**A Research Proposal**

**On**

# Layer-By-Layer Coating of Alginate Matrices with Chitosan–Alginate: Impact of High Molecular Weight Chitosan on Survival and Targeted Delivery of Probiotic Bacteria after Oral Administration

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## 1. Introduction

Probiotics are live bacteria used as nutraceuticals. Most popular probiotic bacteria are *Bifidobacterium breve, B. lactis, B. longum, Lactobacillus lactis, L. acidophilus, L. plantarum, L. casei, L.fermentum, L. rhamnosus, L. salivarius* and *Streptococcus salivarius* for various therapeutic purposes. The gastrointestinal microflora resides in human intestinal tract and provides numerous beneficial effects to the host.[1] World Health Organization (WHO) as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host'.[1] There are more than hundred probiotic species available in human who are responsible for human health and immunity. So consumption of probiotic bacteria is best for average healthy person and incredibly important to wellness. The healthy bacteria live in colon and strengthen the bowel wall to improve mineral absorption as well as regulate hormone production.[2] Thus probiotics have therapeutic and preventive property for various diseases and metabolic disorders.

Oral delivery of probiotic bacteria improve gut health and have been shown to be helpful for childhood diarrhea, irritable bowel disease and bowel infections, so currently used in the treatment of diarrhea, inflammatory bowel disease, and ulcerative colitis.[9] Probiotic bacteria lose its viability in harsh conditions, so free cells are encapsulated in various materials by physicochemical or mechanical processes to increase its viability. Majority of Probiotic bacteria must be kept alive because loss of viability effectively lowers the efficacy of the administered supplement. Stability and viability of Probiotics products are big challenge in the world of nutraceuticals.

The aim of this proposed research is to review and develop microencapsulation methods by using layer-by-layer (LBL) coating of alginate and chitosan. Although alginate-chitosan system is recognized but the size of microcapsules should be revised and needs further research and development. Chitosan act as a stabilizer for alginate by forming polyelectrolyte and improve viability of microencapsulate probiotic bacteria (LBL-coated matrices). Numbers of experiment are performed to increase viability of *B. breve*. Encapsulation of *B. breve* was carried into alginate matrix then alginate-chitosan followed by coating in multilayer of alternating alginate and chitosan which improve survival of probiotic bacteria.

In result number of layers were increased from 1 to 5 on the surface of the alginate matrix to observe the impact of layers on probiotic bacteria and observed increase in the volume of 130.61 ± 21.16%.[4] Swelling and softening of the material were decreased strength of multilayer encapsulated cells.[4] In comparison to uncoated alginate capsules and 5-layers coated capsule strength was reduced from 5.46 ± 0.31 N to 0.97 ± 0.35 N, but highest recovery of viable cells were observed in 3-layers coated matrix in vitro gastric conditions.[4] Although softening of material is a big challenge in stability of encapsulated cells but re-hardening of the capsules can be possible in $CaCl_2$ solution. In the research, low molecular weight chitosan (103 kDa, degree of deacetylation: 85.6%) was used for multilayer coating for alginate microcapsules.[4]

In the previous research it was found that the high molecular weight chitosan microcapsule gave higher survival rate (46%) than low and medium molecular weight chitosan (36%).[8] High molecular weight chitosan form thicker membrane into alginate–chitosan system.[8] Previous studies have proved that efficiency of encapsulated cells affected by molecular weight of chitosan associated with solution viscosity.[19] Crystallinity of high molecular weight chitosan is initially increased and then decreased with increase of degree of deacetylation.[20] High molecular weight chitosan is less permeable than low and medium molecular weight chitosan.

Viability and targeted delivery of probiotic bacteria can be improved by using of high molecular weight chitosan in Layer-by-layer coating of alginate matrices. The aim of this proposed work is to study it in a more fundamental way previously reported.

## 2. Research Title:

Layer-by-layer coating of alginate matrices with chitosan–alginate: Impact of high molecular weight chitosan on survival and targeted delivery of probiotic bacteria after oral administration.

## 3. Literature Review:

Probiotics are a group of bacteria described by the Food and Agriculture Association of the United Nations (FAO).[1] Global market worth $32.6 billion was predicted by 2014,[1] while market size exceeded USD 35.0 billion in 2015,[6] and expected to witness more than 7.4% CAGR to 2024.[7] *Bifidobacterium* or *Lactobacillus* is typically saccharolytic (capable of metabolizing sugars), are used commercially.[9] According to the review of literature, either probiotic or probiotic mix play an important role in human health, which health promoting property associated with cells viability and stability. The oral administration of most bacteria lowers the efficacy of the

administered supplement because high acid and bile salt concentrations present in GIT result in a large loss of viability.

Microcapsulation is a method to protect the cell (active ingredient) from its surrounding environment as well as an opportunity to control release of these cells across the intestinal tract. This technology is based on the immobilization of bacteria into a polymer matrix (capsule) to increase their viability. It also provides sufficient permeability for nutrients and prevents entry of hostile molecules.[15] Calcium cross-linked alginate is basic encapsulation material for *B.breve*.[10] Alginate is a naturally occurring polysaccharide. Which is composed of randomly 1–4 linked β-D-mannuronic acid and $\alpha$-L-guluronic acid, Guluronic acid interacts very strongly with divalent cations.[11,12] The cross linking between the carboxylate anions of alginate guluronate units and the calcium ions form an 'egg-box' junction.[13,14] Alginate beads are very porous and acid sensitive.[17] *Bifidobacterium longum* and *Bifidobacterium breve* encapsulated in alginate microcapsules during exposure to gastric juice and showed reduced viability.[1] Mixing /coating of other polymer compounds or modification by using different additives in alginate structure improve probiotic encapsulation effectiveness.[18] So Alginate is coated with xanthum gum / pectin / whey protein /chitosan-alginate/ poly-l-lysine-alginate/ palm oil and poly-L-lysine / chitosan.

In vitro studies demonstrated that chitosan-alginate system showed good results than alginate alone in applications for oral delivery of probiotic bacteria because chitosan stabilized the alginate microcapsules in stomach pH. It provides protection in simulated GI conditions. Chitosan is a linear polysaccharide it is polymerized by means of a cross-link formation in the presence of anions and polyanions. Chitosan composed of glucosamine units a membrane is formed by positively charged amino groups of chitosan and negatively charged carboxylic acid groups of alginate.[17] This membrane reduces leakage of entrapped materials from the particles.[16] It appears chitosan-alginate system is physically stable to protect the encapsulated bacteria from harsh conditions such as mechanical stress, heat, enzymatic degradation and can provide better survival in the harsh stomach condition as well as in the intestine. But the numbers of viable microorganisms are reduced by the low pH of the stomach thereby reducing the efficacy of the administration. In other hand cells must be alive in order to exert its therapeutic and preventative effects. To improve stability and viability of encapsulated probiotic bacteria high

molecular weight chitosan is used instead of low and medium molecular weight chitosan and found high survival rate.[8] This is because of high molecular weight chitosan forms thick layer than low and medium molecular weight chitosan.

The potential benefit of chitosan coat is improved by multilayer alginate–chitosan coat on alginate matrices using a layer-by-layer coating system. On repeated injection of chitosan alone, it was appeared that chitosan was able to associate to the previous layer of chitosan.[4] Multilayer chitosan-alginate system showed higher responses than chitosan alone. In other hand alginate –chitosan alternate layer showed higher response than chitosan alone multilayer. The complex showed good stability in the pH of the stomach as well as in simulated intestinal fluid at pH 7.2 .[4] These results indicated that stability of MCAMs associated to the surface of the alginate matrix. As number of layers increased on the alginate matrix from 1 to 5 there was an increase in the thickness and swelling of the materials. A final increase in the volume of 130.61 ± 21.16%, this swelling was met with a decrease in strength.[4] Survival rate for 3-layer coated matrices was high and it gave the highest recovery of viable cells.

## 4. Research Aim and Objectives:

This research study is aimed to develop a new method to increase stability and viability of microencapsulated probiotic. To achieve the desired aim following research objectives are set:

**i.** To investigate Layer-by-layer coating of alginate matrices with chitosan–alginate by using high molecular weight chitosan.

**ii.** To investigate effect of high molecular weight chitosan and alginate coats on stability and viability of probiotic.

**iii.** To investigate size of microcapsules on using high molecular weight chitosan in respect to stability of probiotic products.

## 5. Materials:

For the observation of chitosan behavior in different manner materials will be used Sodium alginate (19–40 kDa), low molecular weight chitosan (103 kDa, degree of deacetylation: 85.6%), high molecular weight chitosan (310kDa, degree of deacetylation:>75%),[5] and fluorescein isothiocyanate (FITC) these will be purchased from Sigma-Aldrich (Gillingham, UK), and the bacteria will be used in the research work is *Bifidobacterium breve* NCIMB 8807. The bacteria will be purchased from the National Collection of Industrial Food and Marine Bacteria (Aberdeen, UK).

Wilkins–Chalgren (WC) anaerobe agar (to grow the bacteria) and phosphate-buffered saline (PBS) will be purchased from Oxoid (UK). The Biacore™ CM5 chip will be purchased from GE Healthcare (Hatfield, UK).[4]

## 6. Methods: Experimental model of LBL chitosan-alginate matrix:

### I. Biacore™ analysis of alginate–chitosan multilayer:[4]

For the analysis of alginate-chitosan multilayer Biacore™ 3000 instrument with a CM5 chip will be used. For representation of chitosan layers on alginate matrix a CM5 chip will be activated by carboxymethylated dextran and will be docked. The channel will be used twice primed with a 50 mM phosphate buffer (pH 6.0). 0.4% (w/v) Chitosan solution (in 0.1 M acetic acid will be adjusted to pH 6.0 with 1 M NaOH, 20 µL) will be injected into the channel at a flow of 15 µL $min^{-1}$. And the system will be allowed to stabilize for 1 h. This will be first layer of chitosan with alginate matrix. For the second layer 0.04% (w/v) alginate solution (20 µL) will be injected and leave it for 10 min. to stabilize. Simulated gastric solution (0.2% (w/v) NaCl, will be adjusted to pH 2.0 with 1 M HCl) or the simulated intestinal solution (0.68% (w/v) monobasic potassium phosphate, will be adjusted to pH 7.2 with 1 M NaOH) will be used to test the stability of layers over 90 minutes. Alginate–chitosan and chitosan only multilayer will be produced using the same batch of chitosan and eluent until 3 injections.[4] The process will be repeated for only 3 alternate layers in proposed work.

### II. Preparation of layer-by-layer coated alginate matrices:[4]

To prepare LBL coated alginate matrices 2% (w/v) alginate solution (1 mL) will be extruded using a syringe and pump (2 mL min−1) into a 0.05 M $CaCl_2$ solution (50 mL) to form alginate matrices with the $CaCl_2$ solution. After 30 min. the solution will be filtered for LBL coating. 0.4% (w/v) chitosan solution (in 0.1 M acetic acid will be adjusted to pH 6.0 with 1 M NaOH, 10 mL, 10 min) and 0.04% (w/v) alginate solution (10 mL, 10 min) will be used alternate for coating.[4]

### III. Determination of strength and swelling of multilayer coated alginate matrices (MCAMs) during processing:[4]

A light microscope (Leica DM2500) and image analysis software (ImageJ) will be used to measure diameters of uncoated alginate matrices and coated with 1–3 layers of alginate and chitosan during the preparation of the formulation. Texture analysis (Texture Analyzer, Stable Microsystems) will be conducted with a P\1K steel probe at a rate of 1 mm $s^{-1}$, using a trigger force of 0.01 N.

It will determine compressive strength (the point at which the gel will be seen to fracture on the graph of compressive force against distance) of multilayer coated alginate matrices (MCAMs). Rehardening of gel will be done on exposure of the capsules to 0.05 M $CaCl_2$ (50 mL, adjusted to pH 6.0 with 0.1 M HCl, 30 min).[4]

### IV. Determination of coat thickness of multilayer encapsulated cells by confocal laser scanning microscopy (CLSM):[4]

The thickness of the coat on the outside of the alginate matrices will be determined by CLSM. pHrodo/FITC labeled *B. breve*. will be prepared for CLSM and will be used to prepare alginate-chitosan microcapsules [3]. FITC (fluorescein isothiocyanate)-labeled chitosan will be prepared. Chitosan solution 1% (w/v) (in 0.1 M acetic acid, 100 mL) will be added to dehydrated methanol (100 mL) then a 0.2% (w/v) FITC solution (methanol, 50 mL). The reaction will be allowed to proceed in darkness at room temperature (3 h) before precipitation into 0.1 M NaOH (1 L). This precipitate will be then dialyzed in deionized water (4 L, will be replaced daily) until FITC will not present in the dialysis jar, as determined by UV-spectrofluorometry (Jasco FP-6200, $\lambda_{exc}$: 488 nm, $\lambda_{emi}$: 515 nm). The sample will be lyophilized until complete dehydration. The lyophilized product will be re-suspended to a concentration of 0.4% (w/v) in 0.1 M acetic acid, and will be adjusted to pH 6.0 with 1 M NaOH. This solution will be used to produce MCAMs. These matrices will be imaged using a Leica SP2confocal microscope ($\lambda_{exc}$: 488 nm), which will be allowed the visualization of chitosan on the surface of the gel. From these images, the coat thickness will be measured using the image analysis software ImageJ. The thickness of the coat as a function of the number of layers can be determined in order to confirm the association and aid the understanding of matrix structure.[4]

### V. Microencapsulation of *B. breve* using multilayer chitosan-alginate matrices system:[4]

For encapsulation of *B. breve* into multilayer chitosan-alginate matrices, *B. breve* will be speckled onto WC agar in an anaerobic chamber (37 °C, 48 h). A single colony from grown *B. breve* will be inoculated into TPY (Tryptone-Phytone-Yeast) broth (10 mL) and will be incubated (37 °C, 22 h). After growth to an $OD_{600}$ of ~2.0, the cells will be centrifuged (3200 rpm, 10 min), the supernatant will be removed and the cell pellet will be washed with PBS. The cell pellet will be resuspended in 2% (w/v) alginate solution (10 mL) previously sterilized by microfiltration (0.4 μm). This solution

will be used to produce MCAMs. The chitosan solutions will be used in the experiments will be sterilized by microfiltration (0.4 µm).[4]

### VI. Viability of free and multilayer microencapsulated *B. breve* in a simulated gastric solution:[4]

A 100 µL sample will be taken (from grown *B. breve* in TPY broth) for enumeration by the serial dilution and plate count method. These cells will be diluted in a series of vials containing PBS, before spreading on solid WC agar. Colonies will be counted after 48 h growth in an anaerobic cabinet (37 °C). After enumeration, the cell suspension will be centrifuged (3200 rpm, 10 min) and the supernatant will be removed, followed by washing with PBS (1 mL). The remaining pellet will be suspended in the simulated gastric solution (10 mL) and will be incubated (37 °C, 2 h, with shaking at 100 rpm). Samples will be taken and enumerated at 1 h intervals. 3 batches (1mL for each batch) of MCAMs containing *B. breve* will be produced from the same broth of cells for encapsulation. Two of these batches (*B. breve*) will be placed into the simulated gastric solution and will be incubated (37 °C, with shaking at 100 rpm) and rest one batch will be placed directly into a 100 mL PBS solution and will be incubated (1 h, with shaking at 100 rpm). By using a stomacher (Seward stomach 400 circulator, 20 min) half dissolved MCAMs will be homogenized and enumerated to give a starting cell concentration. After 1 and 2 h incubation, one of the two batches of matrices in the simulated gastric solution will be removed from the gastric solution. These matrices will be dissolved and enumerated using the same method as for the starting cell concentration reading.[4]

### VII. Release of cells from multilayer chitosan-alginate matrices under *in vitro* GIT solutions:[4]

MCAMs matrices will be placed first into the simulated gastric solution (10 mL, pH 2.0) and will be incubated (37 °C, 60 min, with shaking at 100 rpm). Samples will be taken from this solution to determine number of cells by using a light microscope and a hemocytometer on a Nikon Microphot-SA microscope at 0, 30 and 60 min. The MCAMs will be removed after 60 min. incubation from the gastric juice by filtration and will be placed into the simulated intestinal solution (pH 6.0, 50 mL) and will be incubated again (37 °C, 60 min, with shaking at 100 rpm). The cells will be counted at 0 and 60 min. before removal by filtration and resuspension in the simulated intestinal solution (pH 7.2, 50 mL) after that will be incubated (37 °C, 180 min, with shaking at 100 rpm). At 0, 60, 120 and 180 minutes in buffer, the cells will be counted as before and matrices will be produced and placed instantly into PBS (100 mL) and will be incubated (60 min,

with shaking at 100 rpm). These capsules will be placed into a stomacher (Seward stomach 400 circulator, 20 min), by the help of a microscope and a hemocytometer the cells will be counted to give the starting cell numbers for each experiment.[4]

## 7. Year wise plan of work:

### I. First year:

Standardization of experimental techniques, procurement of chemicals, equipments and *B.breve*

| Group | Number of chitosan layer (alone) | Parameters to be assessed |
|---|---|---|
| 1 | Alginate matrix + 0 | Layer analysis, strength and swelling of multilayer matrices, coat thickness, viability of free and encapsulated cells, release of cells in vitro GIT solutions |
| 2 | Alginate matrix + 1 | |
| 3 | Alginate matrix + 2 | |
| 4 | Alginate matrix + 3 | |

### II. Second year:

| Group | Number of alginate-chitosan layer (alternate) | Parameters to be assessed |
|---|---|---|
| 1 | Alginate matrix + 0 | Layer analysis, strength and swelling of multilayer matrices, coat thickness, viability of free and encapsulated cells, release of cells in vitro GIT solutions |
| 2 | Alginate matrix + 1 | |
| 3 | Alginate matrix + 2 | |
| 4 | Alginate matrix + 3 | |

### III. Third year:

Statistical Analysis of Data, Communication of papers for publications, Thesis preparation and submission of thesis.

## 8. Aimed to be achieved:

Although probiotics bacteria improved human health over the years but control release in GIT is not satisfactory which needs to be improved. LBL-coated matrices have a good stability but multilayer (n>3) coating increase swelling and softening of microcapsules. Further research is necessary to reduce size of microcapsule to increase effectiveness. It would be of interest to evaluate the high molecular weight chitosan to obtain microcapsules with the best properties.

## 9. Contribution:

The present study is designed with an aim to define the chitosan-alginate matrices system. To determine strength and swelling of multilayer coated MCAMs by using high molecular weight chitosan. The results of this proposed research will be improvement in the formulation of better probiotic products, development of live vaccines and protection and release of cells implants. It

shall broaden scope of new methods for microencapsulation of living cells for the purpose of their protection and triggered release. The mechanisms of probiotics have a broad scope in treatment of AIDS.

In terms of utilization by the pharmaceutical companies, research outcomes of this project may play a vital role in the development of probiotic products with improved survival and targeted delivery of probiotic bacteria after oral administration. It is evident that the probiotic market has a strong future as the consumers demand products enriched with these ingredients with foods, beverages and supplements.

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