IN-VITRO RELEASE OF BOVINE SERUM ALBUMIN (BSA) FROM ALGINATE-INULIN HYDROGEL

[1]NORSYAZWANI SOLEHAH NORUDIN, [2]HAJARATUL NAJWA MOHAMED

Faculty of Manufacturing Engineering Technology, TATI University College 24000 Kemaman Terengganu, Faculty of Chemical Engineering Technology, TATI University College 24000 Kemaman Terengganu
E-mail: [1]solehahsyazwani@yahoo.com, [2]hajaratulnajwa.mohamed@gmail.com

Abstract - This study investigates the controlled release of a model of protein drug, Bovine Serum Albumin (BSA) from alginate-inulin hydrogels produced through ionotropic gelation method. The amount of inulin has been the main factor affecting the stability and efficiency of hydrogels in protecting the BSA from gastric degradation in the stomach and absorption in intestinal tract. The encapsulation efficiency of BSA was increased as the amount of inulin incorporated into the hydrogel was increased. The in-vitro dissolution of these hydrogels demonstrated that only a trace amount of protein was released in simulated gastric fluid, SGF (pH 1.2). However, after changing into simulated gastric fluid, SIF (pH 7.4) 100% of protein was released within 1.5 hours. The swelling index of alginate-inulin hydrogels was lower in acidic (pH 1.2) compare in alkaline phosphate buffer (pH 7.4) indicating pH sensitive swelling behavior. These hydrogels were also characterized by SEM and TA for hydrogels surface morphology and hydrogels strength, respectively. Moreover, among all the formulations produced, formulation 2% (w/v) ALG- 5% (w/v) inulin has shown the best results with the most suitable sustained protein release pattern during SIF exposure and also demonstrated the highest hydrogel strength after SGF exposure. These findings suggest that sodium alginate-inulin hydrogels could be advantageous as oral administration of protein drugs to prolong efficacy and improve bioavailability.

Index Terms - Alginate, Inulin, Protein release, Controlled delivery.

I. INTRODUCTION

Recently, the development of delivery system for oral administration of protein and peptide is a field of great interest for treatment of diabetes mellitus to overcome the limitation of daily injections. The repeated injections lead to other side effects and also decrease the patient compliance. Therefore, oral administration becomes the most preferable and less invasive route for protein delivery. This administration route prevents the occasional hyperinsulinemia observed by subcutaneous administration, since the principal organ in glucose homeostasis is the liver and this should be the prime target for intervention [1]. However, oral administration has some difficulties due to the protein instability that caused most of protein pharmaceuticals are administered through injection. Acid catalyzed degradation in the stomach, proteolytic breakdown in the GI tract, poor permeability across the gastrointestinal mucosa and first-pass metabolism during transfer across the absorption barrier and in the liver become the major problems that must be overcome for the efficient delivery of proteins into the absorption site [2]. Many attempts have been conducted in order to improve the stability and efficiency of protein in human body. The encapsulation using biodegradable polymer becomes the excellent approach to improve the efficiency of protein drugs. 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 [3]. Biodegradable natural polymers are becoming increasingly important in the design of controlled-release drug delivery systems, particularly for the delivery of protein and peptide drugs [4]. Among this natural polymer, alginate has received much attention as the core material to encapsulate the protein drugs due to outstanding physical properties. Being biodegradable, biocompatible, non-toxic and mucoadhesive macromolecules, this natural polysaccharide has been widely used in the formulation of several drug delivery systems [5].

Alginate is an anionic polysaccharide consisting of various ratios of guluronic and mannuronic acid units linked by glycosidic bonds and can produced hydrogel beads through ionotropic gelation by the addition of divalent cations in aqueous liquid such as calcium [6]. Through this technique, alginate as an ionic polymer undergo ionotropic gelation and precipitate due to electrostatic interaction oppositely charged species to form the hydrogel. However, alginate alone could not be enhanced as the carrier to deliver protein drugs into gastrointestinal tract due to the low drug encapsulation efficiency and rapid release of the loaded molecules [7]. In order to improve the efficiency of protein drug, another polysaccharide is incorporated into the system to combine with alginate. Inulin has been proposed to combine with alginate due to many positive health effects and shows potential as stabilizing agent in the macro capsule. Inulin is a naturally occurring polysaccharide found in many plants and it consists of β 2-1 linked D-fructose molecules having a glucosyl unit at the reducing end [8]. The combination between alginate and inulin will

control the protein release passing through the gastrointestinal tract.

In this study, the combination of alginate and inulin might provide stability and efficiency of hydrogel produced by protecting the protein drug from degraded in acidic environment and its absorption in the small intestine. The purpose of this study is to investigate the release activity of bovine serum albumin, BSA from existing alginate hydrogel compared with newly alginate-inulin hydrogel in different pH medium. Particularly this study focuses on the effect of alginate-inulin formulations on the protein encapsulation efficiency, PEE (%), swelling rate and in-vitro release during exposure to simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). Scanning electron microscopy (SEM) and texture analyzer (TA) have been used to investigate the hydrogels surface morphology and mechanical strength of hydrogels respectively. Therefore, the hydrogels were aimed to protect the BSA during exposure to acidic condition in stomach and once reach the targeted site (small intestine), the hydrogels will slowly degrade and release the BSA

II. MATERIALS & METHODS

A. Materials

Sodium alginate (ALG), inulin powder was obtained from Sigma-Aldrich Co, St. Louis, Mo, USA. Bovine serum albumin (>98%) was purchased from Vivantis Technology Sdn. Bhd. All other chemicals are of analytical reagent (AR) grade.

B. Hydrogels Preparation

The sodium alginate-inulin hydrogels containing BSA was prepared by using ionotropic gelation method where calcium chloride ($CaCl_2$) was used as a cross-linker in ionotropic gelation. Sodium alginate was fixed at (2% w/v) and the mixture of inulin (5-15% w/v) were allowed to dissolve in deionized water containing bovine serum albumin (BSA) (3mg/ml). The alginate-inulin hydrogels were prepared under magnetic stirring until the solution becomes homogenous and hydrogels were introduced into ultra-sonicated for 15 min to remove bubbles. Approximately 1 mL of resulting solution was injected through a syringe needle (23G) from a distance of 10 cm into 40 ml of $CaCl_2$ (0.2 M) solution. The hydrogels were hardened in $CaCl_2$ for 30 minutes in order to form rigid beads. Then, the wet hydrogels were filtered and washed with distilled water. Finally, the hydrogels were dried at room temperature for overnight and dried hydrogels kept safe in refrigerator until used

C. Determination of Protein Encapsulation Efficiency, PEE (%)

Alginate-inulin hydrogels containing 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%. Samples were withdrawn after incubation and analyzed for protein content by UV-VIS spectrophotometer assayed at 595 nm according to Bradford's method [9]. The amount of protein contained in the hydrogels was estimated by using digestion method. The percentage of loading efficiency was determined from the following equation:

$$Loading\ efficiency\ (\%) = (L/Lo)\,x\,100 \quad (1)$$

Where (L) was the actual amount of protein loaded within a known amount of hydrogel according to experimental data meanwhile (Lo) was theoretical amount of protein loaded.

D. Swelling behavior

100 mg of dried hydrogels were placed into 20 mL of simulated gastric fluid, SGF (0.1 N HCl, pH 1.2) for 2 hours and simulated intestinal fluid, SIF (phosphate buffer, pH 7.4) for 1.5 hours. The condition during incubation period was maintained at 37± 1 ºC with 110 rpm shaking. The swelled hydrogels were removed every 30 minutes and the sample was blotted with a filter paper to remove the excess moisture and their swelling rate (%) was recorded. Swelling rate was calculated using the following formula:

$$S\% = \frac{Wt - Wo}{Wo} \times 100 \quad (2)$$

($S\%$) was the swelling rate,(Wt) was the weight of hydrogels after swelling and (Wo) was the dry weight of hydrogels

E. In-vitro protein release studies

The release of encapsulated proteins was carried out in two different pH solutions which mimicking stomach to small intestine transit. The studies were carried out in bottle in shaking water bath. The shaker speed was set at 50 rpm speed and thermostat controlled bath was set at 37 °C. Alginate-inulin hydrogels containing BSA were tested for protein release in 5 ml of simulated gastric fluids, SGF (0.1 N HCl, pH 1.2) for 2 h then replaced by stimulated intestinal fluid, SIF (phosphate buffer, pH 7.4) for the next hours until the hydrogels were disintegrated. Every 30 min, 0.5 ml of aliquots were collected and the reading of protein release was recorded by using Bradford's method. An equal volume of same dissolution medium was added to maintain a constant volume. The percentage of protein release from the hydrogels was calculated.

F. Surface Morphology Analysis

The surface morphology of the dried formulated hydrogels has been studied by scanning electron microscopy (SEM) using (JEOL JSM- 6360LA).

Hydrogels were mounted on aluminum stub using a double sided adhesive tape. Subsequently, the hydrogels were gold coated with a sputtering coater (JEOL JEE-420) to make them electrically conductive and their morphology was examined. Samples were viewed at an accelerating voltage of 10KV using a second detector at high vacuum mode.

G. Determination of Hydrogels Strength

The mechanical strength of the hydrogels was investigated using the method from a previous study reported by Edward-Levy and Levy (1999). The analysis of mechanical behavior of hydrogels was carried out using texture analyzer (T.A. HD plus, Stable Micro System, UK) fitted with a 5 kg load cell and equipped with a delrin cylindrical probe of 5 mm in diameter. The probe was positioned to touch the hydrogels, recorded as initial position and the probe flattened the hydrogels. The compression of the hydrogels 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: 0.5 mm, Strain: 50%, Trigger type: Auto (force), Trigger force: 5 g. The probe was removed when the hydrogels was compressed to 50% of its original height. The maximum force (g) at 50% displacement representing the strength of the hydrogels was recorded. The hydrogels strength was examined before and after being exposed to simulated gastric fluid(SGF) and intestinal fluid (SIF). Wet hydrogels were exposed to 20 mL of SGF for 120 min and 30 min in SIF. A single wet hydrogel was tested each time and 5 replications were performed on each sample.

III. RESULTS AND DISCUSSION

A. Compositions and PEE (%) of alginate-inulin hydrogels

TABLE 1. Composition of the prepared Alginate-Inulin formulations

Formulations	Alginate (% w/v)	Inulin (% w/v)	$CaCl_2$ (M)	PEE (%)
(2-0)	2	0	0.2	48.3
(2-5)	2	5	0.2	55.8
(2-10)	2	10	0.2	60.7
(2-15)	2	15	0.2	63.9

The physical screening has been applied to estimate the range of encapsulating matrices compositions using alginate and inulin to encapsulate BSA. After finalizing the compositions produced, result shown in Table 1 has been proposed to conduct the analysis. The concentration of alginate was fixed with 2 % (w/v) while several concentration of inulin was used with 5 % to 15 % (w/v) in order to produce the rigid hydrogels. Meanwhile, the Figure 1 indicates the diagram of alginate-inulin hydrogel produced. By using the ionotropic gelation technique, roughly spherical and regular shaped of hydrogels were obtained (Fig. 1). Further trial has been carried out by using high concentration of alginate and inulin unfortunately the hydrogels were not well formed. According to [10], alginate and chitosan concentration was fixed at 2 % (w/v) and 0.5 % (w/v) respectively to obtain the good result. Besides that, it was observed that, with a sodium alginate concentration above 1% during the preparation of hydrogels, the viscosity of the external phase was increased so that the formation of drops was strongly hindered [11]. Similarly, in this study alginate was fixed at 2 % (w/v) in order to form the rigid hydrogels. At this time, the hydrogels were successfully formed in all compositions.

Furthermore, the protein encapsulation efficiency of the alginate-inulin hydrogels varied from 48.3% to 63.9%. In the present study, protein encapsulation efficiency, PEE (%) test was conducted to examine the optimum protein densities that able to be encapsulated in the alginate-inulin hydrogel. It was observed that the protein encapsulation efficiency is increased when 5 to 15 % (w/v) of inulin was used. This was probably due to the high viscosity of solution which leads to the strong interaction between alginate and inulin caused high encapsulation yield. Meanwhile, pure alginate hydrogels show lower encapsulation efficiency and this might due to the low viscosity of solution which produced less compact gel matrix. These resulted the hydrogel could not able to retain large amount of protein. Similar finding was reported by [12] who carried out that drug loss during preparation of alginate alone was due to the leaking of drug through the beads pores contribute to the decreasing of encapsulation efficiency. These findings indicate that the concentration of inulin influence the protein encapsulation efficiency and it appears with the increasing of inulin concentration, the protein encapsulation was increased.

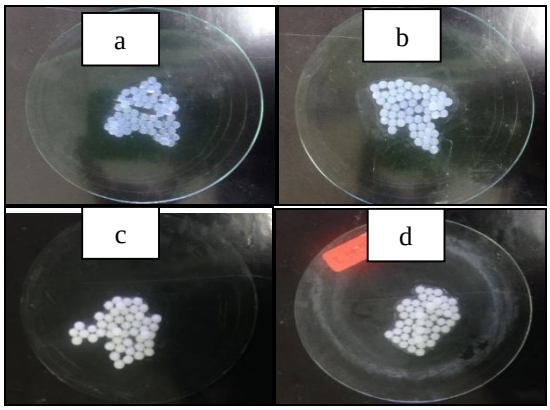


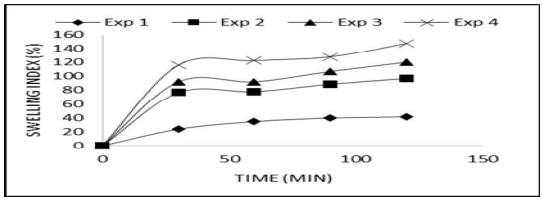
FIGURE 1. Alginate-inulin beads a) 2% (w/v) ALG b) 2% (w/v) ALG-5% (w/v) INU, c) 2% (w/v) ALG-10% (w/v) INU, d) 2% (w/v) ALG-15% (w/v) INU

B. Swelling Behavior

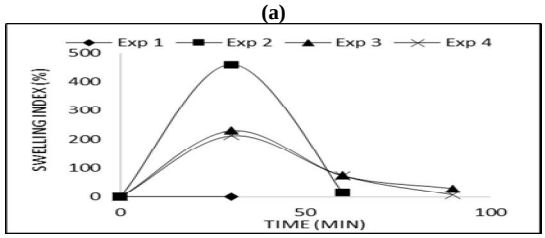
The stability and swelling behavior of alginate alone

and alginate-inulin hydrogel during exposed to SGF (pH 1.2) and SIF pH (7.4) treatments are shown in Fig. 2 (a) and 2 (b) respectively. The result indicates that the swelling index was lower in acidic condition (Figure 2a) compared in alkaline phosphate buffer (Figure 2b) indicating pH sensitive swelling behavior. At acidic 0.1 N HCl solution, all the formulations showed a significant increase of swelling rate until the minutes of 120. Pure alginate (experiment 1) recorded lowest swelling index in acidic condition and it might due to the hydrophilic characteristic of alginate. [13] had reported that in a 0,1 N HCl solution, alginate hydrogel undergoes a decrease in diameter hence resulted alginate shrinks at low pH [14]. This behavior caused the water do not able to penetrate into the hydrogel

However, it was observed that the swelling index was increased after changing into phosphate buffer solution pH (7.4). Experiment 1 (pure alginate) swelled rapidly and reached to highest swelling rate caused it could not withstand after 30 minutes of SIF exposure. It was due to the loss of Ca^{2+} hence resulting the crosslinking of hydrogel decreases and destabilized. Meanwhile, alginate-inulin hydrogel performed contradict result in buffer solution with reduced of swelling index. Experiment 2 reached the highest swelling rate at 60 minutes in SIF, followed by Experiment 3 and Experiment 4. Even though the water uptake of Exp 2 hydrogels was high in SIF, the hydrogels maintained the viscous-gel structure for a longer period and underwent slower erosion process compared to hydrogels of other formulations. Experiment 3 and Experiment 4 hydrogels underwent erosion process after 90 minutes and slowly disintegrated. The results of swelling behavior suggested that these hydrogels have ability to swell less in acidic pH of stomach as they subsequently travel in gastrointestinal tract and swell more in alkaline medium of upper intestine.



(a)



(b)

FIGURE 2. Swelling index of alginate-inulin hydrogel a) in simulate gastric fluid (SGF), (b) in simulated intestinal fluid (SIF) Exp 1) 2% (w/v) ALG, Exp2) 2% (w/v) ALG-5% (w/v) INU, Exp3) 2% (w/v) ALG-10% (w/v) INU, Exp 4) 2% (w/v) ALG-15% (w/v) INU

C. In-vitro Protein Release

In-vitro protein release of alginate and alginate-inulin hydrogels was investigated at pH 1.2 and pH 7.4. The result is depicted in Fig. 3. It was observed that protein was released slowly at pH 1.2. Less than 10% of protein was released in all formulations throughout 2 hours in SGF incubation. Experiment 4 recorded (9.5%) of protein release while only a trace amount of protein release (7%) with experiment 2. Alginate alone (experiment 1) showed (8.1%) followed by experiment 3 with (9.3%) of protein released after 2 hours in acidic condition. As a result, the minor release was recorded with all the samples due to the main material which is alginate that is hydrophilic polysaccharide and insoluble under acid condition hence results the retarded of BSA release through the hydrogels pore. In addition, the incorporation of inulin into alginate matrices increases the hydrogels viscosity and produce the synergistic interaction which enhance the stability and efficiency of hydrogels in acidic environment. Inulin has an unique oligo-or polysaccharide since its backbone does not incorporate any sugar ring which cause it free to move and finally contribute to the increase of flexibility of its molecules [15]. This phenomenon will produce stronger interaction with protein at acidic pH preventing the protein release.

As the pH increased to 7.4, protein release tremendously increases up to 100% within 1 hour. It was due to the deprotonation of carboxyl groups in alginate [17] and resulted the hydrogels become progressively ionized. This behavior leads to the fast release of BSA in alkali medium. Experiment 4 showed the highest release of BSA (94%) after 30 minutes in SIF incubation followed by experiment 1 (43.4%). Experiment 2 and experiment 3 showed consistently release of protein until 1 hour's incubation. The fastest release of experiment 4 might due to the high viscosity of solution leading to fast release of protein. Inulin has a higher molecular weight than mono and disaccharides, with that it has higher glass transition and melting temperature, and is more viscous when dissolved. The higher molecular weight also correlates with a lower solubility [16]. The pattern of protein release in this study indicates that the protein release was minor during exposure to acidic environment (pH 1.2) but in alkali condition the protein release was higher and the time taken for 100% release recorded within 1.5 hours. Therefore, it can be assumed that most of BSA were able to reach the targeted site (small intestine) and released all the content during the transit time. The incorporation of inulin combined with alginate obviously delays the release of a protein drug in the alkaline pH (7.4) after acid treatment. This could be attributed to the presence of inulin make the beads becomes stronger and stable than alginate alone in gastrointestinal condition.

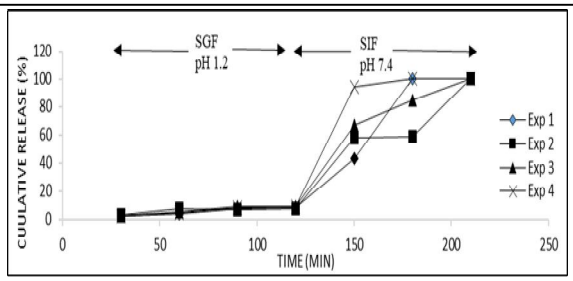
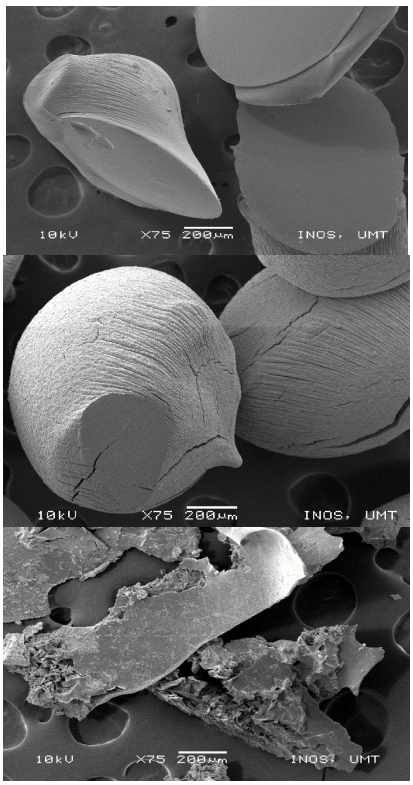
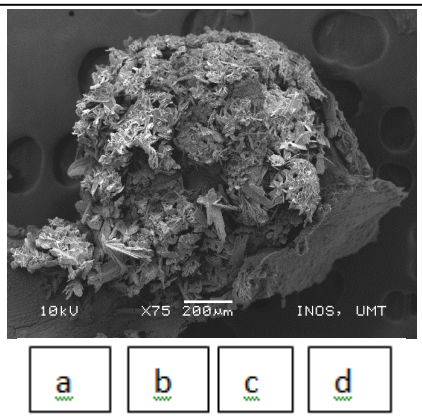


FIGURE 3. Protein release of alginate-inulin hydrogel Exp 1) 2% (w/v) ALG, Exp2) 2% (w/v) ALG-5% (w/v) INU, Exp3) 2% (w/v) ALG-10% (w/v) INU, Exp 4) 2% (w/v) ALG-15% (w/v) INU

D. Beads Morphology (SEM)

The shape and morphological analysis alginate-inulin hydrogels containing BSA before and after SGF and SIF was examined by scanning electron microscopy (SEM). The SEM observation of alginate-inulin beads were performed at x75 magnification and shown in Fig 5 (a-c). It was observed that, before exposure to SGF and SIF (Fig 5a), the hydrogels of were clearly showed the compact structure with spherical shape. Moreover, the beads surface exposed cracks and wrinkles which indicated to partly collapsing the polymeric gel network during drying process [17]. Meanwhile, it can be seen that on alginate-inulin beads, poymeric debris was recorded which might due to the phase separation hsppened when mixture of alginate and inulin was dropprd into cross-linking solution containing Ca^{2+} [18]





In addition, after 2 hours of incubation, hydrogels exposed to SGF incubation (Fig 4b), it can be seen that the size of hydrogels of all formulations slightly increase due to the phenomenon of swelling activities. Nevertheless, as expected the spherical shape of hydrogels was lost after changing into SIF solution (Fig 4c). The beads showed large erosion on the surface due to disintegration activities that occurred at alkaline condition. Moreover, the beads revealed that the surface was rough and many pores were observed.

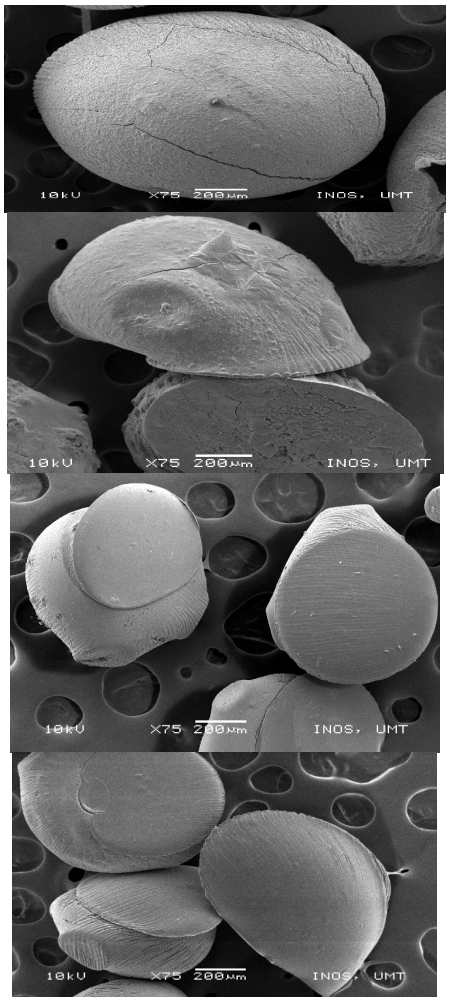


Figure 4(a). EM of alginate-inulin hydrogels before exposure a)2% (w/v) ALG, b) 2% (w/v) ALG-5% (w/v) INU, c) 2% (w/v) ALG-10% (w/v) INU, d) 2% (w/v) ALG-15% (w/v) INU

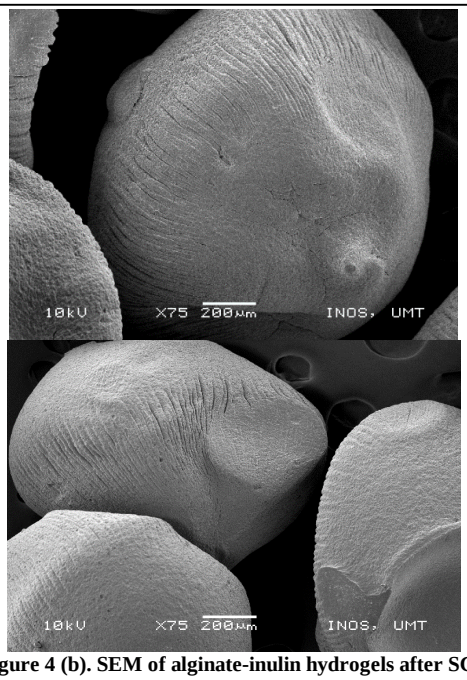


Figure 4 (b). SEM of alginate-inulin hydrogels after SGF exposure
a)2% (w/v) ALG, b) 2% (w/v) ALG-5% (w/v) INU, c) 2% (w/v) ALG-10% (w/v) INU, d) 2% (w/v) ALG-15% (w/v) INU

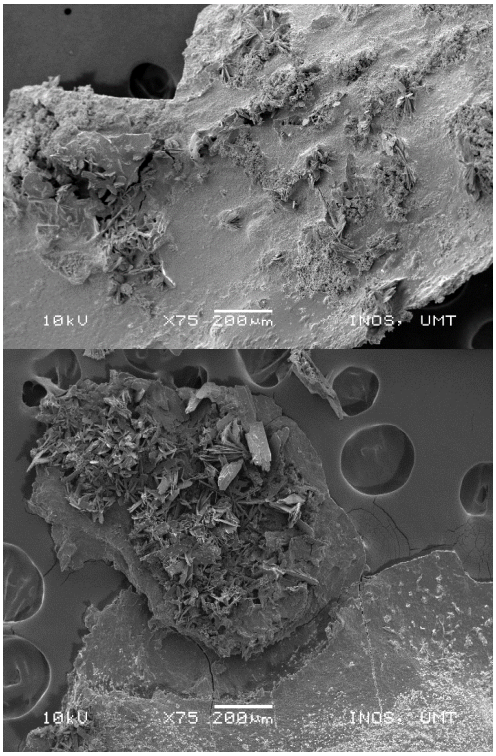


Figure 4 (c). SEM of alginate-inulin hydrogels after SIF exposure
a)2% (w/v) ALG, b) 2% (w/v) ALG-5% (w/v) INU, c) 2% (w/v) ALG-10% (w/v) INU, d) 2% (w/v) ALG-15% (w/v) INU

E. Hydrogels Strength Analysis

hydrogels strength was analyzed before and after being exposed to SGF and SIF. Table 2 represents the hydrogels strength of all formulations and it can be seen that the hydrogels strength before exposure were relatively high. Experiment 1 and experiment 2 show the highest bead strength with 36.04g and 36.80g respectively. Followed by experiment 3 with 30.48g. Meanwhile, experiment 4 that has highest inulin content among the others recorded the lowest hydrogels strength (27.26g). It was probably due to the inhomogeneous structure hence resulted in low hydrogels strength. In addition, after being exposed to simulated gastric fluid, SGF for 2 hours, the hydrogels strength has been reduced. It was supported from the previous research that mentioned the incubation of calcium alginate hydrogels at acidic pH 1.2 has caused the calcium ions to be displaced from the polymer network and calcium alginate hydrogels is converted to the unionized form of alginic acid [19]. Similary, in this study, the alginate has been used as the main matrices of encapsulation and resulted in dissociation of ionic linkage and reduction in the gel strength [20]. Even though the beads strength was reduced after incubation of beads at acidic pH (1.2), the beads were still intact with constantly values for hydrogels strength and protected the protein from release into gastric region.

However, after being exposed to alkaline conditions the hydrogels were broken after compression and resulted in error readings. At alkaline condition, the carboxylic acid group in alginate was deprotonated and produced carboxylate ion with negative charge which leads to dissociation of hydrogen. This phenomenon causes the formation of negative charge along the backbone of polymer chain [21] and leading to swelling of hydrogels Furthermore, beads can rapidly decrease in mechanical strength upon swelling with water [22]. Finally, hydrogels were hydrolyzed and broken.

Table 2. Hydrogels strength of alginate-inulin hydrogels

Hydrogels compositions	Before SGF/SIF	After SGF	After SIF
Exp 1 (2-0)	36.04	27.06	
Exp 2 (2-5)	36.80	36.28	
Exp 3 (2-10)	30.48	29.22	-
Exp 4 (2-15)	27.26	26.95	

CONCLUSION

In conclusion, encapsulation of BSA using combination of alginate and inulin through ionotropic gelation method has successfully enhanced and retained the protein release to the targeted area (small intestine). The formulation shows a good performance where the protein encapsulation efficiency and swelling ability were increased. The time taken of BSA released in physiological saline was also improved (90 minutes). At acidic condition (pH 1.2), minor released of BSA was recorded as the beads underwent a low degree of swelling. On the other

hand, at alkaline condition (pH 7.4), beads started to swell and released all the content at targeted site (small intestine). Therefore, the data presented here shows that alginate-inulin beads might be a potential delivery system for the encapsulation of protein

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