Matroc, Wrexham, UK) driven at its fundamental resonant frequency of 1.6 MHz. The base was designed to hold a 4 ml rounded-end tube with 12 mm o.d. on the axis of the sound field so that the level of blood in a full Vacutainer reached a level 2 mm lower than the top of the transducer (Cousins et al., 2000). 50 µl blood was added to 4 ml of iso-osmotic technical agar (1:80) in a Falcon tube (Becton Dickinson). The tube was placed into the sonication chamber, filled with water at 37^{o}C to prevent the gel setting before banding occurred. Ultrasound was applied continuously and the gel set after 4.5 minutes of treatment. Slices of 10 µm thickness were taken from planes of the gel were cells had visibly concentrated and the slices were examined by light microscopy.

The cylindrical flow through system. A ceramic transducer made of PC4D PZT (Morgan Matroc, Wrexham, UK) was used for these experiments. The transducer was driven at both its fundamental (1 MHz) and at the third harmonic (3 MHz) of its fundamental frequency by a RF amplifier (ENI model 240L, Rochester NY). The transducer set for the flow through experiment allowed a capillary tube of 1mm I.D. to be inserted in the aperture on the lower part of the transducer. A robber tubing was connected to the upper end of the capillary tube to allow the flow through of the fluid gel suspension. A syringe was connected at the upper end of the tubing to create a reservoir of fluid gel. Gravity induced the gel to slowly flow through the tubing system at a speed of 10-20mm s^{-1}. When the suspension reach the cylindrical resonator the treatment took place re-arranging the particles accordingly to the frequency used. The particles maintain their position until the gel was immersed into a curing Ca^{++} ion bath. The rod of gel forms immediately after entering into the curing solution and can be easily recovered.

Image analysis

Light microscopy. 8-10 micron thick horizontal sections of the gel blocks were cut at room temperature, recovered and mounted on common microscopy slides. Photographs were taken using conventional Kodak film.
Confocal microscopy. An epifluorescence microscope (Zeiss Axiovert) with a laser confocal

system (MRC 1024;Bio_Rad Laboratories, Hemel Hempsted, UK), comprising a 15mW Krypton/Argon (Kr/Ar) laser. This laser could be excited at wavelengths of 488-, 568- and 647-nm. Three photomultiplier tubes with 522DF35, 585EFLP and 680DF32 emission filters were

used for preliminary studies. Image processing was carried out with LaserSharp PC software (Bio-Rad Laboratories). We also used a Molecular Dynamics confocal microscope, with Image Space software run on Silicon graphics workstations.

Gel matrices

The acoustic properties of the standing wave resonant systems in presence of the gel matrices are similar to those detected in presence of water (data not shown).

Agar: 3% w/v malt extract Agar (0.9g of agar) and 1% w/v technical agar was prepared in distilled water. For the experiment with red blood cells, 1 % w/v technical agar (Oxoid Ltd, Basingstoke, UK) was made up using physiological saline solution. The solutions were sterilised at 121^0C for 15 minutes and stored at 37^0C until they were used in the experiments.

Alginate gel. It was used in a concentration of 3% in water. The ionotropic nature of the alginate allowed the immediate gelation as the fluid suspension came in contact with Ca^{++}

Particle in suspension

We employed two biological models, yeast and red blood cells, that have been previously used in analytical applications of the acoustically enhanced particle sedimentation principle (Cousins et al., 2000; Radel et al 2000) and an inorganic model latex particles, because of their auto-fluorescent nature.

S.cerevisiae and *Lactobacillus brevis* were separately cultivated for 24h in malt extract broth (Oxoid) and MRS broth (Oxoid), respectively. Cells were inoculated and incubated at 30^0C on an orbital shaker table set at 150rpm. Prior to sonication both populations were mixed thoroughly using a magnetic stirrer. The final concentration of the yeast in the suspension was ~ 4.6×10^7 and the bacterial concentration was 2×10^8 cells per ml.

Red blood cells (RBCs): Blood samples were taken from a volunteer, by veinipuncture, into 4ml Vacutainers containing 95U lithium heparin (Becton Dickinson, Oxford, UK) immediately before experiments.

Latex particles. Auto-fluorescent latex particles with a diameter of 9 microns (Polymer Labs) were diluted in technical agar at appropriate concentration.

RESULTS AND DISCUSSION

According to acoustic theory, cell/particle manipulation during acoustic treatment primarily depends on the effect of both longitudinal and transversal acoustic field versus single particle interaction forces, which characterise the acoustic field applied to the suspension. Since we have recently introduced a novel immobilisation technique to directly study the effects of ultrasonic standing waves on yeast, we were committed to explore the potential of this technique. Mainly the goal was to overcome the limitation due to the neuro-toxic nature of the polyacrylamide gel used in an early stage of the work to investigate cell spatial arrangement (fig 1 adapted from Gherardini et al., 2001).

This paper represents a successful attempt for further exploiting the potential of such a technique. For the first time we demonstrated that it is possible to manipulate biological and non-biological particles suspended in biologically friendly gels, such as agar (Fig 2), by using ultrasound standing waves and create a predictable heterogeneous particle distribution within the matrix. As a consequence, it is possibility to enable the growth of immobilised cells controlling their access to nutrient and regulating their environment. This could be widely exploited in cell encapsulation technique where selective manipulation of specific population of cells is often required (Friel et al. 1999).

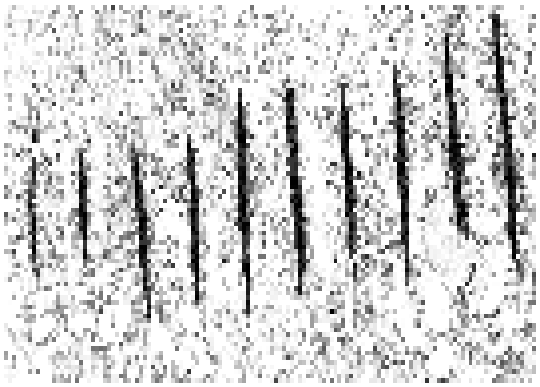

Fig 1.: Light microscopy, (x20). Standing wave ultrasound-induced arrangement of yeast cells in polyacrylamid gel.

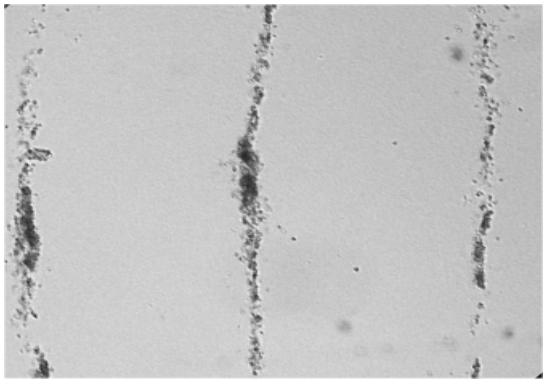

Fig 2.: Light microscopy, (x80). Standing wave ultrasound-induced arrangement of yeast cells in agar gel

Ultrasonic-induced gel immobilisation was applied to gather more information about some ground-breaking results obtained with the filtration devices. Recently we have used the plane wave resonator as a filter to selectively separate yeast and bacteria for the first time from a mixed suspension (Fig 3, a) In the filtered solution a nine fold enrichment of bacteria over the concentration of yeast cells was detected. The use of the immobilisation of the cells during acoustic treatment directly demonstrated the cause of the selective retention.

The image of the immobilised cells showed that only yeast cells were retained effectively in the areas on low pressure (the bands, Fig 3, b), while the rod shaped bacteria remained almost homogeneously dispersed, therefore being able to pass through the filtration system (Lawler et al, 2001). These results suggested that performance of the filtration systems varies in relation to the shape and the size of the particles in suspension. A direct investigation of the particles behaviour under acoustic treatment could lead to improve the design of the acoustic systems.

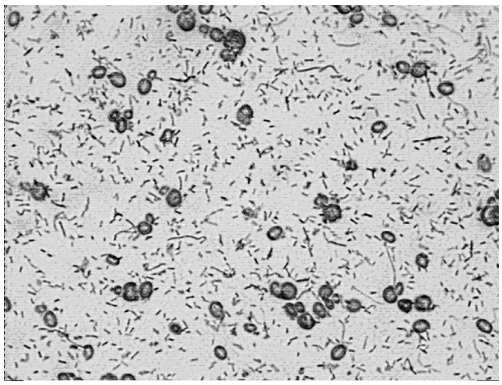

Fig 3 a.: Light microscopy, (x120). Arrangement of yeast (round shape) and bacteria (rods) before ultrasound treatment

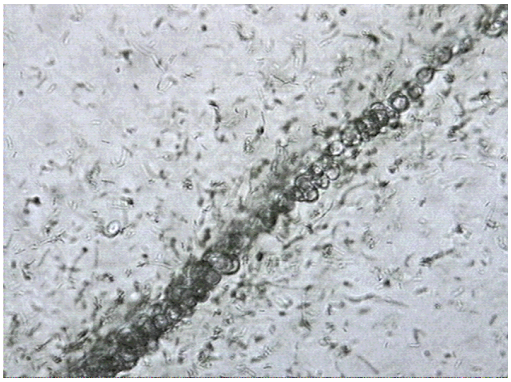

Fig 3 b.: Light microscopy, (x120). Ultrasound-induced arrangement of yeast (round shaped) and bacteria (rods). The treatment traps the yeast along the bands, while the bacteria remained dispersed

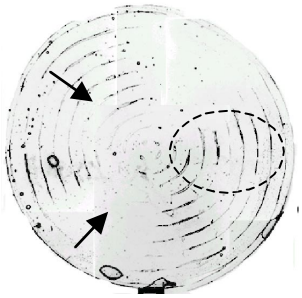

Fig 4.: Light microscopy, (x20). Composed image of the whole surface of the circular gel section. During the acoustic treatment the red blood cell are arranged along the planes of minimal pressure by the primary longitudinal forces. The cells are further concentrated in defined radial areas (see slashed line encirculation) by the transversal forces, perpendicular to the propagation direction (arrows) of the generated sine wave.

The versatility of the immobilisation technique was tested also with several resonators designed to create a cylindrical acoustic field. The ultrasonic device employed in these experiment for the manipulation of red blood cells has previously been described as a potential alternative to centrifugation for plasma purification and red blood cell concentration (Cousins *et al*., 2000).

Gel immobilisation technique enabled, for the first time, direct observation of the spatial arrangement of cells in the sound field within the chamber of the cylindrical resonator (Fig 4). Light microscopy showed that the red blood cells (RBC) were heterogeneously distributed after the treatment, with discontinuous areas of high RBC concentration. There appeared to be "hot spots" where the cells clumped more readily. The highly ordered arrangement of the RBCs reflects the high efficiency of the tubular transducer system, optimised for the separation of human blood.

Finally, the technique was evaluated to check its potential when using micro-encapsulation conditions. This time, the active chamber of the resonator, that is the active volume that contains the gel treated with ultrasound, was only 1mm in diameter. The frequency used (1MHz, Fig 5) allowed only one nodal plane to be generated within the field. In this case, auto-fluorescent particles were used to allow confocal microscopy analysis. The image of the sample collected and analysis by light microscopy suggested that the cells re-arranged along the minimum pressure areas, confirming the expectations. Moreover, it was demonstrated clearly that the cell position is dependent on the frequency used. Confocal microscopy image analysis of the gel treated using the third harmonic (3 MHz, Fig 6) of the resonant frequency clearly showed the image of the horizontal section of the areas with the cell distributions. Three concentric circles can be identified coincidental with the three nodal pressure expected.

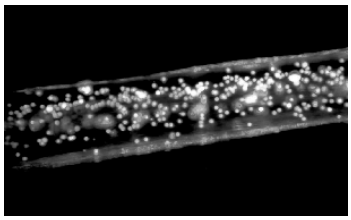

Fig 5.: Light microscopy, (x40). Auto-fluorescent particles arrange by ultrasound in a rod of alginate gel. The particles are concentrated along one surface of minimal pressure, as the system was driven using the fundamental harmonic.

Fig 6.: Confocal microscopy. Longitudinal (left) and horizontal (right) electronic sections of auto-fluorescent particles in a rod of alginate gel. The particles are concentrate along three surfaces of minimal pressure as the resonator was driven using the third harmonic.

CONCLUSION

The ultrasound-base immobilisation technique is a versatile tool for the study of the events occurring during the sonication and for the study of the effect of the treatment on the particle in suspension. The possibility of employing a bio-friendly gel matrix that allow cell to reproduce means that ultrasound can be exploited also to manipulate cells in encapsulation technology. The design of the transducer and the dimensions of the devices used to perform gel immobilisation do not represent limitations, as cylindrical field micro- immobilisation was performed successfully. We believe that micro manipulation of cells in gel matrix that results in a highly ordered and predictable arrangement of cells might find future application in the production of gel capsules, designed to host bio-materials. The effort of a future collaboration will be to assess this technique with special emphasis on improving spatial arrangement of the internal materials to achieve better immuno-isolation of the encapsulated cells.

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