A CLSM method for the dynamic observation of pH within alginate matrices

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INTRODUCTION

Alginate matrices form the basis of many bioencapsulation systems. The popularity of this polysaccharide is a result of its ability to form ionic gels, allowing for the encapsulation of sensitive materials without the need for potentially damaging agents. These gels also allow scope for modification through coating, or by the incorporation of other polymers or exipients into the matrix.

Within our group, and throughout the world, alginates have been used to encapsulate probiotic cells with the intention of protecting them during passage through the gastrointestinal tract (Cook 2012) It has also been seen that by coating these matrices with the cationic polysaccharide chitosan, the survival of cells in gastric solutions could be further improved (Cook 2011, Cook 2013a). There is, however, limited understanding of the processes occurring in the microcapsules during acid exposure, and the exact mechanism of cell protection is often postulated, but never proven.

It was the intention of this research to produce a method by which the pH within alginate matrices could be determined, allowing a greater understanding of the way these matrices may protect cells. The method used involved the labelling of a probiotic strain, *Bifidobacterium breve,* with pH-sensitive fluorophores. The observation of these labelled cells within alginate matrices by confocal laser-scanning microscopy then allowed for the pH within the capsules to be observed during exposure to an acidic solution (Cook, 2013b).

MATERIAL AND METHODS

B.breve NCIMB 8807 was purchased from the National Collections of Industrial Food and Marine Bacteria (Aberdeen, U.K.). Alginate, fluorescein isothiocyanate (FITC) and low molecular-weight chitosan were purchased from Sigma-Aldrich (Gillingham, U.K.). pHrodo succinimidyl ester was purchased from Invitrogen (California, USA). Wilkins-Chalgren (WC) anaerobe agar and phosphate-buffered saline (PBS) were purchased from Oxoid (UK). Alginate was purified by microfiltration (0.45 µm sartorius filter) before use; all other reagents were used without further purification. Materials other than alginate and chitosan were sterilized by autoclaving; alginate and chitosan were sterilized by microfiltration.

Preparation of pHrodo/FITC labelled B. breve
B. breve was grown in TPY broth (10 mL) and incubated (37 °C, 22 hrs) with shaking. After incubation the cells were isolated by centrifugation. The resulting pellet was then resuspended in PBS (pH 9, 10 mL). To the suspension, pHrodo succinimidyl ester (1 µL of a 10 mg/mL stock solution in DMSO) was added and the solution left in darkness (37 °C, 30 mins) with shaking. The cells were then isolated and the supernatant removed before washing with, and resuspending in, PBS (pH 8, 10 ml). The cells were then isolated and washed, before addition of FITC solution (1 µL of a 10 mg/ mL solution in water). The reaction was allowed to proceed in darkness (37 °C, 30 mins) with shaking. The cells were then isolated, washed and killed by submergence in boiling water.

Preparation of alginate-chitosan microcapsules containing pHrodo/FITC labelled B. breve
A 2 % w/v alginate solution (1 mL) was added to pHrodo/FITC labelled *B. breve* pellets produced as above and vortexed to ensure complete mixing. This solution was then extruded into a 0.05 M calcium chloride solution (50 mL), and was left to harden for 30 minutes before filtration. In the case of chitosan-coated alginate microcapsules these were then placed into chitosan solution (0.4 %, 10 mL) and left to stand (10 min). These chitosan-coated alginate microcapsules were then removed from the mixture by filtration.

Calibration of microscope
In order to extract the pH values from the CLSM images a calibration curve was first constructed. pHrodo/FITC-labelled *B. breve* produced previously was resuspended into PBS (1 mL) that was adjusted accurately to: pH 2, 2.5, 3, 4, 5 and 7.2. These new suspensions were placed onto a cover slip and imaged using a Leica SP2 CLSM. Samples were excited with 488 nm and 546 nm lasers sequentially, corresponding to the excitation wavelengths of FITC and pHrodo, respectively. From these 8-bit images the pixel intensity of the cells was determined using the onboard software (Leica Confocal Software).

Measurement of pH within alginate-chitosan microcapsules
A single alginate or alginate-chitosan microcapsule containing pHrodo/FITC labelled *B. breve* was placed onto a purpose designed cover slip (consisting of a 50mm petri dish with a central section removed which was then replaced with a thin glass cover slip),

submerged in simulated gastric solution (pH 2, 100 µL, 37 °C) and imaged using a CLSM at 488 nm and 543 nm excitation wavelengths sequentially at 0, 1, 5, 10, 15, 30, 45 and 60 minute intervals during incubation at 37 °C.

RESULTS AND DISCUSSION

The labelling of *B.breve* with the pH-sensitive fluorophores FITC and pHrodo was successfully achieved. The cells were exposed to a range of pHs, then, using a CLSM, these two fluorophores could independently be excited and the ratio of the fluorescence intensities related to pH.

Previously labelled cells were encapsulated into alginate or alginate-chitosan matrices by an established extrusion method. These matrices were exposed to a simulated gastric solution at pH 2 for one hour, and visualised by CLSM at regular time intervals. The images taken could then be manipulated by MATLAB to colour code the pixel intensities across the image, allowing for the observation of pH within the matrix. It was seen that in both alginate and alginate-chitosan matrices there was a slow encroachment of low pH from the periphery of the microcapsule over time when exposed to gastric solution (Figure 1.). As a result, the pH within the centre of the matrices was greater than the external solution, and therefore less harmful to any encapsulated material. The chitosan-coated alginate matrices showed a larger region of near-neutral pH at the centre of the matrix than was seen in the uncoated matrices. This is consistent with the improved survival of probiotics when in acid after encapsulation in these chitosan-coated materials.

These results suggest that the protection from acid given to probiotics by encapsulation in alginate matrices is a result of the increased pH within the material. This increase in pH is likely a result of buffering, reducing the activity of the acid within the matrix, or by the slow diffusion of acid into the materials when exposed to simulated gastric solution. When alginate drops below its pKa (approx. 3.3), an acid-gel is formed, which may be less penetrable to the acid. Coating with chitosan may reduce the porosity of the material, and therefore rate of acid diffusion.

CONCLUSIONS

A CLSM method has been developed for the dynamic observation of the pH within alginate, and chitosan-coated alginate matrices, using labelled probiotic cells. Matrices containing labelled cells were observed in simulated gastric solution by CLSM, revealing an increased pH within the centre of the material, which was greater in the chitosan-coated sample. These images give a greater understanding of the behaviour of these systems when used for protecting probiotic bacteria from acidic solutions.

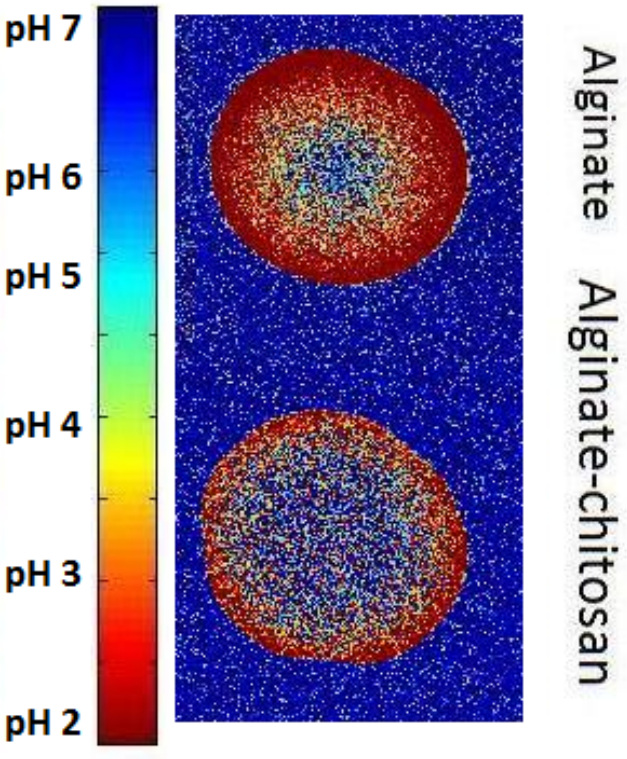

Figure 1 : 'pH map' of alginate and alginate-chitosan matrices after exposure to a simulated gastric solution at pH 2.

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