Artificial cell microcapsule for oral delivery of live bacterial cells for therapy: design, preparation, and in-vitro characterization.

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Abstract Purpose: Bacterial cells can be engineered to synthesize a wide array of disease modifying substrates such as cytokines, vaccines and antibodies; however, their use as an orally delivered therapeutic is limited by poor gastrointestinal (GI) survival and instigation of immunogenic response. Artificial cell microcapsules have been well studied as a means to overcome such problems, however, presently obtainable microcapsules have limitations. This study summarizes a novel microcapsule design specifying its preparation and GI stability in-vitro. **Method:** Multilayer APPPA microcapsules were designed, prepared and characterized in-vitro for bacterial cell oral delivery using *Lactobacillus reuteri* cells as a model. Microcapsule structural integrity, mechanical stability, and GI survival studies were performed in simulated gastric (SGF) and intestinal (SIF) fluids in various pH conditions at 37.2° C and compared with presently available alginate/poly-l-lysine/alginate (APA) microcapsules. HPLC was used for the microcapsule membrane permeability study. **Results**: Results show that APPPA microcapsules can be prepared for bacterial cell encapsulation and are stable in simulated GI conditions. No microcapsule damage was reported when exposed to SGF and SIF for 12 hours at 250 rpm mechanical shaking at 37.2° C. In addition, 93.2±2.3% and 98.9±0.6% of microcapsules were undamaged after 24 hours in SGF and SIF respectively. Microcapsule pH stability results show that 92.8±3.1% of microcapsules remained intact at pH 1, 3, 5, and 7 and no damage was observed at pH 9.0 when challenged for 24 hours. When exposed for 3 hours with 250 rpm shaking at 37.2° C, no damage of the microcapsules in SGF and SIF at pH, 1,3,5,7, and 9 was observed. Compared to APA microcapsules, APPPA membranes showed superior GI stability and permeability for cell encapsulation. **Conclusion:** Novel APPPA microcapsules have superior features for oral delivery of live bacterial cells and they can be used for various clinical applications. However, further study such as membrane permeability, cytotoxicity, immune protection capacity, and suitability for live bacterial cell oral delivery in-vivo is required.

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INTRODUCTION

Bioencapsulation technology offers several advantages and has shown promising results for the treatment of a number of diseases (1-7). For all these applications, appropriate performance of the microcapsules is critically dependent on the properties of the capsular membrane (8-10). Considerable research interest has been dedicated to the encapsulation of bacterial cells for the growing and promising potential in therapeutic applications such as in kidney failure uremia, cancer therapy, diarrhea, cholesteremia, and other diseases (11-17). However, success of microcapsule oral delivery of live bacterial cells for therapy depends on the suitability of the microcapsule membrane for GI delivery. For example, a microcapsule can be disrupted by many different means during its intestinal passage; it may be fractured by enzymatic action, chemical reactions, heat, pH, diffusion, mechanical pressure and other related physiological and biochemical stresses. The safety of microcapsules is even more important when live cells are intended for use in the intestinal system by oral administration. This is because the live cells must be protected during the encapsulation process and microcapsules must reach the GI intact. In addition, the survival of the live cells during their passage must be ensured. Thus, the microcapsule membrane must be provided with sufficient permeability for nutrients, and secretion and excretion products, to pass through, yet prevent the entry of hostile molecules or cells from the host, for example, products of the host's immune response, which could destroy the encapsu-

lated bacterial cells (7; 18-20). The problems inherent with oral delivery, therefore, have made the goal of oral delivery of live bacterial cells very challenging.

Several delivery systems for oral delivery of live bacterial cells have been proposed. For example, microcapsule immobilization of bifidobacteria to protect them against adverse effects of acid during oral administration has been proposed (21; 22). Rao et al. described a method to encapsulate freeze-dried *B. pseudolongum* using cellulose acetate phthalate (CAP) coated with beeswax, showing that encapsulated *B. pseudolongum* survived the simulated gastric environment in larger numbers than non-encapsulated cells (21; 22). Encapsulation of bifidobacteria in butter oil and whey based medium was proposed but was shown to be ineffective in preventing acid injury to bacteria in both low acid and high acid environments (23). Calcium alginate and k-carrageenan–Locust bean gum gel beads are the two most commonly used polymers for immobilizing viable cells (24; 25). However, alginate beads are not acid resistant, and it has been reported that the beads undergo shrinkage and decreased mechanical strength (25-27). In order to overcome this, coating bacteria by cross-linking with a carboxyvinyl polymer career has been suggested. The carboxyvinyl polymer was, however, shown to be effective only for intestinal delivery and release (28). K-Carrageenan–Locust bean gum gel beads are less sensitive to acid than alginate and hence used for lactic fermentation. However, 2 major limitations preclude their use. First, the formation of k-carrageenan–Locust bean gum beads requires high potassium ions. The latter could potentially damage the cells of *B. longum* during lactic fermentation (29; 30). Second, it has been pointed out that, as potassium ions are important in maintaining electrolyte equilibrium, their inclusion in the diet in large amounts would not be recommended (31; 32). Gellan–xanthan beads are not only acid resistant but are also stabilized by calcium ions (33), suggesting that they could be a good candidate for immobilizing bifidobacterial cells and protecting them against acid injury (34). However, they do not protect against immunorejection, which is a primary requirement for probiotic oral delivery of live cells for therapy. Similarly, agarose capsules prepared by emulsification/internal gelatinization for oral delivery of Bacillus Calmette-Guerin (BCG) cells, although stable for up to 12 months in-vitro, have not been shown to be suitable for oral delivery as agarose membranes do not provide immunoprotection (33; 35). Thus, agarose microspheres with various polymer coatings have been proposed (36). Among other formulations, gelatin and polymer coated gelatin capsules have been studied for oral delivery of live bacterial cells (37-40). The latter (with 20% w/v of the polymer) has shown promising results in vitro. On the other hand, concerning uncoated gelatin capsules, a radiological study among human volunteers has shown that they disintegrate within 15 minutes of ingestion (40-42). A host of other formulations using poly(lactide-co-glycolides), carrageenan, alginate-poly-L-lysine, starch polyanhydrides, polymethacrylates, polyamino acids, enteric coating polymers, etc. have been proposed, but they exhibit poor GI survival and lack immunoprotection (37-40; 43-50).

Although numerous encapsulation systems have been studied, to date, the most promising formulation is the encapsulation of calcium alginate beads with poly-L-lysine (PLL) forming alginate-poly-L-lysine-alginate (APA) microcapsules (4; 7; 51-53). The APA microcapsule has been used successfully to limit the major problem of immuno-rejection related to the use of live cells for therapy and other biomedical applications. The presently obtainable APA microcapsules, however, have posed limitations for general use, via oral administration, because of their inadequate stability in the gastrointestinal (GI) tract (4; 52; 54-57). To overcome this, APA microcapsules were modified by others using a higher concentration of alginate cross-linked with barium instead of calcium, and the alginate was fabricated as a gelled bead without solubilizing the core microcapsule (58). This modification prolonged the stability of the capsule for systemic delivery applications in canine models, but not for oral delivery. Another related limitation of the APA membranes is the potential for hydrolysis on enzymatic action during their passage within the GI tract, making them less suitable for oral delivery (59; 60). Given these limitations of the presently available APA microcapsules, and their biomedical applications, development of microcapsules suitable for oral delivery is necessary. In the present study, we use a novel approach towards the design of APPPA microcapsule formulation and test their GI stability in-vitro.

MATERIALS AND METHODS

Preparation of Ca-alginate beads

Ca-alginate beads were first made using an Inotech Encapsulator IER-20 (Inotech Biosystems Intl. Inc.) by extruding a sodium alginate solution (Sigma-Aldrich low viscosity, 1.5% w/w,) into 120 ml stirred solution of 0.1M $CaCl_2$. The beads were prepared using a 300μm encapsulator nozzle at a frequency of 1160HZ, 7.93ml/min syringe pump speed and a voltage of 1.000Kv using a 60 ml syringe.

Preparation of APPPA microcapsules

Ca-alginate beads were exposed to PLL solution (Sigma, Mw=27400 d, 0.1% w/w) for 15 mins, washed with saline; then in pectin solution (Sigma, degree of esterification: 25%, 0.1% w/w) for 15 mins, washed with deionized water; and subsequently soaked in PLL solution (0.1% w/w) for 15 mins, washed with deionized water; finally put it in alginate solution (low viscosity, Sigma-Aldrich 0.05% w/w) for 10mins. The resulting microcapsules were washed with deionized water and stored in 4° C.

Preparation of APA microcapsules

APA capsules were prepared according to the standard protocol (7) with several modifications. Briefly, Ca-alginate beads were suspended in a solution of PLL (0.1% w/w) for 15 mins, washed with ion-free water and then immersed in alginate solution (0.05% w/w for 10 mins. The resulting microcapsules were washed with deionized water, stored in 4°C, and used in the experiments.

Mechanical stability test

For mechanical stability evaluations, 150±30 APPPA microcapsules were exposed to various test fluids (SGF, SIF, microcapsule storage solution, varying pH solution), in a 25 ml conical flask and exposed to various mechanical shaking speeds and stress and time periods in an Environ Shaker at 37.2° C. Samples were withdrawn and analyzed for physical damage using an optical light microscope. For comparative mechanical stability studies, similar quantities of APA microcapsule were used.

Microcapsule permeability study

Preliminary studies for APPPA microcapsule permeability were performed and compared with alginate beads and APA microcapsules. For this 4.00 mg of BSA was dissolved in 1.0 ml of 1.5% (w/w) alginate solution with a BSA/alginate ratio of 26.6 %.(w/w) and alginate beads containing BSA were obtained. Using the above-mentioned procedure, APPPA, APA microcapsule containing BSA was also prepared. The BSA leakage was analyzed by exposing BSA containing APPPA microcapsules, alginate beads, and APA microcapsules to deionized water in a 25 ml flask with 150 rpm shaking in a environ shaker at 37.2° C for 24 hours. After 24 hours of exposure, samples were withdrawn and BSA concentration was measured using an HPLC (Varian Inc. Canada) UV detector at 280nm. For this HPLC BSA permeably study, a Biosep-SEC-S3000 column, 50mM NaH_2PO_4 buffer mobile phase (pH=6.8), and flow rate of 0.5ml/min were used.

RESULTS AND DISCUSSION

Microcapsule design strategy for oral delivery of live bacterial cells: Design, and preparation of APPPA microcapsules

The biomatrix used for developing the microcapsules is of primary importance for addressing the complex problems associated with oral delivery. They should provide mild conditions for encapsulation, be nontoxic to the cell and host, be biocompatible, have sufficient membrane permeability, be impermeable to antibody-sized molecules, and have the ability to overcome the acidic and enzymatic environment of the stomach and GI tract. Available synthetic polymeric materials, which are excellent for the oral delivery of drugs, would not be good candidates, as they would not satisfy the above-mentioned conditions. Alginate and pectinate are potentially good candidates for designing microcapsule formulations for the delivery of live bacterial cells (61). The molecular design strategy and the schematic diagram for the APPPA microcapsule are shown in Figure 1.

We designed and formulated novel alginate/poly-l-lysine/pectin/poly-l-lysine/alginate (APPPA) microcapsules that show special features not available in present formulations of microcapsules. To prepare these novel microcapsules we have taken advantage of the known chemistry that pectin can be cross-linked with ions and poly-l-lysine and can then be made available in the membrane formulation of alginate/poly-l-

lysine microcapsules. For this, we first cross-linked sodium alginate with calcium ions through ionotropic gelation.

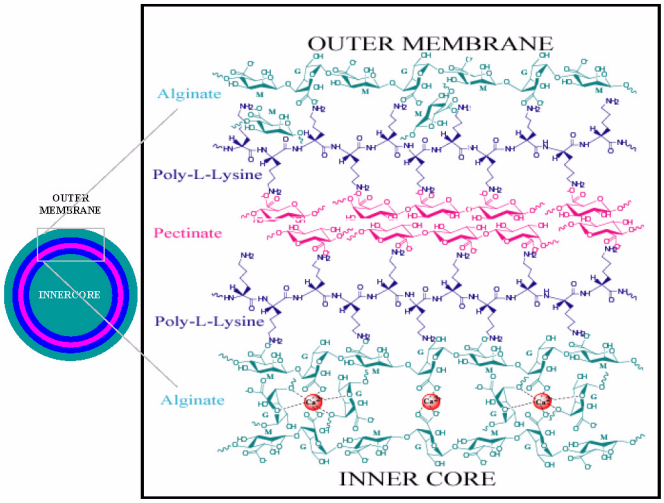

Figure 1: Molecular membrane design for Alginate-Polylysine-Pectinate-Polylysine-Alginate (APPPA) membrane artificial cell microcapsule structure.

Then a PLL coating serves as an immunoprotective membrane layer. The subsequent pectin membrane provides better acid stability and improved strength. The additional alginate coating at the outer surface of the microcapsules ensures the optimal biocompatibility. Characteristic of a multi-layer or "sandwich" structure, this formulation is likely to make the proposed APPPA membrane stronger and more stable, and therefore may be able to solve the problems in GI applications for the current APA microcapsules.

Using multi-step preparation procedures described above we can obtain APPPA microcapsules. The preparation process for the APPPA microcapsules involved the formation of calcium- alginate beads, multiple coatings of poly-l-lysine, pectin, poly-l-lysine, and alginate. We were able to obtain an extremely spherical shape, narrow size distribution and high homogeneity of the microcapsules. The size of the microcapsules obtained depends on the nozzle used; in this study, using a 300 µm nozzle resulted in the diameter of the microcapsules to be in the range of 400±25 µm (Figure 2a).

Results show that APPPA microcapsule membranes were able to retain *Lactobacillus reuteri* bacterial cells (Figure 2b).

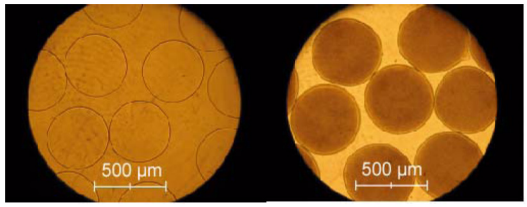

Figure 2: Optical micrographs of APPPA. Left: APPPA microcapsules without bacterial cells, Right: APPPA microcapsules containing with Lactobacillus reuteri (LP80) cells.

Results show (data not shown) that bacterial cells were able to survive during the encapsulation process and grow normally when obtained supernatant was plated after breaking of the microcapsule membrane.

GI stability of APPPA microcapsules

To evaluate the stability of microcapsules for oral therapy, knowledge of encapsulant dynamics is required under relevant physiological conditions that represent the different phases of digestion. In many applications, capsules are subjected to mechanical stresses exerted by their environment that induce deformation and potential break-up; thus capsular mechanical strength is believed to be paramount and should not be compromised. In this study, a shake method was used and the percentage of microcapsules undamaged was observed by microscope to provide information on the mechanical resistance of the microcapsules in various GI conditions. Figures 3 and 4 show that no damage occurs when microcapsules were challenged for 12 hour mechanical shaking at 250 rpm at 37.2° C; neither in simulated gastric fluid (SGF) nor in simulated intestinal fluid (SIF).

93.2±2.3% of microcapsules was undamaged in simulated gastric solution and 98.9 ± 0.6 % microcapsules remained intact in simulated intestinal solution after shaking up to 24 hours. These results suggested that APPPA microcapsules were stable both in simulated gastric solution and in simulated intestinal fluid.

Figures 5 summarized the microcapsules undamaged percentage in various pH conditions after shaking for 24 hours at 250 rpm at 37.2° C.

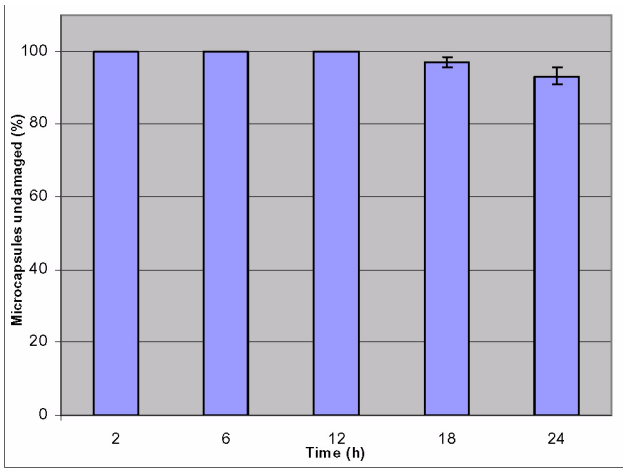

Figure 3: Mechanical stability of APPPA microcapsules in simulated gastric fluid (SGF) after shaking at 250 rpm (pH= 1.5, 37.2 °C).

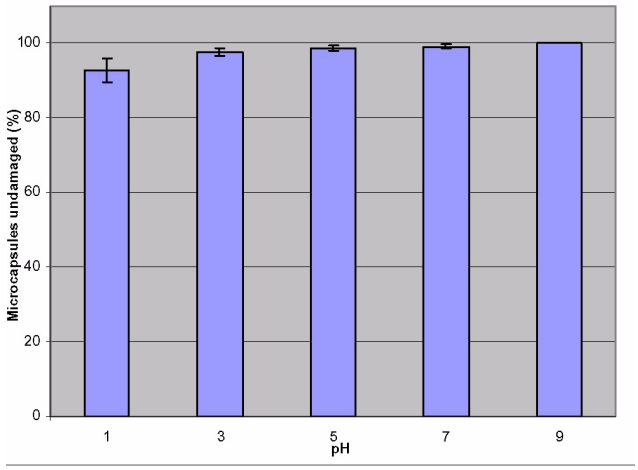

Figure 5: Mechanical stability of APPPA microcapsules in various GI pH conditions after shaking at 250rpm for 24hrs at 37.2 °C.

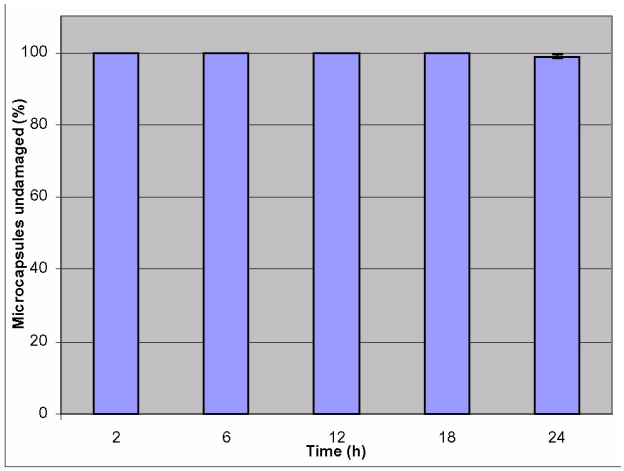

Figure 4: Mechanical stability of APPPA microcapsules in simulated intestinal fluid (SIF) after shaking at 250 rpm (pH= 7.5, 37.2 °C).

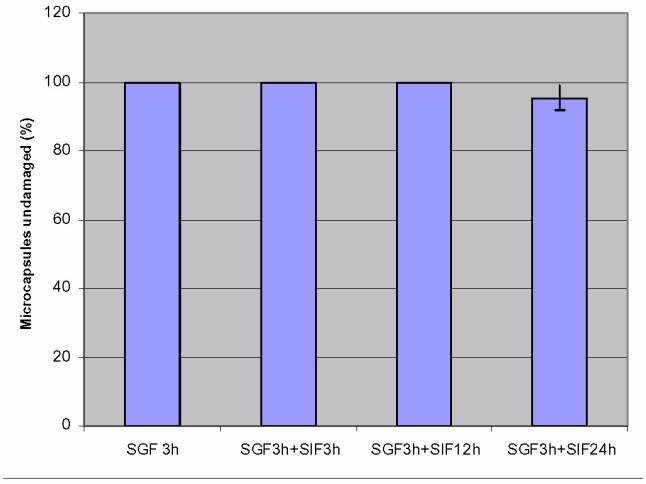

Figure 6: Mechanical stability of APPPA microcapsules in simulated GI condition after shaking at 150rpm at 37.2 °C.

In all these pH values, more than 92.8 ± 3.1% of microcapsules were found undamaged or remained intact and there was no damaged observed in the solution of pH 9.0. These results further demonstrated that the membranes were stable at the different levels of pH (1, 3, 5, 7 and 9) commonly found in the human GI tract. Results (Figures 6 and 8) show that no damage occurs when microcapsules were in simulated gastric fluid (SGF) for 3 hours and in simulated intestinal fluid (SIF) for 3 hours mechanical shaking at 150 rpm at 37.2° C.

However, the microcapsules were swelled in SIF. There were 95.4 ± 3.6% APPPA microcapsules undamaged after shaking up to 24 hours in SIF.

These results suggested that APPPA microcapsules were stable in GI condition.

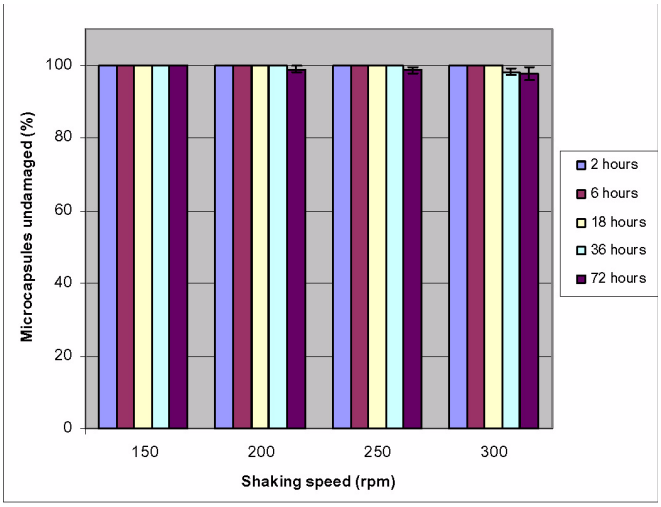

Figure 7: Mechanical stability of APPPA microcapsules in storage solution.

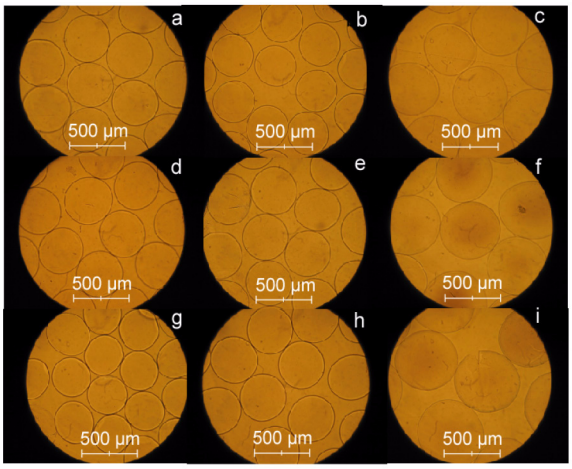

Figure 8: Optical micrographs of APPPA and APA microcapsules. a, APPPA microcapsules; b, APPPA after shaking at 150 rpm, 37.2 °C in SGF for 3hrs. c, APPPA after shaking at 150 rpm, 37.2 °C in SGF for 3hrs and in SIF for 12hrs. d, APPPA after shaking at 150 rpm, 37.2 °C in SGF for 24hrs. e, APPPA after shaking at 150 rpm, 37.2 °C in storage solution for 72hrs. f, APPPA after shaking at 150 rpm, 37.2 °C in SGF for 3hrs and in SIF for 24hrs. g, APA microcapsules. h, APA after shaking at 150 rpm, 37.2 °C in SGF for 24hrs. i, APA after shaking at 150 rpm, 37.2 °C in SGF for 3hrs and in SIF for 24hrs.

Table 1 summarizes the results of mechanical stability of the microcapsule in various GI fluids. Results show that 93.1 ± 3.1% of APPPA microcapsules were undamaged or remained intact (Table 1, Figure 8d) after shaking for 24 hours in SGF solution at 150 rpm at 37.2° C compared to 90.2 ± 3.5% undamaged or intact APA microcapsules (Table 1, Figure 8h). Similar results were obtained in a GI environment; 95.4 ± 3.6% APPPA microcapsules were found undamaged or intact (Table 1, Figure 8f) when microcapsules exposed to simulated gastric fluid (SGF) for 3 hours and to simulated intestinal fluid (SIF) for 24 hours at a mechanical shaking of 150 rpm at 37.2° C compared to 88.9 ± 4.3 % undamaged or intact APA microcapsules (Table 1, Figure 8i). These in-vitro GI stability results showed that the APPPA membrane is stronger and more stable compared to the currently obtainable and most popular APA microcapsule system in SGF and SIF fluids.

Table 1: Comparative mechanical stability of APPPA and APA microcapsules in simulated GI conditions after shaking at 150rpm at 37.2 °C

Microcapsule type	Undamaged percentage of microcapsules in SGF (%)		Undamaged percentage of microcapsules in SGF and SIF (%)	
	3hrs	24hrs	SGF3hrs+ SIF 3hrs	SGF3hrs + SIF24hrs
APPPA	100	93.1±3.1	100	95.4±3.6
APA	100	90.2±3.5	100	88.9±4.3

Microcapsule mechanical stability

Experiments were designed to test the microcapsule stability. For this APPPA microcapsules were immersed in it storage solution (0.1 M $CaCl_2$ solution) and shaken in an environ shaker at different rpm for different times at 37.2° C and then the resulting microcapsules were observed under an optical microscope. No morphological damage on microcapsule membranes was observed after mechanical shaking for up to 72 hours at 150 rpm. Moreover, at 300 rpm mechanical shaking for 72 hours 97.7 ± 1.8% microcapsules microcapsule were found to be intact (Fig. 7, Fig. 8e). These results showed that APPPA microcapsules have good mechanical stability in storage solution.

Microcapsule permeability study

In vitro studies were performed to evaluate immune protection capacity of the APPPA microcapsule membrane and compared with alginate beads and the APA microcapsule membrane using the bovine serum albumin (BSA) molecule and high-performance liquid chromatography (HPLC). Results are shown in Fig. 9.

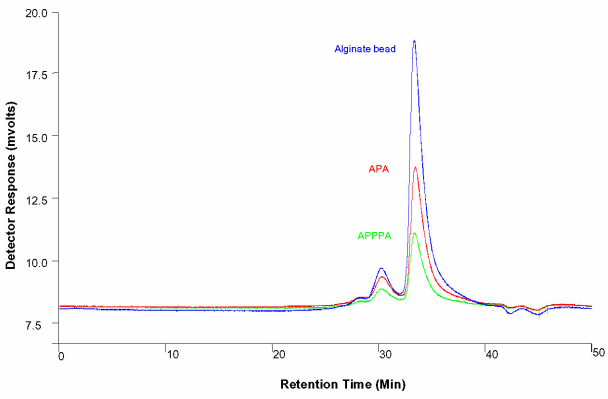

Figure 9: HPLC BSA leakage studies of the APPPA membrane microcapsule compared with alginate beads and presently available APA membrane microcapsules after 24h of shaking (150rpm) at 37.2°C. Blue: Alginate beads (top), Red: Traditional APA microcapsule (middle), Green: Proposed APPPA microcapsule (bottom), solvent used: saline.

Results show that after shaking for 24hours at 37.2° C and 150rpm, about half the amount of BSA was released from the APA microcapsules as compared to the alginate beads; and about half the amount of BSA was leaked from APPPA as compared to the APA microcapsules, suggesting the superior immuno-protective potential of the APPPA membrane. The APPPA microcapsule membrane would protect live bacterial cells form the host immune system; immune responsible molecules are bigger in molecular weight (e.g. IgM 950 KD, IgE 190 KD, IgA 170 KD, IgD 160 KD, IgG 150 KD) compared to the BSA molecule (mole. wt. 68 KD (11)) tested in this HPLC study and would be impermeable to the APPPA microcapsule membranes. These results suggest the permeability of APPPA microcapsules is lower than alginate core and APA beads giving it a competitive advantage over APA microcapsules.

CONCLUSION

Results show the design of multi-layer APPPA microcapsules. This multi-layer APPPA microcapsule displayed superior stability in simulated GI conditions suggesting the possibility of a GI application. These capsules also have shown to display good mechanical stability in storage solutions. Mechanical stability and permeability studies suggest that APPPA microcapsules are more stable, stronger, and display better selective permeability compared to the most popular APA microcapsule systems. It is anticipated that this research of membrane design will provide formulations that would have sufficient resistance to GI tract interactions and allow safe and effective oral delivery of live bacterial cells for various clinical applications. Further research, however, is required to substantiate these results, in particular molecular membrane permeability, immune protection property studies, and cell loading capacity, toxicology, and *in-vivo* affirmation of stability.

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