The Formulation of Low Methoxyl Pectin-Based Polyelectrolyte Complexes for Colonic Coating of Probiotic Cells

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Abstract. Polyelectrolyte Complex (PEC) was formed by a cross-linking process of different charges of two or more polymers with cation. The structure of a three-dimensional network of PEC facilitated encapsulation of probiotic cells. Some natural polyions belonging to polysaccharides and proteins were used for the PEC formation extensively. This study was aimed to obtain a positively charged polyelectrolyte complex as a colonic coating for probiotic cells using Layer-by-Layer (LbL) technique. This research was undertaken in completely randomized design with three replicates. Treatments comprised formulas containing the composition of Low Methoxyl Pectin (LMP), lysine and diethylamine at different levels of chitosan concentration (T1: chitosan of 0.050%, T2: chitosan of 0.075%; T3: chitosan of 0.100%) compared with control (T0: chitosan of 0%). Results showed that the treatments contributed to viscosity and nitrogen content of PEC. This research gained the best result on T3, where the use of chitosan of 0.100% in the formula made up of LMP, chitosan, lysine and diethylamine characterized by stable positive charges, nitrogen content of 0.00688% and viscosity of 18.53 cP. The application of LMP polyelectrolyte (LMPpe) complex of the T3 for probiotic cells coating resulted in good viability until it reached colon with population of 7.67 log cfu/mL (4.67x10^7 cfu/mL).

1. Introduction
Inter-polymer complex (IPC) hydrogels have been widely developed for the purpose of vehicle materials or substances of various systems delivering active materials or life cells. The polymeric networks or hydrogels are well tolerated, biocompatible and are more sensitive to changes in environmental conditions [1].

The IPC hydrogels known as polyelectrolyte complex (PEC) obtained by cross-linking of two or more polymers [2]. The PEC is prepared by electrostatic interactions between oppositely charged polyions. Mixing oppositely charged polyions in solution will result in their self assembly due to the formation of strong, but reversible electrostatic links. Direct interactions between the polymeric chains lead to the formation of PEC networks with non-permanent structures, avoiding the use of covalent cross-linkers. The interpolymer chain interactions in combination of correct polymers are physical in nature and reversible but can provide the required properties for optimal material delivery [3].
The IPC hydrogel provides free-space volume facilitating encapsulation of different materials in the structure of a three-dimensional network. Encapsulation is aimed to protect components sensitive to adverse conditions [4]. According to [5], polymer hydrogels may be used for micro- and nanoencapsulation of bioactives, including cells. One of encapsulation techniques is the Layer-by-Layer (LbL). The technique constitutes consecutive adsorption of differently charged polymers on the surface of a bioactive producing layers whose thickness reaches the nano-scale. Cells may be encapsulated with functional ingredients through the LbL. Life cells’ properties may be well-modified using LbL technique [6]. In bacteria, adhesion of polymer layers on the surface of cell membrane is facilitated by positive charges of first layer [7].

Various properties of IPCs such as porosity, bio-adhesiveness and elasticity can be controlled by the appropriate choice of the network-forming polymers [8]. Properties of a hydrogel as an encapsulation matrix are affected by characteristics of the material used. In encapsulation of probiotics, protective ingredients should be able to facilitate viability of cells. Some polysaccharides are utilized extensively for PEC formation. Nature provides abundant poly-ions, two of which are pectin and chitosan.

Pectin is polyanion used for cross-linking to formulate PEC. Pectin is highly water soluble, absorbed in the upper gastrointestinal tract (GIT), and completely degraded by the colonic bacterial enzymes. Chitosan is also one of the promising biopolymers. It is soluble at low pH, hence there is a need to make enteric coated formulation to protect it from stomach’s environment. The amino groups on the C2 position of the repeating glucopyranose units are positively charged at low pH values (below its pKa value), it can interact electrostatically with anionic groups of other polyions to form polyelectrolyte complexes.

Complexes protecting protein compounds hold biocompatible and biodegradable properties and more effectively restrict release of entangled materials. Designed pectin-chitosan polyionic complexes, which form through interactions between the carboxyl groups of pectin and the amine groups of chitosan [9]. The amine groups on pectin are located on the galacturonic acid residues on the pectin backbone and resulted in increased surface charge (as confirmed by Zeta potential measurements) [10]. The complex protects the protein, has biocompatible and biodegradable characteristics, and limits the release of entrapped materials more effectively. The polyelectrolyte, in addition to different charged polysaccharides, also may be generated by polysaccharides with positively charged molecules or compounds such as proteins and amino acids. The mixture of proteins and polysaccharides allows coacervation complex formation [11, 12]. Lysine is an essential amino acid containing positive charges on ε-amino groups. Lysine is alanine substituted by propylamine on β-carbons. Amino groups indicate a high reactivity. Since pH affect ionization of functional side groups on biopolymers, it has a critical role in controlling protein-polysaccharide interactions [13]. At below isoelectric point of protein, protein is positively charged, which results in strong electrostatic attraction between two biopolymers with opposite net charges [14]. Based on the literature review, this research’s objective was to gain a positively charged polyelectrolyte complex as a coating material for probiotic cells through the LbL encapsulation technique.

2. Materials and methods

2.1. The formation of LMP polyelectrolyte (LMPpe) for a probiotic cells coating

A polyelectrolyte complex of the probiotic cells coating was synthesized on the basis of methods presented by [15] with some modifications (materials, concentrations, volumes, temperatures). The 0.4% of LMP solution was prepared using magnetic stirrer (500 rpm) for 16 hours at room temperature. The 3.5-mL of 0.4% LMP solution (pH 4) and 3.2 mL of chitosan (T1=0.050%; T2=0.075%; T3=0.100% of treatment concentrations) at pH 5 were put into a sterile beaker glass (volume of 25 mL), mixed using magnetic stirrer for 20 minutes. The 0.6-mL of 0.1% lysine was dropped into the mixtures [16] while stirring continued for 1 hour. Mixtures were transferred into sterile bottles, added to the solution of 0.35 M diethylamine (pH 2) [15] and the final volume was adjusted to 10 mL [16], then shaken until homogen (±5 minutes) to form LMPpe. The encapsulant production was also carried out
without chitosan as a control (T0). The experiment was conducted in completely randomized design in three replicates. Parameters observed were viscosity, nitrogen content and zeta potential.

2.2. The probiotic cells coating process using LMPpe
The probiotic cells coating using the LbL technique was done on a combination of [17] and [18] methods, with some modifications (i.e. materials, temperature, speed and duration of centrifugation). The 0.75 mL-suspension of Bifidobacterium longum cells (as model of probiotic cells harvested in the early stationary-phase of h-6) in 11 mM PBS (pH 7.4) was inserted into a centrifuge tube, and added to 1.5 mL of LMPpe. The mixture was left at 37°C for 15 minutes allowing it to be adsorbed completely. The LMPpe-coated cells were centrifuged (speed of 4000 rpm, temperature of 4°C) within 15 minutes, then the supernatant was removed. The LMPpe-coated cells were washed two times with sterile aquadest, and sonicated (temperature of 25°C) within 10 minutes then the liquid was removed. The coated cells were resuspended in the sterile PBS (adjusted pH 7.4).

2.3. The assay of LMPpe-coated probiotic cells
2.3.1. The assay in Simulated Gastric Fluid (SGF).
Pepsin enzyme dissolved in 0.5% NaCl sterile up to a concentration of 3 g/L (pH 1.5 adjusted with 0.5 M HCl sterile) [19] was used as SGF. The 1 mL of suspension of LMPpe-coated probiotic was added to 10 ml of the SGF, warmed in an incubator shaker (170 rpm, 37°C) and sampled at min-0, 10, 40, and 60 respectively. After pepsin was inactivated by 0.5 M of NH₄CO₃ until pH 7.5 was reached [20], samples were subjected to a release from encapsulant according to [21]. Briefly, 1 ml of samples were added to 10 mL of the sterile PBS solution (pH 7), incubated within 1 hour in an incubator shaker (100 rpm, temperature of 37°C).

2.3.2. The assay in Simulated Intestinal Fluid (SIF).
Pancreatin enzyme dissolved into 0.5% NaCl sterile solution up to a concentration of 1 g/L and added to 4.5% bile salt (pH 7.5 adjusted with 0.5 NaHCO₃ sterile solution) [22] was used as SIF. From the 60-minute tube (the end of SGF sampling), 1 ml of sample was taken and put into tubes containing 10 mL of the SIF (tubes for 10, 15 and 30 minutes) [23]. Samples were incubated in an incubator shaker (170 rpm, 37°C) and were sampled at aforementioned time. After proteolysis was inactivated with a 10% w/v concentration of TCA [24], cells were subjected to release from encapsulants.

3. Results and discussion
3.1. Characteristics of Low Methoxyl Pectin polyelectrolyte (LMPpe)
The LMPpe was resulted from electrostatic interaction between positive and negative charges of its cross-linking forming components in ionic gelation process to form a hydrogel. This step showed that LMPpe was formulated using LMP and chitosan as well as components producing positively charged amino groups (NH₃⁺) of lysine and diethylamine. The LMPpe charges are presented in Table 1.

Based on Table 1, it was indicated that all treatments show negative and or positive potential, and not zero point. According to [25], when polyelectrolytes are mixed in such a ratio that there is an excess of one charge (either positive or negative), a non-stoichiometric complex is formed that are usually soluble. Further, Table 1 presents that negatively charged was still appear in T1 (chitosan of 0.050%) and T2 (chitosan of 0.075%). This is due to the fact that there were COO⁻ groups (negatively charged carboxylic groups) originated from pectin chains or other negatively charged molecules of chitosan, lysine and diethylamine freely dissociated (unbound) remained in solutions of the polyelectrolyte complex. In control (T0), all measurements resulted of the negatively charged poly-ions. Seemingly, positively charged molecules of lysine and diethylamine had not met electrostatic reactions with all negative charges of carboxylic groups. The amino group at the C2 position of a chitosan glucopyranose
units’ chain has a positive charge at low pH. This group electrostatically interacts with the anionic group of other poly-ions (commonly negative charges of carboxylic acid) to create a polyelectrolyte complex.

**Table 1.** The zeta potential point of the formulations.

| Observations | T0 | T1 | T2 | T3     |
|--------------|----|----|----|--------|
| 1            | (-2.07); (-2.18); 4.15; 3.74; 5.17; 2.40; 1.07; 0.925; | | | |
|              | (-2.22) (-2.55) | 1.00 | | 0.0115 |
| 2            | (-1.69); (-1.63); 3.62; 3.05; 6.83; 2.74; 0.865; 0.120; | | | 0.261 |
|              | (-2.01) (-3.30) | (-4.42) | | |
| 3            | (-1.74); (-2.02); 2.95; 3.21; 3.64; 2.50; 1.49; 1.14; | | | |
|              | (-2.18) 3.01 | | 2.26 | 1.15 |

Remarks: T0 = without chitosan; T1 = chitosan of 0.05%; T2 = chitosan of 0.075%; T3 = chitosan of 0.100%

A relatively stable LMPpe potential in the T3 (chitosan of 0.100%) engendered positive charges in all measurements. Thus, in terms of the charge profile the T3 indicated the better result rather than the other treatments and the control. According to [26], positive charges of the material may favor occurrence of other negatively charged materials’ adsorption.

The reaction results of pectin and chitosan’s cross-linking and the quaternisation process making use of lysine and diethylamine were confirmed by the Kjeldhal titration method to find out the nitrogen content in the system. Nitrogen contents in LMPpe complexes were affected by treatments of different chitosan content in the formulation (Figure 1). It was suggested that as chitosan concentrations in T2 and T3 formulas were higher than T0 and T1, 0.00767% and 0.00688% respectively. Chitosan is an amino polysaccharide, a polysaccharide containing amino groups, either NH$_2$ or NH$_3^+$ groups depending on levels of its environment acidity [26]. As a coating material, LMPpe that contains chitosan, lysine and diethylamine provides nitrogen ions able to be utilized by probiotics for metabolism.

![Figure 1](image1.png)

**Figure 1.** Nitrogen content of formulation of polyelectrolyte based on LMP, chitosan, lysine and diethylamine (Remarks: T0 = without chitosan; T1 = chitosan of 0.05%; T2 = chitosan of 0.075%; T3 = chitosan of 0.100%).

The viscosity is one of polyelectrolyte complex hydrogel profiles controlled by appropriateness of network-forming polymer selection. Figure 2 illustrates the polyelectrolyte complex hydrogel’s viscosity of the experiment. Significantly the treatments (P<0.05) affected LMPpe complex’s viscosity in which as the chitosan concentration in the formula was greater, the viscosity value was higher. The T3 engendered higher viscosity (18.53 cP) compared to T0 (16.13 cP), T1 (16.00 cP) and T2 (17.33 cP). Viscosity of T1 and T2 formulas were relatively similar and apparently, the use of chitosan of 0.05% (T1) had not altered the levels of solubility from that in the T0.
In T3, slightly positively charged was measured that likely was due to the fact that in acidic conditions (chitosan of pH 5, LMP of pH 4) most of amino group’s positive charges had been cross-linked to most of pectin’s carboxyl groups. This process yielded formation of pectin molecules’ cross-linking network shaping a rigid and strong structure [27] indicated by a condition of a more viscous hydrogel. A relatively higher value of viscosity in T3 (18.53 cP) seemingly shapes a robust and strong hydrogel of LMPpe so as expected to be able to promote stronger adhesion to the membrane surface of the probiotic’s cell, beside the presence of electrostatic reaction between the charge of both of them. From the results, the T3 formula (contained 0.100% of chitosan) was selected as a coating material for probiotic cells in the LbL encapsulation technique.

3.2. The in-vitro assay of probiotic cells viability

The growth pattern of probiotic cells were performed before encapsulated (Figure 4). Growth pattern of *B. longum* was presented in a growth curve based on colony and its biomass for 18 hours using sterile
medium of sprout extract 2.5%. The cells colony on the incubation time h-0 around of $10^{10}$ cfu/mL, and in range time of h-1 to h-4 the cells growth were slow producing colony number of 11.413 log cfu/mL. The time range of h-4 to h-6 was performing as cells exponential phase, the cells colony was increasing sharply from 11.413 log cfu/mL (h-4) to 15.124 log cfu/mL (h-6). After that, until h-18 the cells were experiencing stationer phase (range number of 15.181-15.704 log cfu/mL). On the incubation time of h-6, cells were harvested, then separated from medium to be encapsulated. The h-6-cells were used as initial in-vitro observation for free cells also to be encapsulated.

![Figure 4](image_url)

**Figure 4.** The growth curve of *Bifidobacterium longum* based on population number and biomass in medium of sprout extract 2.5%.

The assay of probiotic cells viability to SGF (fluid which contains pepsin and HCl) and SIF (fluid which contains pancreatin and bile salt) was performed after cells were subjected to encapsulation using LMPpe in LbL technique. According to [28], it is in the LbL that inorganic particles and biological cells are able to be utilized as a template for a coating, besides solids of other substances [29]. The LbL encapsulation was constructed by [30].

![Figure 5](image_url)

**Figure 5.** The survivability profile of *Bifidobacterium longum* in SGF and SIF: (a) LMPpe-coated cells; and (b) free cells (uncoated cells).

Encapsulation of probiotics using LbL technique produced particles with positively charged surface (+21.8; +22.3; +22.8; +20.6; +20.3; +20.9 mV). Beforehand, membran surface charge of probiotic cells were negative (-4.13; -5.68 mV). Once the encapsulation was completed, the average diameter of LMPpe-coated cells was found to be 3326.6 nm (initial cell diameter was 1321 nm). Result showed that probiotic cells had been successfully encapsulated.

Bacteria acts as a probiotic if it survives to reach the host-colon and in an adequate number to provide positive effects for health $10^7$ cfu/mL [31]. To be able to reach the colon, bacteria can pass the low-acidic condition in the stomach and the presence of pancreatin in the intestine. The in-vitro SGF and SIF assay was illustrated in Figure 5. It seemed that the number of uncoated cells at min-10 was 15.88 log cfu/mL, whereas the encapsulated cells dropped to only 12.20 log cfu/mL. The viability
decrease considerably varied that may be affected by treatments and compositions of encapsulant [32]. The decline in the population is likely resulted from the harsh condition of process, i.e. centrifugation, leading to cell injury. Probiotics typically are considered facultative anaerob. A drop in number of population in the encapsulation occurred since different handling of probiotics was apparently not completely anaerobic. Mixing of coating materials as well as stirring engender greater oxygen incorporation into the mixture of cells and materials, while oxygen is toxic for anaerobic bacteria [33].

When observed until min-90, either uncoated cells or LMPpe-coated probiotics underwent a decrease in the population. A fall in the number of free cells occurred due to a high acidity may lead to membrane damage and loss of intercellular components (lysis) and consequently resulting in death [34]. The number of free cell’s probiotics at min-90 was 6.88 log cfu/mL and it was 15.88 log cfu/mL at min-10 decreased by 9 log cfu/mL. The LMPpe-coated cells at min-10 was 12.22 log cfu/mL and after the assay in SGF continued by SIF at min-90 it number of 7.67 log cfu/mL (decreased by 4.53 log cfu/mL). The LMPpe encapsulant contributes to survivability of bacteria. Pectin and chitosan act as a barrier protecting from high acidity. Bacteria may tolerate to high acidity if cytoplasmic pH is retained more alkaline compared to extracellular. To maintain pH level, cells should have a membrane comprising barrier limiting proton movement [34]. This fact suggested that encapsulation with the LMPpe potentially enhances viability of probiotic reaching the colon. After the 90-minute assay the population remained 7.67 log cfu/mL (4.67x10^7 cfu/mL).

To confirm that the active side of LMPpe-coated probiotic cells can react electrostatically with negatively charged of material it was subjected by Transmission Electron Microscope. This observation had been published by author [35]. The adherence of LMP nanoparticles (-15.2; -16.6 mV) [35] on the surface of LMPpe-coated cells (potential zeta of +20.37 until +22.30 mV) were presented in Figure 6.

Figure 6. Observation by Transmission Electron Microscope of LMPpe-encapsulated Bifidobacterium longum cells which was electrostatic reaction with negatively charged of LMP nanoparticles [35].

Figure 6 shows that negatively charged of LMP nanoparticles can adhere on surface of LMPpe-encapsulated probiotic cells [35]. The adherence process was applied using LbL encapsulation technique. Based on result, the active side of positively charged of LMPpe-coated cells probably can be used as vehicle for negatively charged of functional materials delivered to the gastrointestinal tract.

4. Conclusions
The research attempted to obtain an LMPpe complex as a coating material for probiotic cells and reported the best result in the T3, i.e. the use of 0.100% chitosan. This formula had been characterized by stable positive charges, nitrogen content of 0.00688% and viscosity of 18.53 cP. The application of the LMPpe complex of the T3 formula in a probiotic cells’ coating resulted in good viability of cells enabling the 7.67 log cfu/mL (4.67x10^7 cfu/mL) population number to reach the colon.
5. References

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