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OPTIMIZATION AND CHARACTERIZATION OF CALCIUM ALGINATE/KONJAC GLUCOMANNAN BEADS AS ORAL PROTEIN DRUG DELIVERY SYSTEM

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ABSTRACT

This work investigates the optimization and characterization of calcium alginate/konjac glucomannan beads by an ionotropic gelation technique for controlled release of bovine serum albumin. The effect of amount of sodium alginate and konjac glucomannan as the factors affecting protein encapsulation efficiency and protein release were optimized and analyzed by using RSM-FCCD. Increase in protein encapsulation efficiency and decrease in protein release were recorded with the increase of both the amount of sodium alginate and konjac glucomannan, used as polymer blend. The optimized beads showed high encapsulation efficiency (63.3 ± 1.35%) with suitable protein release (100% protein release after almost 4 h). The swelling of beads were highly influenced by pH of dissolution medium. These beads were also characterized by FT-IR spectroscopy, SEM and TA for protein-excipients interaction, beads surface morphology and beads strength, respectively. These developed calcium alginate/konjac glucomannan beads could possibly be employed as controlled release matrix for targeted delivery of protein.

Key words: Encapsulation, Sodium alginate, Konjac glucomannan, Controlled protein release, Targeted drug delivery.

INTRODUCTION

Advances in biotechnology over the past few years have driven the production of various clinically useful protein and peptides. Till recent, parenteral route (injection) is the most common way for administering protein drugs [1]. However, the patient compliance with injection regimens is very poor, particularly for disease like diabetes which require 2-5 times injection per day. Thus, oral route remains as the most preferable route to deliver protein drugs due to ease of administration [2].

However, administration of protein and peptide drug through oral route is quite challenging in terms of controlled delivery, targeting formulations and controlled manner. Unlike synthetic pharmaceutical, proteins are more sensitive because of their diffusivity and low partition coefficient [3]. Due to these features, proteins may undergo chemical changes, proteolysis and denaturation during passage through the human gut [4]. Many attempts have been carried out in order to improve oral stability of protein in human body. The encapsulation of protein drugs using different biodegradable and biocompatible polymers have received much attention in recent years [5]. Encapsulation of protein by means is to incorporate a protein drug into a suitable matrix that can provide protection during exposure to the harsh condition of the human gastrointestinal tract [6]. In addition, encapsulation also will help in attaining the controlled release of the drug at the targeted site over a long period of time [7].

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Development of ionotropic gelation technique in producing biopolymeric beads as carrier in drug delivery system has gained great attention recently [8, 9]. Through this technique, beads are formed when ionic polymer such as alginate, konjac glucomannan and gum Arabic undergo ionotropic gelation and precipitate due to electrostatic interaction between oppositely charged species. This is a very simple technique, non-toxic and easy to control in terms of production [8]. Alginates are natural occurring polysaccharides extracted from brown algae that have been investigated since decades ago for their unique characteristics. These characteristics have enabled alginate to be used as matrix for protein delivery. Alginate is a polyanionic copolymer of guluronic and manuronic acids that can form hydrogel beads through ionotropic gelation by the addition of divalent cations in aqueous liquid [10]. Besides proteins [11, 12], the other bioactive agents that can be entrapped into alginate matrices are including cells [13] and DNA [14]. This is due to the relatively mild gelation process.

Even though alginate beads are easy to be prepared through ionotropic gelation method, there is a major problem regarding drug loss during beads preparation due to the porosity of alginate [15]. In addition, alginate is unstable in acidic environment which can cause the decarboxylation of alginate [12]. Therefore, many modifications based on the combination of alginate with other polymers as drug carrier have been investigated [12, 16, 17]. Konjac glucomannan (KGM) is high molecular weight polysaccharide extracted from tubers of *Amorphophalluskonjac* plant. It is non-ionic glucomannan and consists of linear random copolymer of β-(1→4) linking D-mannose and D-glucose, and almost one in 15 of the sugar units are acetylated. Due to its nature that it cannot be hydrolyzed by digestive enzymes in human stomach, KGM is considered as an indigestible dietary fiber that is useful to reduce the risk of developing diabetes and heart disease [18, 19]. KGM could produce strong, elastic, heat-stable gel when heated with mild alkali [20]. Besides that, KGM also has been investigated as drug carrier. Wang and He [12] have reported the usage of KGM in combination with alginate and chitosan as controlled released matrix. Optimization is defined as statistical experimental design methodologies and has been used widely to produce optimum response. Central composite design, which is a response surface design, is one of the most reliable statistical optimization designs [16, 21]. It is very flexible and efficient, offering much information regarding experiment variable effects and overall experimental error in a minimal number of required runs [22].

In this study, an optimized formulation of alginate and konjac glucomannan as encapsulating matrices is determined by using response surface methodology (RSM). Bovine serum albumin (BSA) has been employed as model protein. It is our aim to utilize optimized ALG-KGM beads to deliver BSA orally in the small intestine. Infrared (IR) spectra and scanning electron microscopy (SEM) have been employed to investigate the protein-excipient interaction and the beads surface morphology. We also used texture analyzer (TA) to measure the mechanical strength of the beads. The main site for the drug absorption in man is considered to be small intestine due to its high effective surface area [23]. If any, only little drug absorption will occur in stomach and large intestine. Therefore, the beads was aimed to protect the BSA during exposure to acidic condition in stomach and once reach the targeted site (small intestine) the beads will slowly disintegrated and release the BSA. It seems that ALG-KGM beads have potential use as a carrier for targeted protein drug delivery system.

MATERIALS AND METHODS

Materials

Sodium alginate (ALG) andbovine serum albumin (≥98%) powder was obtained from Sigma-Aldrich Co, St. Louis, MO, USA. Konjac powder (90% purity) was purchased from CN Lab Nutrition, Asian Group (Shaanxi, China). All other chemicals are of analytical reagent (AR) grade.

Beads preparation

The konjac glucomannan-calcium alginate beads containing bovine serum albumin were prepared by using ionotropic gelation method where calcium chloride ($CaCl_2$) was used as cross-linker in ionotropic gelation. Sodium alginate and konjac glucomannan were allowed to dissolve in deionized water containing bovine serum albumin (BSA) (3 mg/ml). The formulations was as follow: sodium alginate (2 – 4% w/v) and konjac glucomannan (0.2 – 0.6 % w/v). The final calcium alginate-konjac glucomannan solution containing BSA were deaerated by ultrasonication for 15 min. Approximately 1 ml of the resulting solution which contained 3 mg of BSA was injected through a syringe needle (23G) into 50 ml of 0.2 M $CaCl_2$ solution for hardening process. The beads were formed instantly and were retained in $CaCl_2$ solution for 30 min in order to form rigid beads. Then, the beads were filtered and washed at least two times with distilled water. The rinsed beads were allowed to dry overnight at room temperature. The dried calcium alginate – konjac glucomannan beads containing BSA were stored in a refrigerator until used.

Experimental design

Response Surface Methodology-Face Centered Composite Design (RSM – FCCD) has been applied to design the experiments, model and optimize three response variables including protein encapsulation efficiency (PEE, %), protein release at 2 h in SGF (%) and time for 100% release (min) in SIF. The amounts of sodium alginate and the konjac glucomannan were defined as the selected independent formulation variables (factors). Each factor was coded at three levels between 1 and +1, where the factor alginate and konjac glucomannan were changed in

the ranges shown in Table 1. Nine experiments were augmented with three replications at the center points to evaluate the pure error and to fit a quadratic model. Minitab™ version 14.0 software (Minitab Inc., PA, USA) was used for regression analysis of experimental data and to plot response surface. The 'response optimizer' in Minitab™was employed to determine the optimum setting for each independent variable that contributed to the optimum predicted responses. In addition, the interaction effect between significant variables were also analyzed by using response surface plots. The polynomial mathematical model generated by central composite design was expressed as follow:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_1x_2 + b4x_1^2 + b_5x_2^2 + \varepsilon$$

Where y is the response, b_0 is the intercept and b_1, b_2, b_3, b_4, b_5 are regression coefficients. $x_1$and x_2 and individual effects; x_1^2 and x_2^2 are quadratic effects; x_1x_2 is the interaction effect, and ε is the residual.

Determination of protein encapsulation efficiency, PEE (%)

The amount protein (BSA) loaded in the beads was estimated by using digestion method. Beads containing 3 mg BSA was dissolved in 20 ml of 0.1 M phosphate buffer saline (PBS), pH 7.4 for at least 18 hours at 25°C ± 0.5°C. The experiment was done in triplicate. BSA content was spectrophotometrically assayed using UV-Vis spectrophotometer at 595 nm (Bradford, 1976). The percentage of protein encapsulation efficiency was calculated by expressing the actual amount of protein loaded (L) divided by the theoretical amount of protein loaded (L_0), as a percentage.

Protein encapsulation efficiency = (L / L_0) x 100

In vitro protein release studies

The release of BSA from various ionotropically gelled calcium alginate-konjac glucomannan/gum Arabic was tested in two different pH solutions which mimicking mouth to small intestine transit. The studies were carried out in glass bottle in shaking water bath. The dissolution rates were measured at 37°C ± 1°C under 50 rpm speed. Beads containing 3 mg BSA were tested for protein release in 5 ml of simulated gastric fluids, SGF (0.1 M HCl, pH 1.2) for 2 h as the average gastric emptying time is about 2 h. Then, the dissolution medium was replaced by simulated intestinal fluid, SIF (phosphate buffer, pH 7.4) and held until the beads were disintegrated. At regular time intervals, 0.5 ml of aliquots was collected and analyzed to determine the protein release from the beads by using Bradford's method [24]. An equal volume of same dissolution medium was replaced to maintain a constant volume. The cumulative percentage of BSA release from the beads in dissolution medium was calculated.

Beads swelling behavior

The swelling behavior of beads was evaluated in two different aqueous media: 0.1 N HCl, pH 1.2 (SGF) and phosphate buffer, pH 7.4 (SIF). Briefly, 100 mg of dried beads were exposed to 20 mL of SGF for 120 min and SIF for 180 min. The condition during incubation period was maintained at 37 ± 1 °C with 110 rpm shaking. The swelled beads were removed at predetermined time interval and weighed after blotting the surface with filter paper to remove the excess moisture. All experiments were done triplicate. Swelling index was calculated using the following formula:

$$\text{Swelling index (\%)} = \frac{\text{weight of beads after swelling} - \text{dry weight of beads}}{\text{dry weight of beads}} \times 100$$

Fourier transform-infrared (FT-IR) spectroscopy

FT-IR was conducted with a Thermo Scientific Nicolet 6700 spectrophotometer (USA). The control of instruments, data collection and primary analysis were processed by OMINIC software. The sample of pure BSA, polymers and dried BSA loaded beads were crushed into powder and 5 mg of the sample was ground completely with 950 mg of spectroscopy grade KBr powder. Then, the mixture was then pressed into a pellet with a die press and placed in the sample holder. Spectral scanning was done in the wavelength region between 400 and 4000 cm^{-1} at a resolution of 4 cm^{-1} with scan speed of 1cm^{-1}.

Surface morphology analysis

The surface morphology of the dried formulated beads was analyzed using Scanning electron microscopy (SEM) (LEO 1455 VP SEM, Oberkochen, Germany). Beads were mounted on aluminum stub using a double-sided adhesive tape. Subsequently, the beads were gold coated with a sputtering coater (Bal-TEC SCD 005, Spulter Coater, Principality of Leichtenstein, Switzerland) to make them electrically conductive and their morphology was examined. Samples were viewed at an accelerating voltage of 20kV, using a second detector at high vacuum mode.

Beads strength determination

The method for determining the strength (g) of the beads was modified from a previous study reported by Edward-Lévy and Lévy (1999). The analysis of mechanical behavior of beads was crried out by using texture analyzer (T.A.HD plus, Stable Micro System, UK) with a 5 kg load cell equipped with a delrin cylindrical probe of 5 mm in diameter. The probe was positioned to touch the beads, recorded as initial position and the probe flattened the beads. The compression of the beads was measured using following conditions: Test mode: compression (g), Pretest speed: 2 mms^{-1}, Test speed: 2 mms^{-1}, Post-test speed: 2 mms^{-1}, Target mode: strain, Distance: 5 mm, Strain: 50%, Trigger type: Auto (force), Trigger force: 5 g. The probe was removed when the beads was compressed to 50% of its original height. The maximum force (g) at 50% displacement representing the strength of the beads was recorded and analyzed by Texture Exponent 32 software program (version 3.0). The bead strength was examined before and after being exposed to simulated gastric fluid

(SGF) and intestinal fluid (SIF). Wet beads were exposed to 20 mL of SGF for 120 min and 60 min in SIF. A single wet bead was tested each time and 5 replications were performed on each sample.

Statistical Analysis

One-way ANOVA was performed to examine significant differences between normally distribution data. Tukey's test was applied to perform multiple comparisons between means within each analysis. Probability level of less than 0.05 was considered as significant ($p < 0.05$). All data was analyzed using MINITAB version 16 (Minitab Inc., PA, United States).

RESULT AND DISCUSSION

Formulation Optimization by central composite design

Planning pharmaceutical formulation without pretrials is very challenging for pharmaceutical researchers [25]. In conventional optimization method, a single factor is varied while the other factors are fixed at a particular set of settings. However, this method is time consuming and less effective as it does not consider the interactive effect of all the main factors. If several factors are to be considered at the same time, their interactions are not noticeable even for the dominant ones. Therefore, using statistical tool such as central composite design is very useful because it helps to study the effect of independent variables influencing the responses by changing them simultaneously. This approach provides statistically reliable results with fewer numbers of experiments and can be applied for the development, improvement and optimization of the biomanufacturing process [26, 27, 28]. Conventional screening has been applied to estimate the range of encapsulating matrices compositions using alginate and KGM to encapsulate BSA. Based on the screening process, a central composite design with total 9 experimental formulations of calcium alginate/konjac glucomannan containing BSA was proposed for two factors: amount of alginate, x_1 and amount of KGM, x_2 used in polymer-blend. The factors and levels with experimental values are reported in Table 1.The effects of these factors on protein encapsulation efficiency (y^1), the amount of protein release in SGF (y^2) as well as time taken for 100% release of protein in SIF (y^3) are presented in Table 2.

Based on the estimated coefficient of the experimental result, the estimated polynomial regression model relating the PEE (%) as response became:

$y_1 = 0.31 + 20.72\ x_1 + 27.69\ x_2 - 2.09\ x_1^2 - 29.87\ x_2^2 + 3.13\ x_1x_2$ [R^2= 0.996; p< 0.1]

The regression model equation for R_{2h}(%) can be predicted as follows:

y_2= $24.00 - 7.23\ x_1 - 9.02\ x_2 + 0.71\ x_1^2 + 0.86\ x_2^2 + 1.00\ x_1x_2$[$R^2$= 0.982; p< 0.1]

The regression model equation relating T_r (min) as response became:

y_3= $-16.70 + 15.53\ x_1 + 73.63\ x_2 + 2.83\ x_1^2 + 39.47\ x_2^2 - 15.63x_1x_2$[$R^2$= 0.978; p< 0.1]

Model building steps have been carried out by excluding non-significant terms (p> 0.1). Starting with full quadratic terms, the most non-significant terms will be eliminated first. The same procedure is applied for the next step until all the non-significant terms are eliminated. However for $y_1$case, all the factors were significant (p< 0.05). On the other hand, after eliminating non-significant terms (p> 0.05), the regression model equation for$y_2$and y_3 responses became:

y_2= $22.76 - 6.89\ x_1 - 5.33\ x_2 + 0.72\ x_1^2$

y_3 = $-46.06 + 32.50\ x_1 + 105.21\ x_2 - 15.62\ x_1x_2$

These models were evaluated statistically by applying one-way ANOVA (p <0.05), which is shown in Table 3. Response Surface Methodology (RSM) software generated three-dimensional (3D) response surface plot and contour plot relating investigated response, PEE (%).

The 3D response surface plot is very helpful in understanding about the main and interaction effects on the factors; meanwhile two-dimensional (2D) contour plot provides a visual illustration of values of the response [29, 30]. The 3D response surface plot relating PEE (%), R_{2h}(%) and T_r (min) are presented in Figure 1 (a, b and c, respectively). Meanwhile, the 2D contour graph relating PEE (%), R_{2h} (%) and T_r (min) are presented in Figure 2(a, b and c, respectively).

Based on 3D response surface plot, it can be noted that PEE (%) increases with the increase of both the amount sodium alginate (x_1) and konjac glucomannan (x_2). On the other hand, the response surface plot relating R_{2h} (%) depicted the decrease in R_{2h}(%)with the increase of both the amount of sodium alginate and konjac glucomannan. In the meantime, the increase in T_r (min) was observed from 3D response surface plot as both the amount of sodium alginate and konjac glucomannan increased.

A numerical optimization method using the desirability approach was used to develop a new formulation of sodium alginate and konjac glucomannan with the desired responses. Constraints like maximizing protein encapsulation efficiency (PEE), minimizing the protein release in SGF (R_{2h}) and maximizing the time taken for protein release in SIF (T_r) were set as the goals to locate the optimum settings of factors in the new formulations. 'Response optimiser' in Minitab™ version 14.0 software (Minitab Inc., PA, USA) was employed for the optimization process. In order to obtain the desired optimum responses, the factors were restricted to 2% (w/v) $\leq x_1 \leq$ 4% (w/v) and 0.2% (w/v) $\leq x_2 \leq$ 0.6% (w/v); while the responses were restricted to 60% $\leq$ PEE $\leq$ 100%, 0% $\leq$ $R_{2h} \leq$ 4% and 90 min $\leq T_r \leq$ 120 min.

The optimized formulation was developed using 4% (w/v) sodium alginate and 0.6% (w/v) konjac glucomannan. The optimized beads containing BSA were evaluated for PEE (%), R_{2h} (%) and T_r (min) for verification. Table 4 displayed the experimental data for

the response and those predicted by the mathematical model. The optimized beads which contain BSA showed PEE value of 63.3 ± 1.35%, R_{2h} (%) value of 2.90 ± 0.68% and T_r (min) value of 112.5 ± 3.53 min. It can be observed that the experimental and predicted values for all the responses were not significantly different (p> 0.05) with the prediction error ranged between 0.43 and 4.92%. This result suggests that mathematical models obtained from central composite design were well fitted.

Protein encapsulation efficiency, PEE (%)

The PEE (%) of all the calcium alginate/konjac glucomannan beads containing BSA was within the range 39.1 ± 2.85 to 63.3 ± 5.50% (Table 2). It was noted that PEE (%) increases with the increase of both the amount of both sodium alginate (x_1) and konjac glucomannan (x_2). The increased of PEE (%) with the increasing amount of sodium alginate and konjac glucomannan could be attributable to the high viscosity of polymer solution. High viscosity of solution can be obtained when the amount of polymer addition increases and through this method, the drug leaching during beads preparation might be prevented and result in high encapsulation efficiency [30]. In this study, high protein encapsulation efficiency, PEE (%) was desired in order to make sure that optimum protein densities were able to reach the target area.

In vitro protein release

Drug release profile of optimized beads is shown in Figure 3. Initially, the release amount of BSA was in the range of 2.9 to 11.2 % in SGF throughout the 2 h incubation (pH 1.2) and this is due to unique characteristics of alginate. Alginate is a hydrophilic polysaccharide and water soluble. However, alginate is insoluble under acidic condition. At low pH, the quantity of positively charged ions is high and they decrease the electrical repulsion between negatively charged alginate molecules [31]. This results to protonation of alginate into insoluble form of alginic acid. Therefore, at acidic pH, penetration of dissolution fluid through the polymer is slowed down and the amount of protein release is minimal. Moreover, the introduction of other polysaccharides into alginate matrices increases the beads viscosity and allows the synergistic interaction which enhances the stability of beads in low pH solution.

Once pH was increased to 7.4, the protein release gradually increased up to 100%. This behavior is due to the deprotonation of alginic acid that occurs at higher pH [32]. It will draw fluid into the beads which led to swelling and disintegration. Consequently, the protein release from the beads occurred rapidly. The pattern of protein release in this study indicates that the trace amount of protein was released during exposure to acidic environment (pH 1.2), but at alkali condition (pH 7.4), the release amount and speed of release were much higher and faster than those in acidic medium. The protein is completely released within 2 hours after pH change. In the previous work reported by Wang and He [12], the alginate-konjac glucomannan beads with chitosan coating demonstrated full released within 3 hours after pH change. This showed that the coating affect the stability of the beads in different pH conditions and able to protect proteins for a longer period of time.

Davis and other [33] reported the transit time pharmaceutical dosage form through small intestine of healthy man is 3 to 4 hours. Another study by Malagelada and collegue [34] showed that the mean value for small intestine transit was 2 to 3 hours. Thus, we assume that the optimize ALG-KGM beads containing BSA were able to reach the targeted site (small intestine) with high bioavailability and release all the content during the transit time.

Beads swelling behavior

The beads stability and swelling behavior during SGF and SIF treatment is shown is Figure 4. The swelling index of the beads was initially lower in acidic pH (0.1 N HCl pH 1.2) compare to that in alkaline phosphate buffer (pH 7.4), indicating a pH-sensitive swelling behavior. Low swelling index in acidic environment was probably due to the shrinkage of alginate. On the other hand, the swelling behavior of beads in SIF could be explained by the ion exchange phenomenon that occurs between calcium ions in the beads and sodium ions in phosphate buffer. Sodium alginate is a polysaccharide with highly hydrophilic properties due to the –OH and –COOH groups present in its chain [35].

This characteristic enables alginate to cross-link with the positive charge ions, Ca^{2+} in $CaCl^2$ during hardening process of the beads. In acidic environment, the ionic strength was stronger due to the stability of negative and positive charges. However, at pH 7.4 (near to neutral), water tends to penetrate into the chain to form hydrogen bond through –OH and –COOH groups and fills up the space along the chain [36]. Finally, calcium ions in egg-box buckle structure diffuse into dissolution medium and alginate beads begin to swell substantially, which causes the disintegration of the beads at higher speed.

FT-IR spectroscopy

The natural polymers (alginate and konjac glucomannan) that have been used in this study as microsphere matrices for drug delivery system are referred as the excipients. A study of interaction between protein and excipients is important to establish polymer compatibility and the effects on the stability of the encapsulated protein.The interaction between various functional groups present in the polymers which contribute to the stability of the encapsulating matrices is also of interest. Konjac glucomannan is a non-ionic polysaccharide and unlike alginate, it is unable to form gel in the presence of multivalent ions. Therefore, there is possibility that konjac glucomannan could be leaked from gel [12]. FT-IR spectroscopy was used to examine the existence of konjac glucomannan within beads after gelling

besides determine the interaction between both polysaccharides as well as protein-polymer compatibility. The FT-IR spectra of sodium alginate, konjac glucomannan, calcium alginate/konjac glucomannan without protein, calcium alginate/konjac glucomannan containing BSA and pure BSA are shown in Figure 5. The FT-IR spectra of sodium alginate showed the characteristic peaks around 3000 – 3600, 1614, 1417 and 1032cm^{-1}, which are assigned to the stretching of –OH , –COO (asymmetric), –COO (symmetric) and C–O–C, respectively.

The FT-IR spectrum of konjac glucomannan showed the stretching peaks of –CH were observed at 2920, 2885, 1415 and 1380 cm^{-1}. The broad band at 3473 cm^{-1}was seen characteristically for the presence of –OH hydroxyl group. The peak of the carbonyl from aceto group was presented at 1730 cm^{-1}. The fragment of C=O was observed at 1657 cm^{-1} and it showed the existence of β, 1-4 linked glucose-mannose. Peaks appeared at 1031 cm^{-1} and 1069 cm^{-1} which corresponded to the stretching of C–O–C. Peaks were seen at 879 cm^{-1} and 814 cm^{-1} and they showed the presence of mannose in konjac glucomannan structure. In the FT-IR of alginate-konjac glucomannan beads without BSA, some peaks became weak or disappeared due to the interaction as well as superposition between groups of alginate and konjac glucomannan [12]. The stretching of –CH at 2920 cm^{-1}and 2885 cm^{-1} in konjac glucomannan could not be seen. In addition, the carbonyl of aceto group at 1730 cm^{-1} of konjac glucomannan also disappeared. The absorption band of –COO at 1614 cm^{-1}in alginate may have combined with the C=O bonds at 1657 cm^{-1} in konjac glucomannan and formed a new peak at 1630 cm^{-1}. The peak of –COO (symmetric) in alginate at 1415 cm^{-1}may have coupled with –CH stretching of konjac glucomannan at 1380 cm^{-1} and a formed large peak at 1420 cm^{-1}. This phenomenon indicated that konjac glucomannan was contained within beads and had interaction with alginate. Similar findings were reported by Wang and He [12] who showed that the hydrogen bonding and electrostatic interaction occurs between alginate and konjac glucomannan. In the FT-IR of pure BSA, the peak near 1650 cm^{-1} was related to amide I band which resulted from the C=O stretching vibrations of the peptide bond. Likewise, the peaks at 1540 cm^{-1}and 1240 cm^{-1}were assigned to N-H bending vibration/C-N stretching vibration which is also known as amide II band and amide III band, respectively. The broad peak near 3300 cm^{-1}was due to the N-H bending vibration, while the peak near 1400 cm^{-1}resulted from the protein side-chain –COO. Beads containing BSA showed various characteristic peaks of sodium alginate, konjac glucomannan and BSA which indicate no physiochemical interaction between BSA and the polymer used (alginate and konjac glucomannan).

Beads morphology

The shape and morphological analysis of optimized alginate-konjac glucomannan beads loaded with BSA before and after SGF and SIF exposures were visualized using SEM at different magnification and shown in Figure 6-8 (a-b). It was observed that these beads possessed a homogenous and compact structure with oval shape which was the result of the high viscosity polymer blend. At higher magnifications (Figure 6b), bead surface topography exposed cracks and wrinkles, which might due to partly collapsing the polymeric gel network during drying process [37]. Polymeric debris was seen on the beads surface andit was probably due to the procedure of beads preparation such as instantaneous gel beads preparation and development of the polymer blend matrix [38].

After 120 min (2 h) of SGF incubation, beads were still intact with slight differences in their morphology. The size beads were slightly increased which indicated the beads swelling behavior in SGF. Beads showed swollen behavior with small erosion on the surface after SGF treatment indicating the beads experience the early sign of disintegration activity (Figure 7a). This finding conforms to previous studies which demonstrated that alginate-konjac glucomannan matrices had low degree of swelling and erosion in acidic pH [12]. The incorporation of konjac glucomannan into alginate matrices might not give much difference under SGF exposure. This is because the main component of the matrices is alginate which is very stable in acidic condition. Wang and He, [12] reported that KGM was not hydrolyzed in acidic medium and stabilizes the beads. Nevertheless, during incubation in SIF, there was large difference in bead morphology which explained the release profile of BSA from the beads. After 120 min of exposure in SIF (pH 7.4), the beads obviously lost their shape due to the erosion and swelling activities (Figure 8a).

Beads strength analysis

The mechanical properties of beads like alginate are not easy to be measured due to the large amount of water contained inside the beads. In their study, the high compression speed used for analysis was able to minimize the time-dependent behavior [39]. Table V shows the mechanical strengths (g) of optimized alginate/konjac glucomannan beads before and after being exposed to SGF and SIF.

The initial bead strength was relatively high (205.05 ± 8.42 g) and it could be to the high concentration of sodium alginate in the polymer blend. According to the study carried out by Ouwerx and others [40], the gel strength of alginate beads was highly influenced by the concentration of alginate used in the polymer solution. High concentration of alginate results in high viscosity of solution producing more rigid and strong beads. After being exposed to simulated gastric fluid, SGF (pH 1.2) for 2 h, the bead strength has been reduced (128.68 ± 6.21 g). This may have been the result of decarboxylation of alginate at low pH [12]. Sodium alginate contains

carboxylic acid groups (RCOOH) that are protonated in acidic environment as following:

$$[RCOO^-]_{gel} + [H^+]_{aq} \rightarrow [RCOOH]_{gel}$$

This results in low charge density and the content of mobile counterions within the beads which cause the gel to shrink [41]. Even though the beads strength was reduced after SGF exposure, the beads were still intact with relatively high values for bead strength and protected the protein from release into gastric region. These results arein conformity with the similar findings obtained in the current study which showed that only a trace amount of protein was released during 2 h exposure to SGF.

The results in Table 5 show a drastic reduction of beads strengthafter being introduced to SIF (49.22 ± 7.82 g). In alkaline surrounding conditions, the carboxylic acid groups (RCOOH) in alginate are deprotonated and produce carboxylate ion with negative charge ($RCOOH^-$) as following:

$$[RCOOH]_{gel} + [OH^-]_{aq} \rightarrow [RCOO^-]_{gel} + H_2O$$

When carboxylic acid groups are exposed to alkaline solution, the hydrogen is dissociated and causes the formation of negative charges along the backbone of polymer chain. These negative charges repel each other and force the polymer to uncoil. This process also called as chain relaxation. In addition, the negative charges also cause the attraction of polymer to water increases. The loosened bead enhances water molecule penetration leading to swelling and subsequent and physical instability of beads [42]. In this study, the beads strength is targeted to be low after SIF exposure in order to give indication that the beads are ready to release the protein into the intestinal region.

Table 1. Factors and their levels in the experimental design.

Factors	Coded levels		
	-1	**0**	**+1**
Sodium alginate (% w/v)	2	3	4
Konjacglucomannan (% w/v)	0.2	0.4	0.6

Table 2. Experimental plan and result using FCCD.

Experimental formulation	Factors		Responses[a]		
	Alginate (% w/v), x_1	Konjac Glucomannan (% w/v), x_2	PEE (%)[b], y_1	R_{2h} (%)[c], y_2	T_r (min)[d], y_3
F-1	2	0.2	39.1 ± 2.85	11.2 ± 2.78	35 ± 0
F-2	2	0.4	41.8 ± 4.62	9.70 ± 1.25	47.5 ± 3.53
F-3	2	0.6	43.2 ± 2.80	8.30 ± 0.46	67.5 ± 3.53
F-4	3	0.2	7.20 ± 5.45	7.20 ± 1.85	65 ± 0
F-5	3	0.4	54.3 ± 1.86	6.40 ± 1.30	72.5 ± 3.53
F-5	3	0.4	53.8 ± 3.50	6.30 ± 2.74	70 ± 0
F-5	3	0.4	53.7 ± 2.95	6.50 ± 0.86	75 ± 0
F-6	3	0.6	54.6 ± 2.20	5.80 ± 0.65	82.5 ± 3.53
F-7	4	0.2	56.7 ± 3.98	4.90 ± 0.51	92.5 ± 3.53
F-8	4	0.4	60.5 ± 2.20	3.65 ± 0.35	102.5 ± 3.53
F-9	4	0.6	63.3 ± 1.35	2.90 ± 0.68	112.5 ± 3.53

[a]Observed response value: Mean ± S.D (n=2),
[b]PEE (%) = Protein encapsulation efficiency (%),[c]R_{2h} (%) = Protein release at 2h in SGF,[d]T_r (min) = Time taken for 100% of BSA release from the beads in SIF.

Table 3. Summary of ANOVA for the response parameters.

Source	Degree of freedom	Sum of square	Mean square	F-value	*p*-value
a) For PEE (%)					
Regression	5	592.829	118.566	450.45	0.000
Linear	2	33.442	16.721	63.53	0.000*
Square	2	19.500	9.750	37.04	0.001*
Interaction	1	1.563	1.563	5.94	0.059*
Residual error	5	1.316	0.263		
Lack-of-fit	3	1.109	0.369	3.58	0.226
Pure error	2	0.207	0.103		
b) For R_{2h}(%)					
Regression	3	48.018	16.006	185.60	0.000*
Linear	2	46.610	5.187	60.14	0.000*

Square	1	1.407	1.407	16.32	0.005*
Residual error	7	0.604	0.086		
Lack-of-fit	5	0.584	0.117	11.67	0.081
Pure error	2	0.020	0.010		
c) For T_r (min)					
Regression	3	4990.10	1163.368	149.13	0.000*
Linear	2	4951.04	893.490	80.11	0.001*
Interaction	1	39.06	39.062	3.50	0.100*
Residual error	7	78.08	11.154		
Lack-of-fit	5	65.58	13.116	2.10	0.353
Pure error	2	12.50	6.250		

*p-value < 0.1, indicates the significance of the coefficient to the model

Table 4. Comparison of experimental and predicted responses.

	PEE (%)[1]	**R_{2h} (%)[2]**	**T_r (min)[3]**
Experimental	63.30 ± 1.35[a]	2.90 ± 0.68[a]	112.5 ± 3.53[a]
Predicted	63.03[a]	3.05[a]	111[a]
Error (%)	0.43	4.92	1.35

*Values in the same column with different letters were not significantly different (p >0.05).

Table 5. Bead strength (g) before and after being exposed to SGF and SIF.

Beads strength (g)		
Before SGF and SIF	**After SGF[1]**	**After SIF[2]**
205.05 ± 8.42[a]	128.68 ± 6.21[b]	49.22 ± 7.82[c]

*Values in the same row with different letters were significantly different (p < 0.05).

Figure 1. The 3D response surface plot showing the effects of amount of sodium alginate (% w/v) and konjac glucomannan(% w/v)on (a) PEE, (b) R_{2h} and (c) T_r

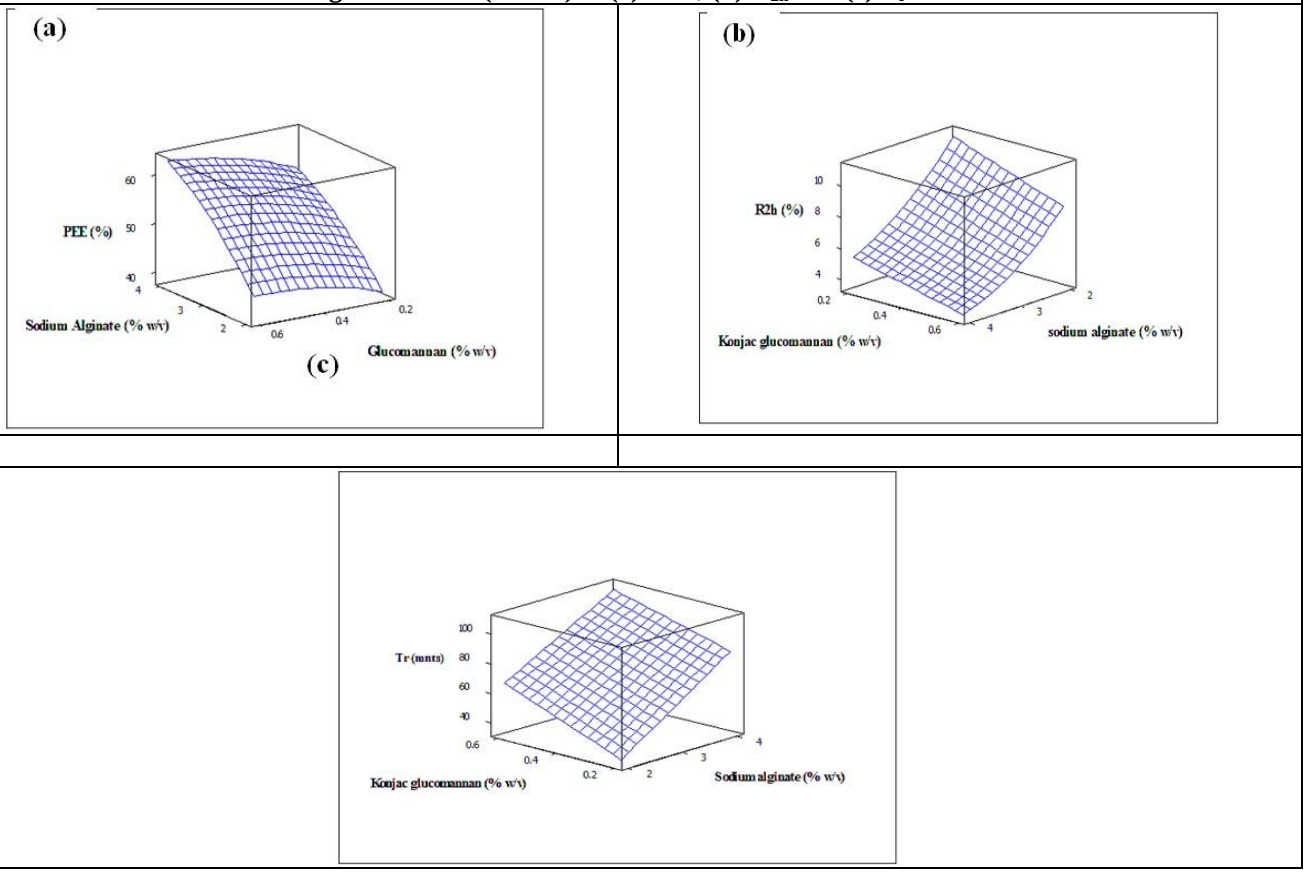


Figure 2. The 2D contour plot showing the effects of amount of sodium alginate (% w/v) and konjac glucomannan(% w/v)on (a) PEE, (b) R_{2h} and (c) T_r

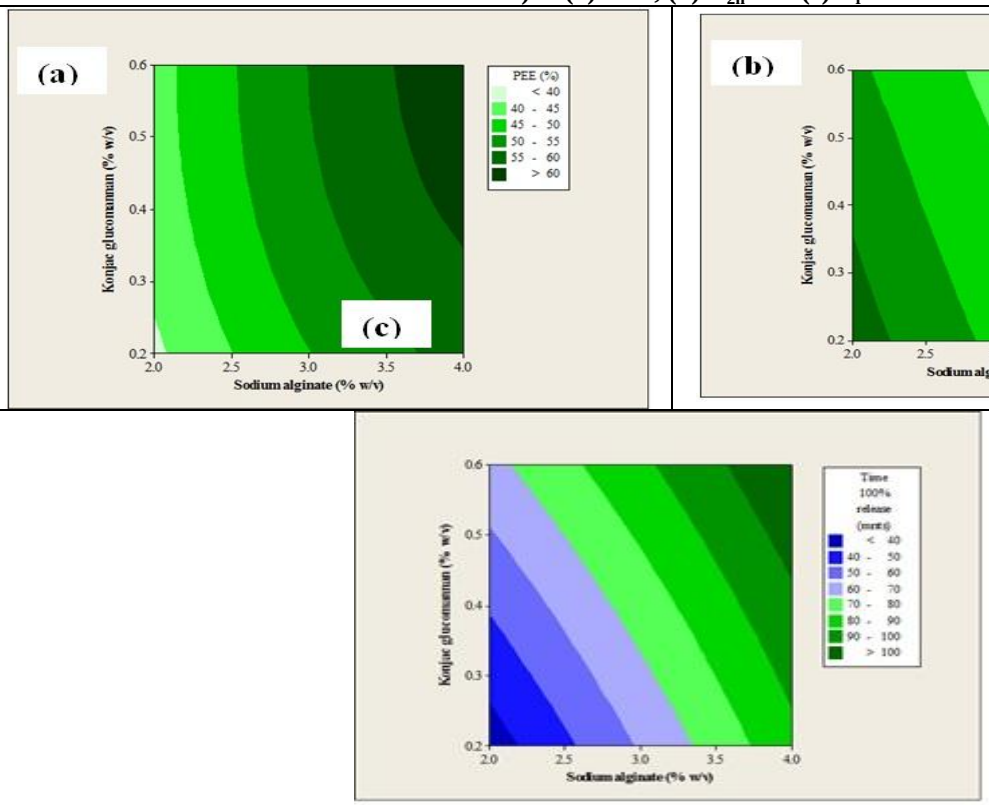


Figure 3. Cumulative release of BSA

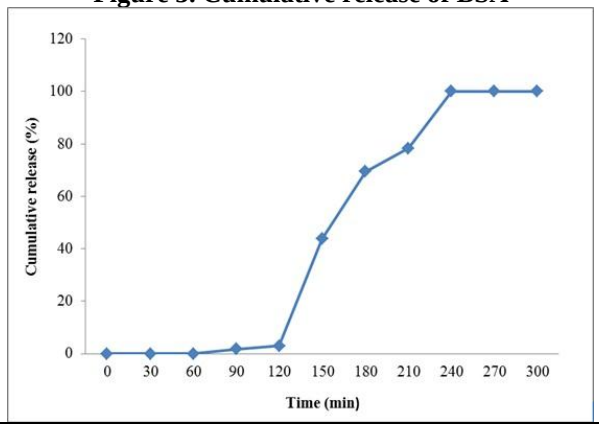


Figure 4. The swelling index of ALG-KGM beads.

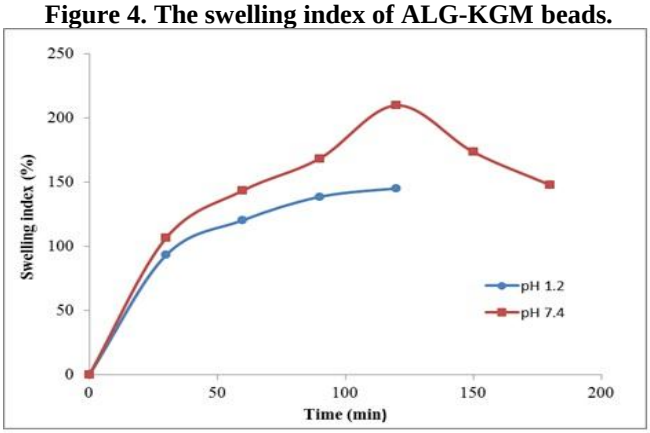


Figure 5. The FT-IR spectra of (a) sodium alginate, (b) konjacglucomannan, (c) calcium alginate/konjacglucomannan without BSA, (d) calcium alginate/konjacglucomannan containing BSA and (e) pure BSA

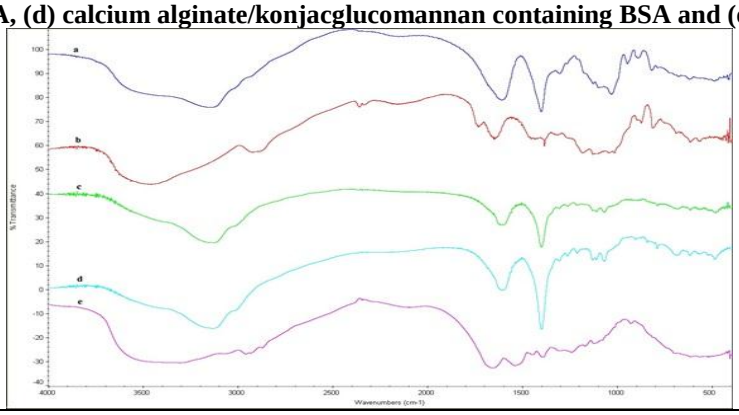


Figure 6. Bead morphology before SGF and SIF exposure

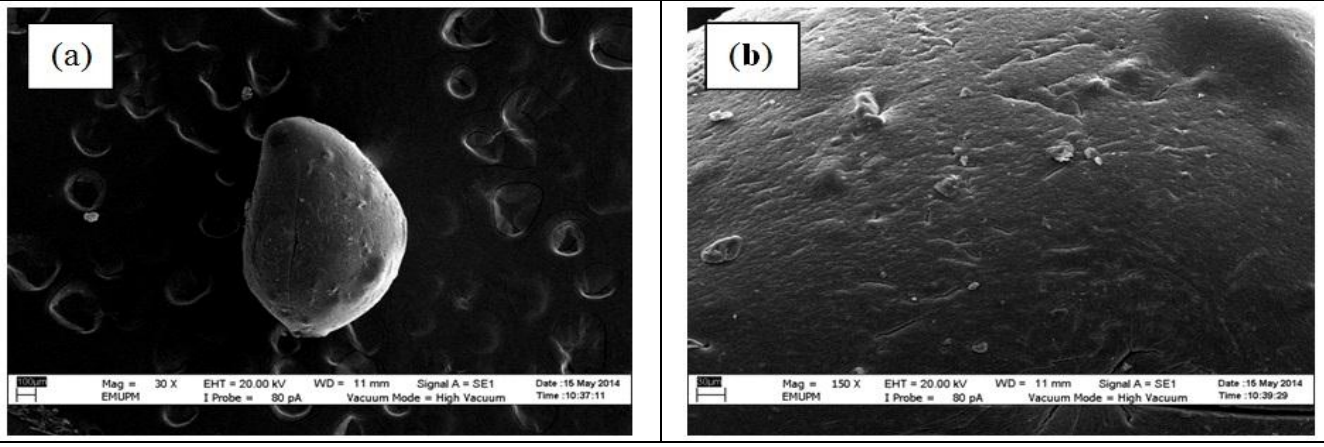


Figure 7. Bead morphology after SGF exposure

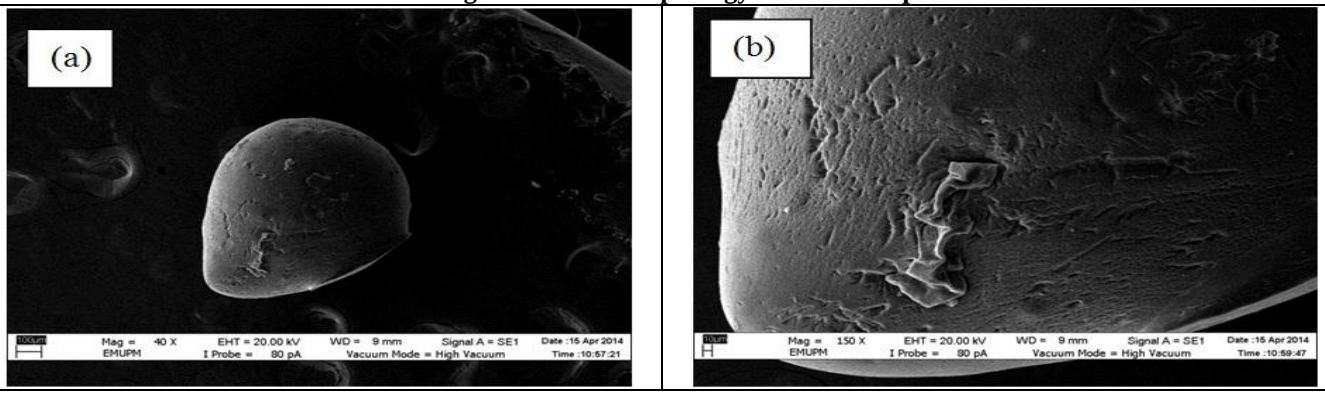
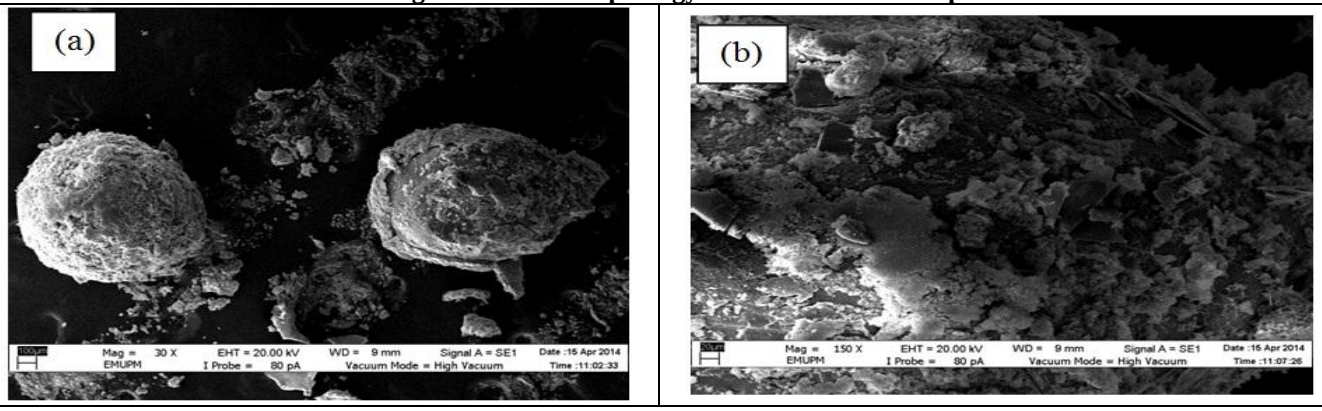


Figure 8. Bead morphology after SGF and SIF exposure



CONCLUSION

Encapsulation of BSA using combination of konjac glucomannan and alginate has successfully improved the survival and protein release to target area which is the small intestine. Therefore, by using these formulations, the oral delivery of protein drugs for the treatment of pediatric patients is now possible. Thus, the pain and discomfort due to frequent injections in everyday treatment can be avoided.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of paper.

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