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# Novel multi-layer APPPA microcapsules for oral delivery: preparation condition, stability and permeability

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Oral therapy utilizing cell microencapsulation has shown promise in the treatment of many diseases. Current obtainable microcapsule membranes, however, show inadequate stability in the gastrointestinal (GI) environment, thus restricting the general application of live cells for oral therapy. To overcome this limitation, we have previously developed a novel multi-layer alginate/poly-L-lysine/pectin/poly-L-lysine/alginate microcapsule (APPPA) with demonstrated improvement on membrane stability over the frequently reported alginate/poly-L-lysine/alginate (APA) microcapsules. In this study, we further examined the effects of preparation conditions on microcapsule formation, and assessed the membrane strength and GI stability. Results showed that increased membrane strength of the APPPA microcapsules was attained by using pectin with low degree of esterification as the mid-layer material, saline as the solvent for the preparation solutions and washing medium, and 0.1 M $CaCl_2$ as the gelling solution for alginate cores. Resistance of this membrane to the simulated GI fluids was also investigated. Permeability of and release profiles from the APPPA microcapsules were found comparable to the APA microcapsules. These findings suggested that the multi-layer APPPA microcapsule formulation may have potential in oral delivery of proteins, live bacterial cells and other biomedical applications.

**Keywords**: Microcapsule, APPPA, Membrane Strength, GI stability, Artificial Cells, Oral Delivery, Pectin

## Introduction

Advancement in cell microencapsulation research has shown promise in delivery of therapeutic products for the treatment of a number of diseases[1-4]. Successful applications require appropriate performance of microcapsules, largely dependent on their core and membrane properties. Previous studies have shown that encapsulated bacterial cells can be delivered orally[5-9] to remove unwanted molecules from the body, as they travel through the intestine and are finally excreted in the stool without being retaining in the body. The microcapsules used for bioencapsulation can be disrupted during the gastrointestinal (GI) passage and fractured by enzymatic actions and other related physiological and chemical stresses. Thus, the microcapsule membrane should be strong enough to withstand the harsh environment in the GI tract and to protect the enclosed cells during the GI passage in order to realize the therapeutic potential.

Although numerous microencapsulation systems have been reported[10-14], the most common formulation for biologic delivery is the alginate-poly-L-lysine-alginate (APA) microcapsules developed in the 1980s[15-19]. These APA microcapsules have been used successfully to limit the major immuno-rejection problems related to the use of live cells[20]. However, they have limited membrane stability and are prone to enzymatic hydrolysis and rapid degradation when exposed to the GI tract, thus making them less suitable for oral applications. Other available microencapsulation systems also have certain limitations and may not be suitable for oral delivery of live biologically active materials for therapy.

Earlier, we have reported that alginate and pectin can be used to design a microcapsule[21]. Both alginate and pectin are naturally-occurring polysaccharides generally recognized as safe by FDA for use as a food ingredient. While pectin is highly methylated in nature, it can be chemically modified to modulate its ester content for a specific application. Pectin is

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*Abbreviations*: APA, alginate-poly-L-lysine-alginate; APPPA, inate/poly-L-lysine/pectin/poly-L-lysine/alginate; aq, aqueous; BSA, bovine serum albumin; DE, degree of esterification; GI, gastrointestinal; HPLC, high-performance liquid chromatography; PLL, poly-L-lysine; SGF, simulated gastric fluid; SIF, simulated intestinal fluid.

reported to possess great stability in acidic conditions, a favorable feature in oral applications[22,23]. The negatively-charged carboxyl groups on pectin molecules can interact with positively-charged poly-L-lysine (PLL) via polyelectrolyte complexation, making it possible to incorporate pectin into the commonly used APA membrane. Based on these properties, we have successfully developed a multi-layer APPPA microcapsule system having some superior features for oral delivery of bacterial cells[24].

In the present study, we further examined the effects of preparation conditions including the type of pectin used, $CaCl_2$ concentration in the gelling bath and the presence of $Na^+$ ion on the formation and stability of the APPPA microcapsules. Some important features that influence their property for potential oral delivery applications were also assessed and compared with the commonly used APA membrane.

## Materials and Methods

### Chemicals

Sodium alginate (low viscosity), poly (L-lysine) hydrobromide (*Mw* 27.4 kD), and model proteins with various *Mw* including bovine serum albumin (BSA), IgG, catalase and thyroglobulin (Table 1) were obtained from Sigma-Aldrich, Canada. Two types of pectin with degree of esterification at 25% (or DE-25) and 63% (or DE-63) were purchased from Sigma-Aldrich, Canada and Fluka BioChemika, respectively. All other reagents and solvents were of reagent grade and used as received without further purification.

### Preparation of microcapsules

The APPPA microcapsules were prepared according to the previously reported protocol[24]. Briefly, Ca-alginate beads were first made using an encapsulator (IER-20, Inotech Biosystems Intl. Inc.) by extruding a sodium alginate solution (1.5 wt %, aq.) into a $CaCl_2$ gelling bath, followed by washing and sequential suspension in PLL (0.1 wt %, aq.), pectin (0.1 wt %, aq.), PLL (0.1 wt %, aq.), each for 15 min and then in alginate (0.05 wt %, aq.) for 10 min to form a 4-layered membrane on the alginate beads, producing the APPPA microcapsules. Washing was performed after each coating to remove unbound biomaterials. The APA microcapsules were prepared by a similar procedure[15,25] by omission of the coating step for pectin and the subsequent PLL incubation in the preparation procedure for the APPPA microcapsules. Unless otherwise specified, the materials used for preparing the microcapsules including alginate, PLL, pectin and $CaCl_2$ were dissolved in deionized $H_2O$, beads were also washed with deionized $H_2O$ prior to each coating step, and the gelling bath contained 0.1 M $CaCl_2$.

To test the effect of alginate gelling on microcapsule stability, $CaCl_2$ solution at different concentrations (0.01, 0.05, 0.1, or 0.2 M) was used as the receiving bath to form the Ca-alginate beads. To compare with the $Na^+$ ion-free protocol, saline replaced the deionized $H_2O$ as the washing medium and the solvent for the afore-mentioned starting materials. APPPA microcapsules containing a mid layer of pectin with low and high DE were also prepared to investigate the membrane stability. The dimension of the resulting microcapsule was measured with an eyepiece micro-meter equipped on an inverted light microscope (LOMO PC, Russia), and averaged from at least 10 beads per batch. The microcapsules were stored at 4°C prior to assessing their properties.

### Osmotic pressure test and mechanical stability of microcapsules

The microcapsule membrane strength was examined by the osmotic pressure test. Specifically, the microcapsules were equilibrated for 30 min in isotonic saline (0.85 wt % aq. NaCl) prior to exposure to a hypotonic medium (1% saline), which led to a high osmotic pressure inside the microcapsules. The sudden influx of water caused the microcapsules to swell and break. The morphology and physical integrity of the microcapsules were examined under the microscope (LOMO PC, Russia), and microphotographs were recorded using a digital camera (Canon Power shot G2). The percentage of broken beads was counted in at least three randomly picked observation fields after 1 h of hypotonic exposure.

### *In-vitro GI stability test*

To examine the potential of microcapsules for oral applications, the simulated gastric fluid (SGF, pH 1.2) and the simulated intestinal fluid (SIF, pH 7.5) were prepared according to United States Pharmacopoeia XXII protocol, and used to test the microcapsule GI stability. Microcapsules were incubated in the SGF at 37ºC with gentle rotation of 150 rpm in an ENVIRON shaker for 3 h, and then transferred into the SIF (37ºC, 150 rpm) for another 2 h of testing. At intervals, the microcapsules were examined by optical microscopy for morphological integrity and changes, and

microphotographs recorded using a digital camera (Canon Power shot G2).

**Protein encapsulation and membrane permeability**

To prepare the protein encapsulated APPPA or APA microcapsules, a mixture of 4 model proteins with various *Mw* (Table 1) was first dissolved in saline and mixed with an alginate solution to give a final alginate concentration at 1.5 wt %. The final protein concentration in the mixture was listed in Table 1. Droplets of the mixture were gelled in a $CaCl_2$ receiving bath (0.1 M). The subsequent coating with PLL, pectin if applicable, and alginate was performed according to the afore-mentioned saline-free protocol.

To confirm the loading of protein in the tested microcapsules, the above freshly made microcapsules were immersed in a sodium citrate solution (10 wt %, aq.), followed by pressing the bead suspension through needles with gradually increasing gauge (from 18 to 27 G) to break the microcapsules and liberate the entrapped proteins. The suspension was collected and centrifuged at 5 000 *g* for 5 min and the supernatant was pressed through a 0.22 μm syringe filter. The clear filtrate was analyzed by a high-performance liquid chromatographic system (HPLC, Varian Inc. Canada) equipped with a column of Biosep-SEC3000 (Phenomenex). The mobile phase was 50 mM phosphate buffer solution (pH 6.8) pre-filtered through 0.22 μm vacuum-driven filter unit (Millipore, Japan), and used at a flow rate of 0.5 mL/min at room temperature. The injection loop was set at 20 μl and UV detection at 280 nm. The mixture of the 4 model proteins in saline solution was used as reference.

To examine the release of encapsulated proteins, known amount of microcapsules were suspended in 2.0 mL saline with gentle rotation (150 rpm) at 37°C. At various time points of 0, 6, 12, 24 and 48 h, a small amount of supernatant was withdrawn, filtered and injected to the HPLC system for analysis under the same settings as described above. Accumulated protein release was determined by HPLC chromatography and data reported from triplicate experiments.

Table 1—Load of proteins in preparation mixture and microcapsules*

| Medium | Load of protein markers (mg/ml) | | | |
|---|---|---|---|---|
| | BSA (68 kD) | IgG (150 kD) | Catalase (247 kD) | Thyroglobulin (670 kD) |
| Alginate mixture | 2.00 | 0.75 | 2.50 | 1.25 |
| APPPA | 0.70 ± 0.04 | 0.42 ± 0.02 | 0.69 ± 0.04 | 0.37 ± 0.02 |
| APA | 0.65 ± 0.04 | 0.30 ± 0.01 | 0.52 ± 0.03 | 0.45 ± 0.03 |

*Mean ± SD from triplicate experiments

## Results

**Effect of type of pectin**

Two types of pectin with low or high DE were tested in this study. As shown in Table 2, the APPPA microcapsules in spherical shape and with smooth surface were obtained by using low DE pectin, whereas high DE pectin was associated with sub-optimal microcapsule morphology with some small cracks on the surface. Stronger membrane strength was observed in the APPPA microcapsules with low DE, as evidenced by less than 20% of these microcapsules broke, in contrast to more than 80% of those with high DE pectin were found destroyed in the osmotic pressure test under the same condition.

**Effect of $CaCl_2$ concentration**

The mechanical stability of the APPPA microcapsules with an alginate core gelled under varied $CaCl_2$ solutions was investigated using an osmotic pressure test. Results showed that gelling in a low level of $CaCl_2$ (0.01 M) led to inferior membrane strength since the APPPA microcapsules were largely ruptured (Fig. 1a). In contrast, increasing the $CaCl_2$ concentration to 0.05 M or 0.1 M in the gelling bath resulted in stronger microcapsule membrane that could withstand the osmotic pressure to a greater extent (Fig. 1b-c). Quantitatively, when the initial Ca-alginate beads were obtained with 0.01 M $CaCl_2$, substantial microcapsule deterioration occurred in the osmotic test, with approximately 80% of the resulting

Table 2—Effect of degree of esterification (DE) of pectin on APPPA microcapsule morphology and membrane stability

| Type of pectin | Microcapsule morphology | Microcapsule diam* (μm) | % Broken microcapsules[#] |
|---|---|---|---|
| Low DE (DE-25) | Spherical in shape smooth surface | 400 ± 20 | 19.7 ± 3.9 |
| High DE (DE-63) | Mostly spherical some cracks on surface | 410 ± 30 | 86.3 ± 7.2 |

*From freshly made microcapsules and expressed as mean ± SD (n=10)

[#]Assessed after the microcapsules were exposed to osmotic pressure. Data are expressed as mean ± SD (n=3)

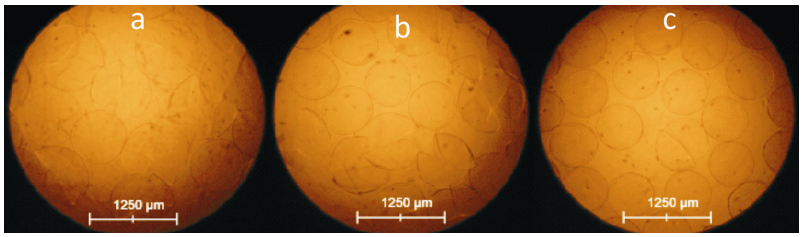


Fig. 1—Optical micrographs of APPPA microcapsules after exposure to hypotonic solution following isotonic incubation in the osmotic pressure test [The core alginate beads of these microcapsules were prepared by extruding an alginate solution into a gelling bath containing $CaCl_2$ of different concentrations: (a) 0.01 M, (b) 0.05 M and (c) 0.10 M]

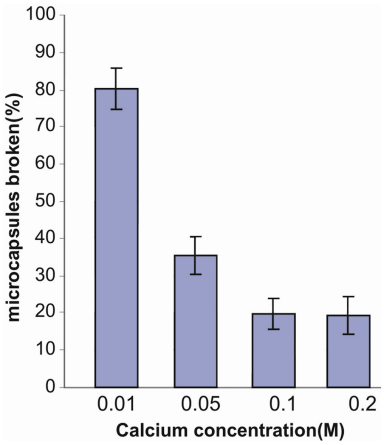


Fig. 2—Osmotic pressure stability of APPPA microcapsules as a function of $CaCl_2$ concentration in the gelling bath [The microcapsules were prepared using low DE pectin and under saline-free conditions]

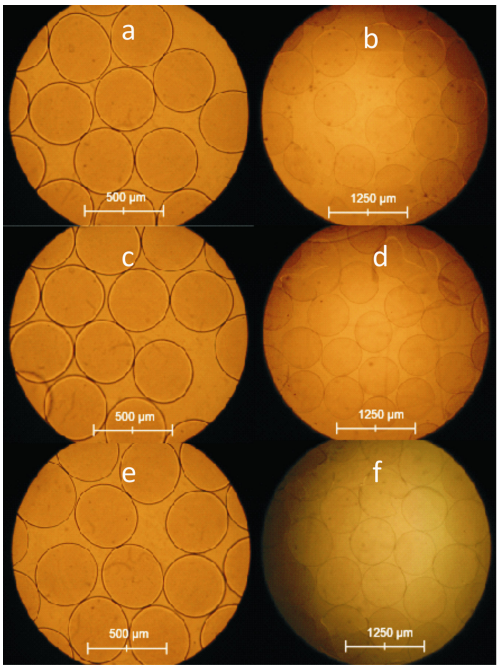


Fig. 3—Optical micrographs of APA (a-b) and APPPA (c-f) microcapsules after preparation (a, c and e) and after exposure to hypotonic solution following isotonic incubation in the osmotic pressure test (b, d and f) [The microcapsules were prepared either in saline-free conditions (a-d) or in the presence of saline (e-f)]

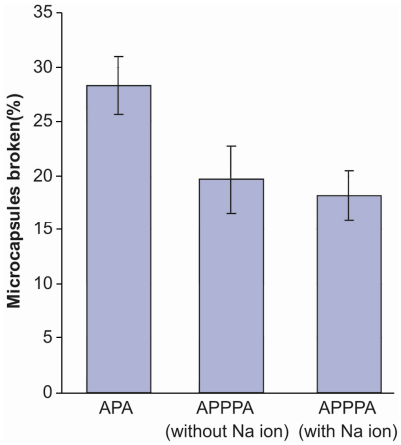


Fig. 4—Osmotic pressure stability of APPPA microcapsules prepared under saline-free conditions or in the presence of saline, and compared to the control APA microcapsules

APPPA microcapsules split (Fig. 2). The number of burst beads drastically decreased with an increase of $CaCl_2$ concentration to 0.1 M, where only 20% of the APPPA microcapsules broke in hypotonic solution. No further increase in membrane strength was observed at 0.2 M $CaCl_2$ concentration.

**Effect of presence of saline during microcapsule preparation**

Experiments were designed to study the effect of $Na^+$ ion during microcapsule formation on the stability of the resulting microcapsule membrane. For this, deionized $H_2O$ was replaced by saline as a solvent to prepare all the solutions for preparing the APPPA microcapsules including the $CaCl_2$ precipitation bath, biomaterial solutions and washing medium. Figure 3 shows the morphology of the APPPA microcapsules prepared under saline or saline-free condition before and after challenged to osmotic pressure. Apparently, the presence of saline in preparation media did not impair formation of the APPPA microcapsules. The membrane strength of these microcapsules was found similar to those obtained under saline-free conditions, with 18.2 ± 2.3% and 19.7 ± 3.1% of beads burst under the same osmotic pressure, respectively. For comparison, the control APA microcapsules displayed a slightly higher % broken beads (28.3 ± 2.7%) (Fig. 4).

***In vitro* GI stability**

To evaluate the membrane resistance to the simulated GI conditions, the APPPA microcapsules were sequentially incubated with the SGF and the SIF. Figure 5 shows that both APA and APPPA microcapsules remained physically intact, when exposed to the SGF, with slightly shrinking of less than 10% over the tested period. Just a few minutes after immersion in the SIF, the microcapsules swelled substantially and became difficult to identify their boundary. Although both APPPA microcapsules prepared with or without saline largely retained spherical morphology, the microcapsules from the saline-free batch displayed a greater extent of swelling, became fairly weak and hardly withstood handling (Fig. 5).

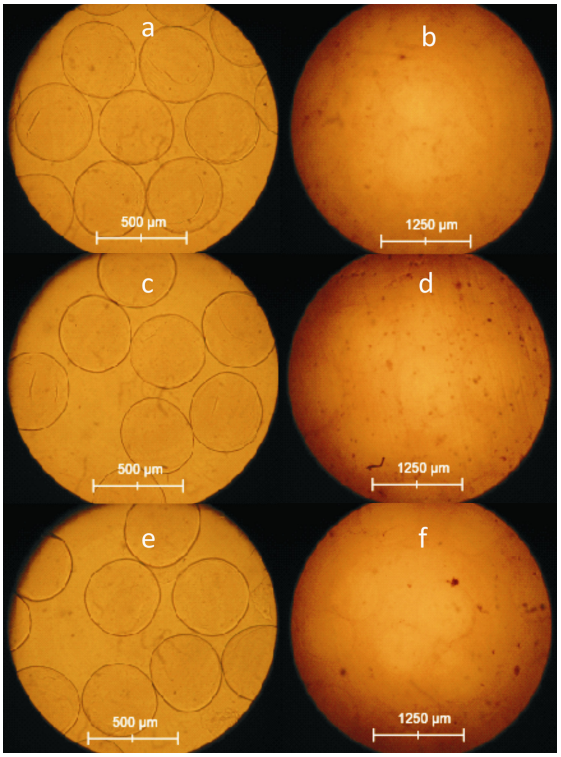


Fig. 5—Optical micrographs of APA (a-b) and APPPA (c-f) microcapsules after preparation (a, c and e) and after exposure to simulated GI fluids (b, d and f) [The microcapsules were prepared either in saline-free conditions (a-d) or in the presence of saline (e-f)]

**Protein encapsulation and membrane permeability**

The present *in vitro* study characterized the permeability of the APPPA microcapsules by egress experiments using a mixture of model proteins as the permeate markers. Results showed that all the tested proteins were successfully encapsulated in the APPPA microcapsules with varied protein loading (Table 1), similar to those encapsulated in the APA beads.

Results from our protein egress experiments showed that at 0.5 h, no protein was detected in the supernatant of the media containing protein-loaded APPPA or APA microcapsules (purple lines in Fig. 6a,b). After 6 h of incubation, release of encapsulated proteins from both APPPA and APA microcapsules was observed by the presence of two small peaks in the chromatograms detected at the retention time of 31.243 min and 33.049 min, which represented BSA and IgG (yellow lines in Fig. 6a,b). Increase in these peaks' height and area was seen over incubation time, indicating more BSA and IgG released into the media as time elapsed. Catalase and thyroglobulin with higher *Mw* (247 kD and 670 kD, respectively) were not detected in the media throughout the testing period for up to 48 h. Based on these observations, the APPPA microcapsule membrane was permeable to molecules of 150 kD or lower, and the molecular weight cut-off lied between 150 kD to 247 kD.

By normalizing with the loading of proteins in the microcapsules, it was found that 32.8 ± 2.9% of the encapsulated BSA was liberated from the APPPA microcapsules at 6 h, and the amount was doubled to

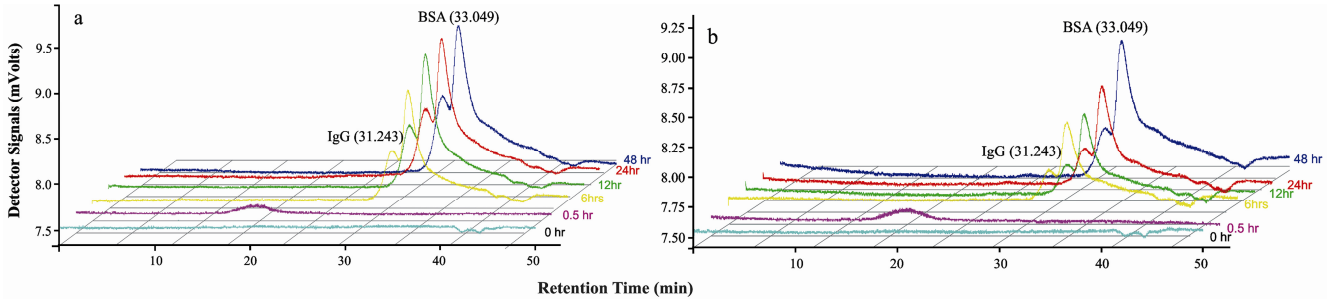


Fig. 6—Overlaid HPLC chromatographs of released proteins from microcapsules. APPPA (a) and APA (b) microcapsules containing a mixture of protein markers (BSA, IgG, catalase and thyroglobulin) with various *Mw* were incubated at 37°C in saline with gentle rotation of 150 rpm. Supernatant was withdrawn at different time intervals (0, 0.5, 6, 12, 24 and 48 h) to determine the release of proteins

Table 3—Percent accumulated protein release from microcapsules*

| Incubation time (h) | % BSA | | % IgG | |
|---|---|---|---|---|
| | APPPA | APA | APPPA | APA |
| 0 | 0 | 0 | 0 | 0 |
| 0.5 | 0 | 0 | 0 | 0 |
| 6 | 32.8 ± 2.9 | 30.1 ± 1.5 | 13.8 ± 1.2 | 14.2 ± 1.2 |
| 12 | 68.6 ± 4.3 | 53.8 ± 3.1 | 28.6 ± 2.4 | 28.5 ± 2.1 |
| 24 | 81.4 ± 4.3 | 78.5 ± 3.1 | 31.0 ± 2.4 | 27.0 ± 1.8 |
| 48 | 91.4 ± 7.1 | 92.3 ± 4.6 | 42.9 ± 2.4 | 45.4 ± 3.0 |

*Percent accumulated release of detected proteins was defined as the total amount released to the medium over the total amount loaded in tested microcapsules. Data are expressed as mean ± SD from triplicate experiments

68.6 ± 4.3% at 12 h. After 24 and 48 h, the released BSA augmented to 81.4 ± 4.3% and 91.4 ± 7.1%, respectively (Table 3). This BSA release profile appeared similar to that for the control protein-containing APA microcapsules. With regard to the encased IgG of higher *Mw*, the release was found much slower than that of BSA. Approximately 14% of the encapsulated IgG was released from both APPPA and APA microcapsules at 6 h, and gradually increased to nearly 30% and slightly over 40% at 24 and 48 h (Table 3).

## Discussion

In many biomedical applications, microcapsules are subjected to mechanical stresses exerted by their environment that could induce deformation and potential break-up of the microcapsules. It is believed that microcapsule mechanical stability is paramount and should not be compromised[23,25]. The preparation details during the microcapsule formation are expected to be important factors in determining its structure and stability. For instance, the type of pectin used to form the mid layer in the sandwich-like membrane structure was found to have a significant effect on the APPPA microcapsules (Table 2). Pectin with low DE contains a higher level of free carboxyl groups than that with high DE, thus may allow for more effective binding with positively-charged PLL, resulting in improved membrane strength of the APPPA microcapsules.

Calcium concentration was another critical element in controlling the stability and permeability of the APPPA microcapsules. Since neither alginate nor pectin can gel or form a cross-linked network in the absence of divalent ions, $CaCl_2$ concentration as low as 0.01 M could not provide adequate cross-linking to stabilize the alginate core and pectin-based membrane. Our results showed that increasing $Ca^{2+}$ ion to 0.1 M in the gelling bath yielded a stronger gelled core and microcapsule membrane, likely because of more cross-linking sites and higher degree of aggregation. Further increase in $CaCl_2$ concentration to 0.2 M was not beneficial as the carboxyl groups in alginate and pectin available for binding may become saturated, and excessive $Ca^{2+}$ ions may possibly be detrimental to the living cells.

For microencapsulation of living cells, a saline protocol is favourable in terms of preserving live cell viability. As well, a substantial quantity of $Na^+$ and phosphate ions is present in physiological conditions that could induce osmotic swelling. Previous study[25] suggested that the presence of non-gelling ions such as $Na^+$ in the gelling bath may leave a high number of free carboxyl groups on the alginate molecules for binding with PLL. On the other hand, the presence of $Na^+$ ion could favor the formation of more homogenous alginate gels, leaving a higher amount of alginate at the surface of the beads with a higher number of binding sites for complexation with PLL. However, resistance of microcapsule membrane may be compromised by the change in the ionic strength of the solvent, which directly affects the conformation of polyamino-acid, alginate and pectin molecules. In particular, the long chains of these biopolymers will transform from a compact coil structure in saline to an elongated conformation in water. The elongated form of PLL was shown to be associated with thinner membrane and lower diffusion rate. Our results demonstrated that the APPPA microcapsules prepared in the presence of $Na^+$ ion displayed improved membrane strength over their counterparts prepared using a saline-free protocol. Results also showed that using saline as solvent increased microcapsule resistance, but did not effectively alter the membrane strength of microcapsules.

The permeability of microcapsules is known to be one of the most important parameters for live cell delivery. In particular, the semi-permeable membrane should allow for diffusion of small particles such as nutrients, oxygen, wastes and therapeutic products, but impede the passage of large molecules and materials such as immune cells, antibodies and leukocytes. Our study demonstrated that BSA and IgG of relatively lower *Mw* could pass through the membrane of the APPPA and APA microcapsules, but the catalase and thyroglobulin having higher *Mw*t could not. These

results suggested that molecular weight cut-off of the multi-layer APPPA microcapsules lied in between 150 kD and 247 kD, which was comparable to the APA membrane. Sustained release of BSA and slower release of IgG molecules were evidenced, indicating potential of the APPPA microcapsules in controlled release applications.

In conclusion, the present study demonstrated that the membrane stability of the multi-layer APPPA microcapsules could be improved by modulating preparation details including the use of pectin with low degree of esterification, saline as the solvent and washing medium, and 0.1 M $CaCl_2$ as the alginate gelling solution. The resulting APPPA microcapsules displayed increased membrane strength and resistance to the simulated gastrointestinal fluids, suggesting the potential in GI applications. However, further study is required to substantiate these results, in particular *in vivo* affirmation of membrane stability.

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