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Enhanced pH-responsive carrier system based on alginate and chemically modified carboxymethyl chitosan for oral delivery of protein drugs: Preparation and *in-vitro* assessment

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ABSTRACT

In this investigation a new series of biodegradable pH-responsive hydrogel microspheres were prepared, characterized and *in-vitro* evaluated as potential carriers for oral delivery of protein drugs. The microspheres are based on ionotropically-crosslinked mixture of sodium alginate and chemically modified carboxymethyl chitosan and coated through polyelectrolyte complexation with chitosan grafted with poly(ethylene glycol). The main objective of the developed microspheres is to survive the harsh acidity of stomach and preferably release peptide and protein drugs in intestine. Both ionotropic gelation and coating process were carried out under mild aqueous conditions, which should be appropriate for retention of biological activity of protein drugs. Swelling studies were carried out for the microspheres at 37 °C in simulated gastric and intestinal fluids. Morphology, size and *in-vitro* biodegradation of the microspheres were also investigated. A model protein drug was entrapped and the *in-vitro* drug release profiles were established. The preliminary investigation of the microspheres developed in this study showed a consistent swelling pattern, high entrapment efficiency and promising sustained release profiles of the model protein drug.

1. Introduction

Oral route can be considered as one of the most comfortable ways for drug administration. However, in case of peptide and protein drugs, the oral route exhibits many inadequacies. For instance, these types of drugs are readily degraded, if taken orally, due to the harsh high acidity of stomach and the enzymatic attack in the upper small intestinal tract. Also, the short half-life of protein drugs and their limited transit time in the gastrointestinal tract represent major challenges (El-Sherbiny, Abdel-Bary, & Harding, 2010; Ramadas, Paul, Dileep, Anitha, & Sharma, 2000). To date, several trials have been reported to overcome these shortcomings and to formulate peptide and protein drugs with maximum oral bioavailability (Dolatabadi-Farahani, Vasheghani-Farahani, & Mirzadeh, 2006; El-Sherbiny et al., 2010; Heller et al., 2002; Imren, Gumusderelioglu, & Guner, 2010; Liu, Jiao, & Zhang, 2007; Mi et al., 2005; Rekha & Sharma, 2008).

Hydrogels are three-dimensional crosslinked hydrophilic polymers, which swell without dissolving when brought into contact with water or other biological fluids (Ju, Kim, Kim, & Lee, 2002). The pH-responsive hydrogels, a class of smart hydrogels, have potential use in site-specific delivery of drugs to the gastrointestinal tract (El-Sherbiny et al., 2010). Unfortunately, the number of polymers appropriate for preparing hydrogels for the controlled release of drugs is quite limited, as compared to the total available synthetic polymers, due to inherent toxicity or lack of certain characteristics such as swelling ability in a specific environment and biodegradability.

Sodium alginate is a natural non-toxic biodegradable polyanionic copolymer. It consists of 1,4-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues arranged either as consecutive blocks or in a random distribution. Alginate has a unique ability of gel formation in presence of divalent cations such as calcium and barium ions (Decho, 1999). Hydrogels based on calcium-crosslinked alginate have been widely investigated for protein drug delivery (Dolatabadi-Farahani et al., 2006; Hari, Chandy, & Sharma, 1996; Murata, Kodama, Isobe, Kofuji, & Kawashima, 2009; Vandenberg, Drolet, Scott, & de la Noue, 2001). However, the swelling of the calcium-crosslinked alginate beads at pH 7.4 is minimal (Lin, Liang, Chung, Chen, & Sung, 2005). This may limit drug release at the intestinal tract. Various trials have been reported to overcome this disadvantage by preparing hydrogels based on alginate with other polymers such as chitosan (Cs) and its derivatives (Dolatabadi-Farahani et al., 2006; Hari et al., 1996; Lin et al., 2005; Vandenberg et al., 2001).

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In an attempt to overcome this shortcoming, a hydrogel matrix was developed in our previous study (El-Sherbiny et al., 2010). This hydrogel was a calcium-crosslinked blend of alginate and a graft copolymer of methacrylic acid onto carboxymethyl chitosan (CMCs). The developed hydrogel beads showed enhanced swelling behavior in intestinal fluid as compared to that of the hydrogels based on alginate alone. However, the swelling extent of most of the developed gel beads formulations was relatively high in simulated gastric fluid (SGF). This led to loss of a relatively significant percent of the uploaded protein drug in SGF. Therefore, in this study, a trial was carried out to develop hydrogel formulations with enhanced characteristics towards more efficient oral delivery of protein drugs.

In the present work, carboxymethyl chitosan (CMCs) was prepared and further modified via grafting of sodium acrylate (AAs) onto its backbone in a mild aqueous medium using ammonium persulphate (APS) as initiator. The main purpose of grafting was to increase the number of carboxylic groups in CMCs. Thus by developing Ca^{2+}-crosslinked hydrogel microspheres based on alginate with CMCs-g-AAs, these carboxylic groups are expected to participate in minimizing the swelling at pH 2.1 (SGF) and maximizing the swelling at pH 7.4 (SIF). Also, some studies showed that polymers containing carboxylic groups have the ability to protect protein and peptide drugs from some protease enzymes such as trypsin and chymotrypsin. This enzyme inhibitory effect was mainly attributed to ability of these polymers to bind with extracellular divalent cations (calcium and zinc) (Ameye et al., 2001). The more reduction in concentration of extracellular divalent ion can also help in opening of tight junctions and consequently improves the paracellular transport of protein drugs across the intestinal epithelium (Luessen et al., 1995). In addition, the mucoadhesive characteristics exhibited by these polymers increase the residence time of oral dosage forms at the epithelial surface and accordingly enhance the drug absorption (Peppas & Sahlin, 1996).

Also, in a comparative study, similar hydrogel microspheres were developed and then coated through two different procedures with poly(ethylene glycol)/Cs graft copolymer (Cs-g-PEG). Coating the microspheres with this biodegradable copolymer (Cs-g-PEG) would enhance the microspheres ability to survive the harsh acidity of the stomach, minimizing the loss of protein drug in gastric fluid and preferably release the drug mostly in the intestine.

2. Experimental

2.1. Materials

Sodium alginate of low viscosity (approx. 250 cps for a 2% w/v solution at 25 °C), acrylic acid and Bradford reagent were supplied by Sigma Chemical Co. (St. Louis, MI, USA). Chitosan and BSA were obtained from Acros Organics (New Jersey, USA). PEG-monomethyl ether (PEG-OCH_3) with M_w of 5000 Da was supplied by Aldrich. Monochloroacetic acid was purchased from Riedel–De Haenag Seelze (Hanover, Germany). $NaBH_4$ was obtained from Fluka, USA and ammonium persulphate (APS) was obtained from AJAX Chemicals, Clyde Industries Ltd (Auburn, N.S.W, Australia). Isopropyl alcohol, DMSO, acetone, methanol, acetic anhydride, chloroform, acetic acid, calcium chloride and all other reagents were purchased form El-Gomhoria Company (Mansoura, Egypt) and used as received.

2.2. Methods

2.2.1. Characterization of chitosan (Cs)

The average molecular weight (M_w) of the Cs under investigation was determined to be 31.8×10^4 D using the Mark-Houwink viscometry method (El-Sherbiny, 2009), in a solvent of 0.1 M acetic acid/ 0.2 M NaCl maintained at 25 °C. Also, the *N*-deacetylation % of Cs was found to be 73.6% as determined by FTIR using the following relationship (Roberts & Domszy, 1982):

$$\%N - \text{deacetylation} = 100\left[1 - \left(\frac{A_1655}{A_3340}\right)\left(\frac{1}{1.33}\right)\right] \quad (1)$$

where, A is the absorbance at the given wave number. These two absorption signals at about 1655 and 3340 cm^{-1} correspond to the amide and the primary amino groups of Cs, respectively. The factor (1.33) represents the value of the ratio of A_{1655}/A_{3340} for the fully N-acetylated Cs. *FTIR* (υ_{max}, cm^{-1}) 3427 (O–H stretching and N–H extension vibration), 2855 (C–H stretching), 1654 (amide C = O stretching), 1426, 1384, 1321, 1156, 1026 and 555.

2.2.2. Preparation of CMCs-g-AAs copolymer

2.2.2.1. Carboxymethylation of Cs. The carboxymethylation of Cs was carried out through a modified method as previously reported in our earlier work (El-Sherbiny, 2009). In brief, 2 g of Cs were transferred to 500 ml RBF and suspended in 50 ml of isopropyl alcohol at room temperature for 2 h. To the swollen Cs suspension, 80 ml of aqueous NaOH solution (60% w/v) were added and then the whole mixture was refluxed at 85 °C for 2 h. Then, 100 ml of aqueous monochloroacetic acid solution (60% w/v) were added over a period of 10 min. The mixture was heated with stirring, at 65 °C for a further 4 h. The reaction mixture was then neutralized using HCl solution (4 M). After removal of the undissolved residue by filtration, the resulting CMCs was precipitated by adding methanol. The product was filtered, washed several times with a mixture of CH_3OH / H_2O (1:1) and dried under vacuum (yield: 83.2%). FTIR (υ_{max}, cm^{-1}) 3433 (O–H stretching), 2951 (aliphatic C–H stretching), 1735 (carboxylate CO assym. stretching), 1628 (amide C=O stretching), 1517, 1384 (CO symmetric stretching), 1156 (C–O–C stretching) and 594; EA, found (%): C, 37.2, N, 5.1 and H, 5.9.

The degree of substitution (DS) of the prepared CMCs was determined using potentiometric titration (Ge & Luo, 2005). A solution of 150 mg of CMCs dissolved in 50 ml of distilled water was adjusted to pH < 2 by adding hydrochloric acid. The CMCs solution was then titrated with 0.1 M aqueous NaOH and the pH value was simultaneously recorded. The NaOH amount was determined by the second order differential method and the DS value was calculated as follow:

$$DS = \frac{161 \times V \times C}{m_{CMCs} - 58 \times V \times C} \quad (2)$$

where, m_{CMCs} is the mass (g) of CMCs, V and C are the volume and molarity of NaOH solution, respectively. The values 58 and 161 represent the molecular weights of the carboxymethyl group and the glucosamine unit of Cs, respectively.

2.2.2.2. Graft copolymerization. The water soluble CMCs-g-AAs copolymer was prepared in a 250 ml two-necked flask using 0.1 g CMCs. Before addition of the predetermined amount of monomer, AA, the monomer was neutralized by using NaOH (4 M) and then completed to the desired volume by deionized water to give a final concentration of 8.5 mM. The components were then mixed through regular stirring for 30 min with bubbling of slow stream of nitrogen gas. Then the flask was placed in a thermostated oil bath at 70 °C. Upon reaching the desired temperature, the aqueous solution of APS initiator, (8 mM based on the total volume of reaction mixture) was added dropwise with stirring. The graft copolymerization was continued for 2 h. The reaction was then stopped by letting air into the flask and rapidly cooling down the reactor. The products were precipitated by pouring the reaction mixture

into excess acetone. The precipitate was filtered off, washed with acetone and the crude product was dried and weighed. The homopolymer formed was extensively extracted in a soxhlet apparatus by refluxing with methanol for 20 h. The residual graft copolymer was washed with methanol, dried and weighed. The percent grafting (*G*%) of the obtained copolymer was calculated as follows:

$$G\% = [(W_g - W_0)/W_0] \times 100 \quad (3)$$

where W_g and W_0 are the weights of copolymer and CMCs, respectively. FTIR (υ_{max}, cm^{-1}): 3424 (O–H stretching), 2949 (aliphatic C–H stretching), 1715 (carboxylate C=O assym stretching), 1574 (amide C=O stretching), 1425, 1320 (PAA), 1179 (C–O–C stretching) and 1089 (polysaccharide moiety); EA (*G*%: 2400%), found (%): C, 32.1, N, 0.28 and H, 4.62.

2.2.3. Preparation of Cs-g-PEG copolymer

Cs-g-PEG was synthesized as described by Harris et al. (1984) and Zhang et al. (2008). First, PEG-aldehyde was prepared by oxidation of PEG-monomethyl ether (PEG-OCH_3) with DMSO/acetic anhydride. In brief, a certain weight of PEG-OCH_3 was dissolved in anhydrous DMSO/chloroform mixture (9:1, v/v) and then a slow stream of nitrogen gas was bubbled through the solution for 10 min. Then, acetic anhydride was added to the mixture under nitrogen with acetic anhydride/PEG-OCH_3 molar ratio of 12 and the mixture was further stirred for 9 h at room temperature under nitrogen. The reaction mixture was then neutralized with 0.5 N NaOH and used directly for the next reaction. The Cs-g-PEG copolymer was prepared through the Schiff base formation. PEG-aldehyde (with a molar ratio of 0.4 to the amino groups of Cs) and Cs were added to a mixture of acetic acid and methanol (2:1, v/v), and the mixture was stirred for 30 min at room temperature. Then an aqueous solution of sodium borotetrahydride ($NaBH_4$) was added slowly to the reaction mixture at pH 6.5 with vigorous stirring at a molar ratio of 8:1 for $NaBH_4$/PEG-aldehyde. The resulting mixture was dialyzed (14,000 Da cutoff) first against 0.05 N NaOH and then against distilled water before drying under vacuum. Pure Cs-g-PEG was finally obtained by removal of unreacted PEG with excess acetone. FTIR (υ_{max}, cm^{-1}): 3379 (NH bending and O–H stretching), 2911 (aliphatic C–H stretching), 1673 (amide C=O stretching), 1095 (C–O–C stretching), 957, 688 and 609.

2.2.4. Preparation of hydrogel microspheres

The alginate/CMCs-g-AAs hydrogel microspheres (formulations PF1–PF4, Table 1) were prepared via ionotropic gelation technique by using calcium chloride solution as the coagulation fluid. Homogenous aqueous solutions of sodium alginate and CMCs-g-AAs (20 ml, 1%) with predetermined compositions (50:50 or 60:40) were prepared and left overnight to degas. Then, the aqueous polymer solutions were dropped into 80 ml of gently stirred (250 rpm) calcium chloride solution (0.05 or 0.1 M) through a 1 ml syringe with a 27 gauge needle at a dropping rate of 1.0 ml/min. The formed particles were allowed to crosslink with the Ca^{2+} in solution for 20 min. Then, the resulting calcium-crosslinked microspheres were collected, washed with distilled water (20 ml) to remove the unreacted calcium chloride , dried, while frozen, under vacuum and then stored until further use.

The coated alginate/CMCs-g-AAs hydrogel microspheres (formulations PF5-PF8, Table 1) were prepared by complex coacervation between the alginate/CMCs-g-AAs particles as a gel core and Cs-g-PEG as cationic polyelectrolyte. Two different methods were applied; the one step method was by dropping the alginate/CMCs-g-AAs aqueous mixture directly into a solution of calcium chloride (0.1 M) mixed with Cs-g-PEG (1% w/v). The two steps method was by dropping the alginate/CMCs-g-AAs aqueous mixture into calcium chloride solution (0.1 M), followed by a thin layer coating step via suspending the microspheres resulting from first step for 20 min in a solution of Cs-g-PEG (1% w/v). The microspheres were allowed to harden for 2 h before washing them twice with distilled water and then allowed to dry under vacuum.

2.2.5. Characterization

The elemental analysis for CMCs, CMCs-g-AAs and Cs-g-PEG copolymers was performed with Carlo Erba EA 1108 elemental analyser (now CE Instruments, Wigan, UK) with a flash combustion technique (Campbell Microanalytical Laboratory, Dunedin, NZ). Also, Cs, CMCs, CMCs-g-AAs and Cs-g-PEG were characterized by FTIR (Nicolet 6700 FTIR spectrometer). The crystallography patterns were investigated using 2D-XRD equipment (Rigaku Micro Max 007 microfocus imitating anode X-ray generator (Cu Kα) coupled with Osmic "Blue" confocal optics and a Rigaku RAxis (VI++) image-plate detector. Images were recorded and analyzed with Crystal Clear (1.3.6-SPI, Pflugrath, JW, 1999, Acta Crystallogr. D50 1718–1725).

2.2.6. Drug entrapment and determination of the entrapment efficiency

Hydrogel microspheres loaded with BSA, as a model for protein drugs, were prepared in the same manner used for plain microspheres. Predetermined amounts of the BSA (30% w/w) were added to the alginate/CMCs-g-AAs aqueous mixture, stirred and then the gelation reaction was carried out by same method described for drug-free microspheres.

The BSA content was determined by dissolving 100 mg of both dried plain and BSA–loaded hydrogel microspheres in 0.2 M phosphate buffer, pH 6.8 under vigorous stirring for 30 min to make about 10 mg/ml solutions (Wittaya-areekul, Kruenate, & Prahsarn,

Table 1
Different composition of the developed hydrogel microspheres.

Formulation code	Alginate (1% aq. soln.) (%)	CMCs-g-AAs (1% aq. soln.) (%)	$CaCl_2$ (M)	Cs-g-PEG soln. (1%)		Equil swelling ratio (SR) ± *SD*		[#]Particle size (diameter, μm) ± *SD*		Entrapment capacity, Ec (%) ±*SD*
				1S[*]	2S[*]	SGF	SIF	Feret's	Sieve	
PF0	100	00	0.1	–	–	3.70 ± 0.1	7.90 ± 0.7	703.1 ± 21	658.1 ± 11	39.6 ± 3
PF1	50	50	0.05	–	–	8.86 ± 4.9	51.8 ± 2.3	819.0 ± 11	745.8 ± 19	31.8 ± 5
PF2	50	50	0.1	–	–	4.81 ± 1.6	49.2 ± 3.4	787.2 ± 14	699.0 ± 18	43.2 ± 3
PF3	60	40	0.05	–	–	8.92 ± 3.5	47.0 ± 2.2	793.6 ± 12	691.2 ± 22	32.0 ± 4
PF4	60	40	0.1	–	–	1.90 ± 0.6	42.3 ± 1.6	781.3 ± 08	708.0 ± 17	47.9 ± 2
PF5	50	50	0.1	†	–	2.70 ± 3.8	44.2 ± 1.3	802.9 ± 19	713.7 ± 08	69.6 ± 2
PF6	50	50	0.1	–	†	5.12 ± 2.7	46.0 ± 1.9	811.0 ± 12	719.2 ± 16	63.0 ± 2
PF7	60	40	0.1	†	–	1.10 ± 0.3	38.9 ± 1.5	787.5 ± 12	681.4 ± 13	83.1 ± 5
PF8	60	40	0.1	–	†	2.10 ± 0.6	34.7 ± 1.0	789.8 ± 19	690.0 ± 10	79.4 ± 2

[*] 1S and 2S: one and two steps coating processes.
[#] Estimated using microscopy with aid of "ImageJ" software

2006). After dissolution, the samples were filtered. Then, the amount of BSA present was estimated by UV–vis spectrophotometry using Bradford method (Bradford, 1976) from the absorption at λ_{max} 595 nm. Using a standard curve of BSA in same buffer solution, the amount of BSA per mg polymer microspheres was then calculated. The solution of plain microspheres was used as absorbance background control. It was found prior studies that there is no absorbance interference from the plain microspheres under the same conditions. The mean values for three replicate determinations and their ±*SD* were reported. The entrapment capacity (*Ec*%) of the model drug, BSA was calculated as follows:

$$Ec\% = (m_r/m_i) \times 100 \quad (4)$$

where m_i and m_r are the amounts (mg) of the BSA initially uploaded and remained in the microspheres, respectively.

2.2.7. Morphology and size determination of microspheres

The surface morphology of the developed hydrogel microspheres was investigated with a scanning electron microscope (Cambridge Stereoscan S-250 mk 3 scanning electron microscope, Cambridge Instruments Ltd., Cambridge, UK) at Massey University, NZ. Samples were placed on aluminum mount, sputtered with gold with a Baltec scd 050 sputter coater, and then scanned at an accelerating voltage of 20 kV.

Measurements of average sieve and Feret's diameters of the hydrogel microspheres were carried out with an optical microscope (Olympus, Germany) at magnification of 4× with aid of ImageJ software (NIH). The diameters were determined as the mean values of 20 randomly selected microspheres ($n = 20$).

2.2.8. Swelling studies

The maximum swelling values of the coated and non-coated alginate/CMCs-g-AAs microspheres were determined by immersing a predetermined weight of the sample in 10 ml buffer solution of pH 2.1 (SGF) or pH 7.4 (SIF) at 37 °C until the equilibrium was attained. Then the weights of the swollen samples were determined after removal of the surface water using tissue paper. The swelling profiles of the coated and non-coated hydrogel formulations prepared using 60% alginate:40% CMCs-g-AAs in presence of 0.1 M $CaCl_2$ (PF4, PF7 and PF8), as example of other formulations, were also determined. In a typical procedure, a certain weight of the dried hydrogel microspheres was immersed in 10 ml buffer of pH 2.1 (SGF) at 37 °C for 2 h and subsequently transferred into another 10 ml buffer of pH 7.4 (SIF) at 37 °C for 8 h. The swollen weights of the hydrogel microspheres were determined at intervals, after blotting the surface liquid. The swelling ratio was calculated by the following relationship:

$$SR = (W_t/W_o) \quad (5)$$

where W_o is the initial weight and W_t is the final weight of the swollen microspheres at time *t*. The data points represent means ± *SD* from three independent experiments. The behavior of PF4, PF7 and PF8 in SGF was also further investigated by determining their cyclic swelling patterns. Briefly, predetermined weights of samples were placed in 10 ml of SGF and the swelled samples were weighed at intervals, after removal of surface liquid, until equilibrium swelling was attained. Then, the swelled samples were completely dried at 20 °C under vacuum and reweighed. This swelling–deswelling process was repeated three times for each sample and the swelling ratio was calculated as in Eq. (5).

2.2.9. In-vitro biodegradation study

A preliminary *in-vitro* biodegradation study was carried out for the hydrogel microspheres in presence of 0.2% lysozyme dissolved in phosphate buffer, pH 7.4 (Nsereko & Amiji, 2002). Briefly, predetermined weights of microspheres (15–20 mg) were transferred to small tubes and incubated in 2 ml of lysozyme solution with shaking in water bath at 37 °C for 3 h until microspheres almost attained equilibrium swelling. The weights of the swollen microspheres (W_o) were determined after removing the solvent. Then 2 ml of fresh lysozyme solution was added to the swollen microspheres. At certain intervals (1, 3, 6, 12, 24, 48, 96 and 144 h) starting from determination of W_o, the steps of removing solvent and weighing were repeated and the final weights (W_t) of microspheres at these intervals were determined. The percent weight remaining (*Wr*%) of the samples due to enzymatic degradation were determined according to the following equation:

$$Wr\% = 100 - [(w_o - w_t/w_o) \times 100] \quad (6)$$

where w_o is the weight of sample after 3 h swelling in lysozyme solution and w_t is the weight of the sample after incubation with lysozyme for a given time *t*.

2.2.10. In-vitro release studies

The *in-vitro* cumulative release patterns of the entrapped BSA were determined by placing the pre-weighed hydrogel microspheres loaded with BSA in 10 ml of buffer at pH 2.1 (SGF) at 37 °C for 2 h and subsequently in 10 ml buffer of pH 7.4 (SIF) at 37 °C for 8 h. At intervals, 100 μl aliquot was withdrawn and analyzed by the Bradford method at λ_{max} 595 nm using a UV–vis. Spectrophotometer (Bradford, 1976). The withdrawn sample was replaced with an equal volume of fresh buffer, to keep the volume of the release medium constant. The data points represent means ± *SD* from three independent experiments.

2.2.11. Statistical analysis

The obtained results were analyzed and expressed as means ± *SD* from three independent experiments. Effects of various parameters on the characteristics of the developed hydrogel microspheres were statistically analyzed by one-way ANOVA using the general linear models procedures of JMP (SAS Institute Inc., 2007). In the statistical analysis of all the data, PF0 was taken as the control formulation. The differences were considered significant at the level of $P < .05$ and the significant differences between means were separated by the least significant difference test.

3. Results and discussion

3.1. Synthesis of CMCs and CMCs-g-AAs

The water soluble carboxymethylated Cs (CMCs) was prepared by a method described in our earlier reported study (El-Sherbiny, 2009) with a DS value of 0.48 as determined by potentiometric titration. The structural changes of Cs and its derivatives (CMCs and CMCs-g-AAs) were confirmed by FTIR as reported in details elsewhere (El-Sherbiny & Elmahdy, in press). The IR spectrum of Cs shows a strong peak at 3427 cm^{-1} which is assigned to the O–H stretching vibration, N–H extension vibration and the intermolecular H-bonds of the polysaccharide moieties. The weak peak at 1654 cm^{-1} is due to the amide C=O stretching. The IR spectrum of CMCs shows a strong new peak at 1735 cm^{-1} representing the carboxylate C=O asymmetric stretching. The signal at 1384 cm^{-1} could be assigned to the symmetric stretching vibration of carboxylate C=O. The C–O absorption peak of the secondary hydroxyl group becomes stronger and moves to 1156 cm^{-1}. This tends to indicate that the substitution occurs mainly at the C_6 position. In case of IR spectrum of CMCs-g-AAs (*G*%: 2400%), the peak appeared at 1319 cm^{-1} is characteristic of poly(AAs). Also, in the IR spectra of the copolymer, the characteristic absorption peak of polysaccharide at around 1100 cm^{-1} became weaker which may be attributed to the high grafting percentage. The IR spectra of CMCs-g-AAs

shows also the absence of clear absorption due to vinyl unsaturation around 1640 cm^{-1}. This tends to indicate the disappearance of the vinylic double bond of AAs monomer due to grafting. The carboxymethylation process of Cs and synthesis of CMCs-g-AAs copolymer are illustrated in Scheme 1.

The incidence of graft copolymerization has been proved elsewhere by several ways (El-Sherbiny & Elmahdy, in press). For instance, the higher weights of the graft product as compared to that of the starting CMCs after extensive removal of the homopolymer (PAAs) has been taken as evidence of grafting. The FTIR spectra of CMCs-g-AAs, as discussed above had both characteristic peaks of CMCs and AAs which can be considered another experimental proof of grafting. Also, the intensity of C=O absorption at 1734 cm^{-1} was increased with increasing the *G*%. The occurrence of grafting has been also deduced from the decreasing of *N*-content upon comparing the measured elemental analysis data of both CMCs and some CMCs-g-AAs copolymers.

3.2. Synthesis of Cs-g-PEG copolymer

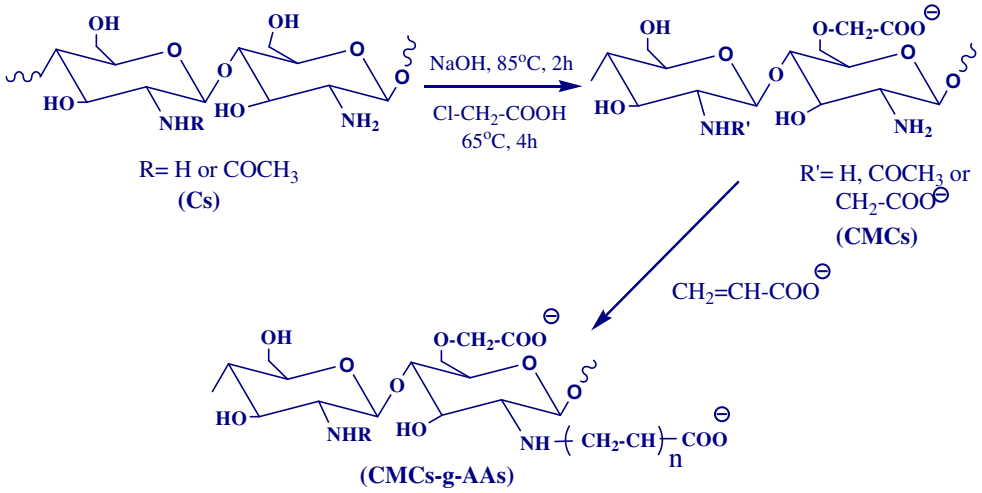
The Cs-g-PEG copolymer has been synthesized as reported by Harris et al. (1984) and Zhang et al. (2008). Chitosan was reacted with a PEG-aldehyde to yield an imine (Schiff base) and then the Cs-g-PEG copolymer was produced through the reduction of this imine with $NaBH_4$ (Scheme 2). Upon using the molar ratio of 0.4 of PEG-aldehyde to the amino groups of Cs, a Cs-g-PEG copolymer sample with a *G*% of 28% (based on gravimetric calculations) was obtained. The final purified Cs-g-PEG copolymer was characterized by FTIR. The peak appeared at 3379 cm^{-1} represents both stretching and bending frequencies of OH and NH groups in the Cs backbone. The absorption peak at 2911 cm^{-1} is attributed to the stretching frequency of the aliphatic C–H bonds. Also, in the FTIR spectra of Cs-g-PEG copolymer, the peak appeared at around 1150 cm^{-1} which is characteristic for the polysaccharide moieties became very weak (shoulder) which may be due to grafting of the high molecular weight PEG chains. Grafting of PEG onto the Cs backbone was also confirmed through the appearance of a very strong absorption peak at around 1095 cm^{-1} which is attributed mainly to the C–O–C bond of PEG side chains.

3.3. 2D-XRD

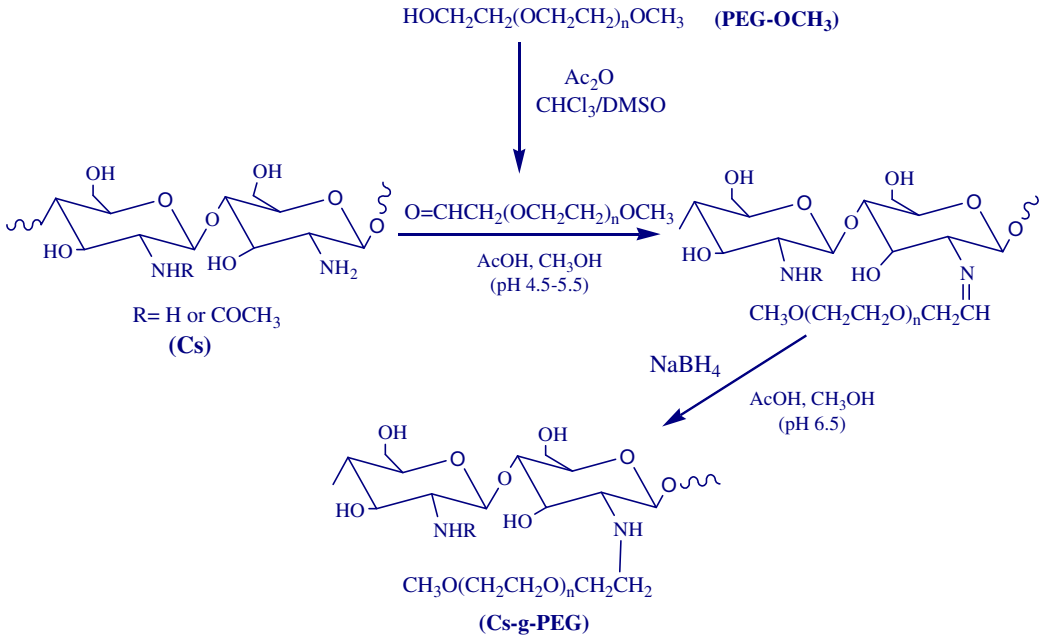
Fig. 1 shows the 2D-XRD patterns of Cs and its derivatives, CMCs, CMCs-g-AAs and Cs-g-PEG. The diffractogram of Cs (Fig. 1a) shows three major crystalline peaks at 2θ values of 8.38°, 11.49° and 18.25° in addition to many weak crystalline peaks. This diffraction pattern reflects a kind of crystallinity for the Cs under investigation. Fig. 1b shows the diffractogram of CMCs, from which it was found that CMCs has many crystalline peaks plus two broad bands corresponding to 2θ values of about 21.43° and 26.33°. The diffractogram of CMCs seems to keep some of the characteristic peaks of Cs. For instance, CMCs still shows the bands at 2θ values of 10.40, 11.49, 16.10, ~18.46° and ~21.43°. Grafting of AAs onto CMCs backbone turned the resulting copolymer, CMCs-g-AAs into an amorphous material. The diffractogram of CMCs-g-AAs copolymer (Fig. 1c) shows some weak peaks at 2θ values of 11.15°, 4.00° and 2.54°. Also, it was noted that the synthesized Cs-g-PEG copolymer (Fig. 1d) is mostly amorphous with development of some small peaks around 2θ of 21°. This resulting amorphous structure of both graft copolymers, CMCs-g-AAs and Cs-g-PEG may be attributed to occurrence of grafting in a random manner along the polymer backbones and consequently destroying regularity of packing of the original polymer chains leading to formation of amorphous structure.

3.4. Preparation of hydrogel microspheres

A significant body of research has investigated the hydrogel matrices based on calcium-crosslinked alginate for drug delivery (Dolatabadi-Farahani et al., 2006; Hari et al., 1996; Murata et al., 2009; Vandenberg et al., 2001). However, many of these types of matrices showed some shortcomings as carriers for oral delivery of protein drugs. These shortcomings include the minimal swelling in intestinal fluid, (SIF, pH 7.4) (Lin et al., 2005) which may limit the drug release in the small intestine. Also, swelling of some of these reported hydrogel matrices was relatively high in gastric fluid (SGF, pH 2.1) which leads to a relatively big loss of the uploaded protein drug in stomach. In an attempt to overcome these inadequacies, we have developed, in an earlier study (El-Sherbiny et al., 2010) a series of calcium-crosslinked hydrogel matrices based on a mixture of alginate and chemically modified carboxymethyl chitosan. The resulting hydrogel beads showed enhanced swelling behavior in intestinal fluid as compared to that of the hydrogels based on alginate alone. However, the swelling extent of these gel beads was still relatively high in simulated gastric fluid which makes them subject to a relatively significant loss of the entrapped protein drug in gastric region. Therefore, in this study, an extension of our previous trial (El-Sherbiny et al., 2010) was carried out by developing of more enhanced pH-responsive microspheres formulations. These developed microspheres were



Scheme 1. Carboxymethylation of Cs and synthesis of CMCs-g-AAs copolymer.



Scheme 2. Synthesis of Cs-g-PEG copolymer.

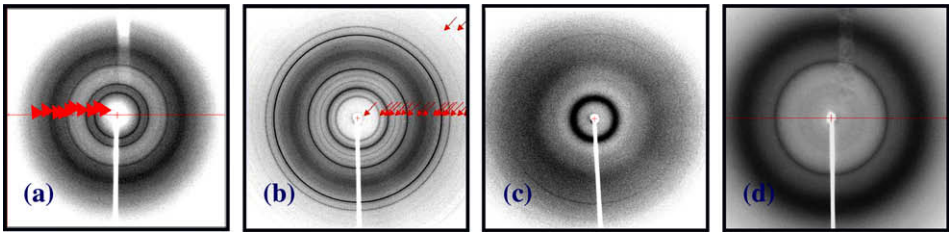


Fig. 1. 2D-XRD patterns of (a) Cs, (b) CMCs, (c) CMCs-g-AAs and (d) Cs-g-PEG.

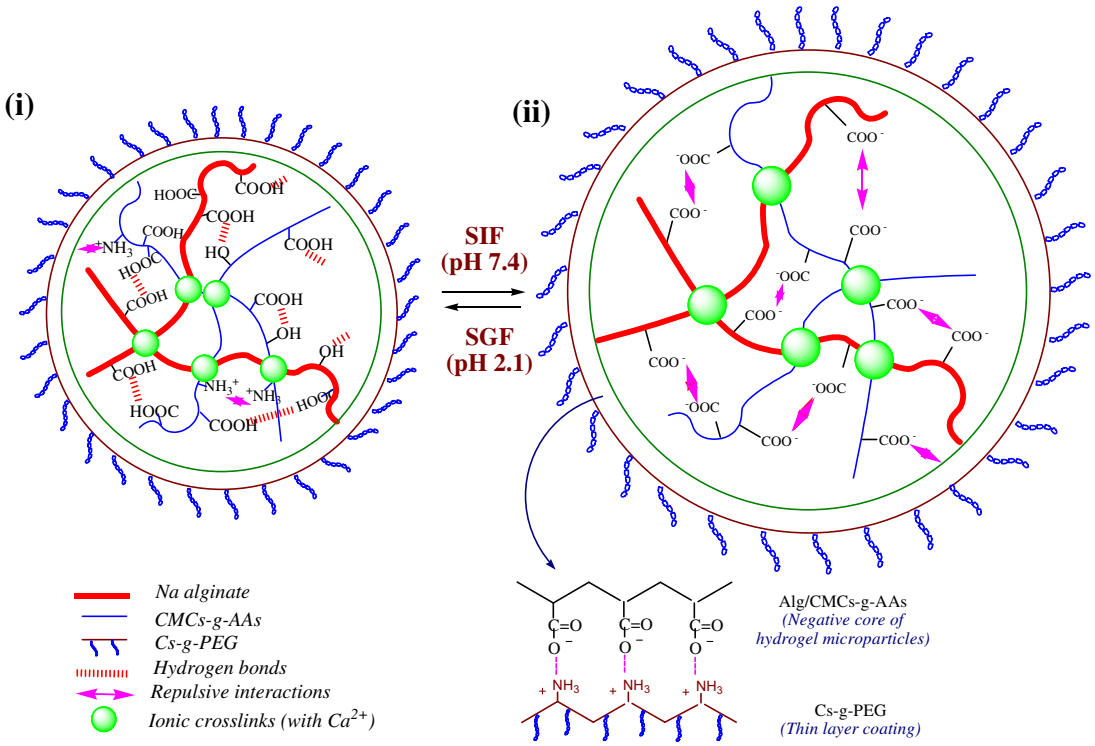
designed in such away to show very limited swelling extent in SGF with a good swelling pattern in SIF.

For developing of the microspheres, CMCs was prepared and further modified via grafting of AAs onto its backbone (*G*%:2400%). The main purpose of this modification was to increase the number of carboxylic groups in CMCs. Thus by developing a calcium-crosslinked hydrogel microspheres based on alginate with this modified CMCs, these carboxylic groups are expected to participate in minimizing the swelling at pH 2.1 (SGF) and maximizing the swelling at pH 7.4 (SIF). This expected role of carboxylic groups in controlling swelling extent of the developed hydrogel microspheres is illustrated in Scheme 3. In addition, coating of the resulting hydrogel microspheres with a polymer of unique polycation characteristics, such as Cs-g-PEG leads to a strong interaction with the negatively charged alginate/CMCs-g-AAs hydrogel core. Consequently, this coating tends to offer a more reduction in the swelling in SGF with a prolonged drug release properties in SIF (Anal, Bhopatkar, Tokura, Tamura, & Stevens, 2003; Shu & Zhu, 2002). This polyelectrolyte complexation coating with Cs-g-PEG copolymer is shown in Scheme 3 and it was carried out through either one or two steps coating procedures.

A trial was carried out during the early stage of this study to examine the ability of CMCs-g-AAs copolymer alone to form hydrogels with Ca^{2+}. It was noticed that the aqueous solution of this copolymer can form hydrogel particles when dropped onto a Ca^{2+} solution. However, the resulting particles had vey random shapes and were not symmetrical. Therefore, the formulations based on CMCs-g-AAs alone were not investigated further in this study. In combination with alginate, as represented in Scheme 3, the copolymer can form strong ionic crosslinks with Ca^{2+} at both pHs, 2.1 and 7.4. At pH 2.1 (Scheme 3i), the free carboxylic groups, which are not involved in the ionic crosslinking with Ca^{2+}, tend to form hydrogen bonds with each other and with OH groups of the sugar moieties. Both of ionic crosslinks and hydrogen bonds act to minimize the swelling at pH 2.1. The repulsive interactions that may occur between the protonated NH_2 groups work to increase hydrogel swelling. However, these repulsive forces are expected to be few, as the number of NH_2 groups remaining free, after carboxymethylation of Cs and its further modification via grafting, tend to be quite limited. In contrast, at pH 7.4 (Scheme 3ii), most of the free carboxylic groups would be ionized. Hence, strong repulsive forces are created by the electrostatic repulsion between these ionized carboxylate groups (COO^-). These repulsive forces are thus responsible for attaining the microspheres higher values of swelling at pH 7.4. Coating the microspheres with a thin layer of another hydrogel produced through polyelectrolyte complexation between the remaining negative charges on alginate/CMCs-g-AAs and the polycationic Cs-g-PEG copolymer can also play a role in enhancing the swelling profile of the microspheres towards minimizing it in SGF and make it more regular in SIF.

3.5. Morphology and particle size measurements

Fig. 2 shows the scanning electron micrographs for some of the developed hydrogel particles. From the figure, the particles are mostly spherical with a relatively rough surface. This roughness



Scheme 3. A schematic illustration of the different types of interactions in the developed alginate/CMCs-g-AAs hydrogel microspheres coated with Cs-g-PEG at both pH 2.1 (SGF) and pH 7.4 (SIF).

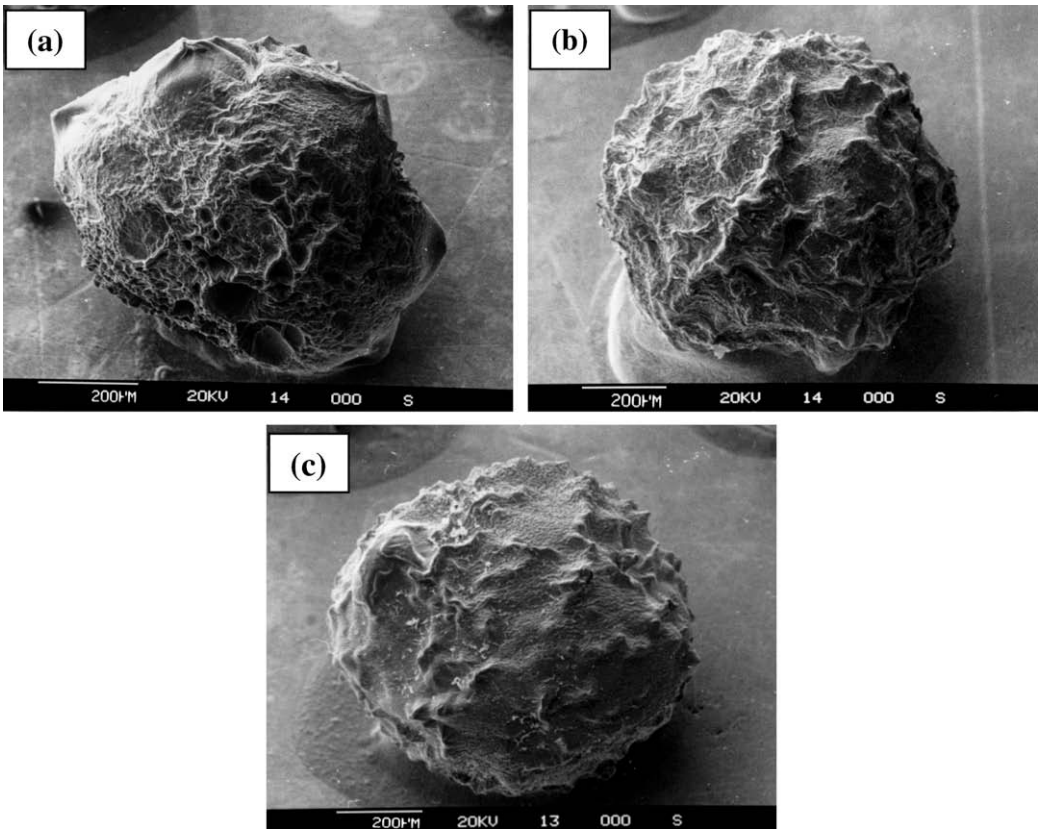


Fig. 2. Scanning electron micrographs of (a) alginate microspheres, PF0 (b) non-coated CMCs-g-AAs/alginate microspheres, PF4 and (c) coated CMCs-g-AAs/alginate microspheres, PF7.

seems to be relatively lower in non-coated CMCs-g-AAs/alginate microspheres (Fig. 2b) than alginate microspheres (Fig. 2a). Coating the microspheres with Cs-g-PEG turns them into more smoother (Fig. 2c). This surface morphology of microspheres can be seen more clearly in Fig. 3. As appeared from the figure and according to surface roughness; alginate microspheres ⩾ alginate/CMCs-g-AAs microspheres one step coated alginate/CMCs-g-AAs microspheres. The microspheres prepared through the two steps cross-linking/coating process were turned to show more dense, integrated and smooth surface (Fig. 3d). This smoothness may be attributed to formation of a thin layer of Cs-g-PEG onto the rough surface of the Ca^{2+}-crosslinked particles during the second cross-linking step.

The particle size can be expressed in different ways. Both Feret's and sieve diameters were estimated for the developed microspheres using optical microscopy. As illustrated in Fig. 4, Feret's diameter is the measured distance between two tangents on opposite sides of the particle whereas, sieve (inner) diameter is the diameter of the maximum inscribed circle (the maximum circle lying completely inside the particle) (Ortega-Rivas, 2005). As the shape of particles become more spherical, both Feret's and sieve diameters tend to attain similar values. As shown in Table 1, the microspheres developed in this study have sieve and Feret's diameters in the range of (658.1 ± 11–745.8 ± 19 μm) and (703.1 ± 21–819.0 ± 11 μm) respectively. Also, from the obtained results, remarkably, Feret's and sieve diameters yielded similar values despite the considerable differences in their measurement principles due to the spherical nature of the developed microspheres. It was found that the size of all the formulations based on CMCs-g-AAs/alginate (PF1-PF8) is significantly larger ($P < .0001$ and .0002 for Feret's and sieve diameters, respectively) as compared to the control formulation, PF0 (based on alginate alone). From Table 1, for a certain particle composition, it appears that increasing the concentration of $CaCl_2$ decreases both Feret's and sieve diameters of the particles. However, this effect of $CaCl_2$ on particle size was found to be statistically significant only in formulations prepared from 50% alginate:50% CMCs-g-AAs. Moreover, The Feret's and sieve diameters were found to decrease insignificantly ($P > .05$) with increasing the percent of sodium alginate in the microspheres. For instance, the Feret's diameter of PF2 (50% alginate) and PF4 (60% alginate) are 787.2 ± 14 and 781.3 ± 08 μm, respectively. With the concentration of $CaCl_2$ and microspheres composition kept constant, it seems from Table 1 that, coating of microspheres with Cs-g-PEG copolymer tends to increase their size. For instance, PF5 and PF6 showed a relatively larger Feret's and sieve diameters than PF2. However, this increase in microspheres size upon coating was found statistically insignificant. In addition, it was found that the

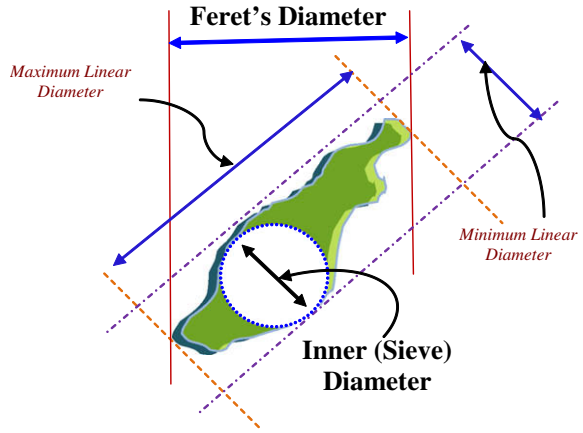


Fig. 4. A schematic illustration of Feret's and sieve (inner) diameters of an irregular particle.

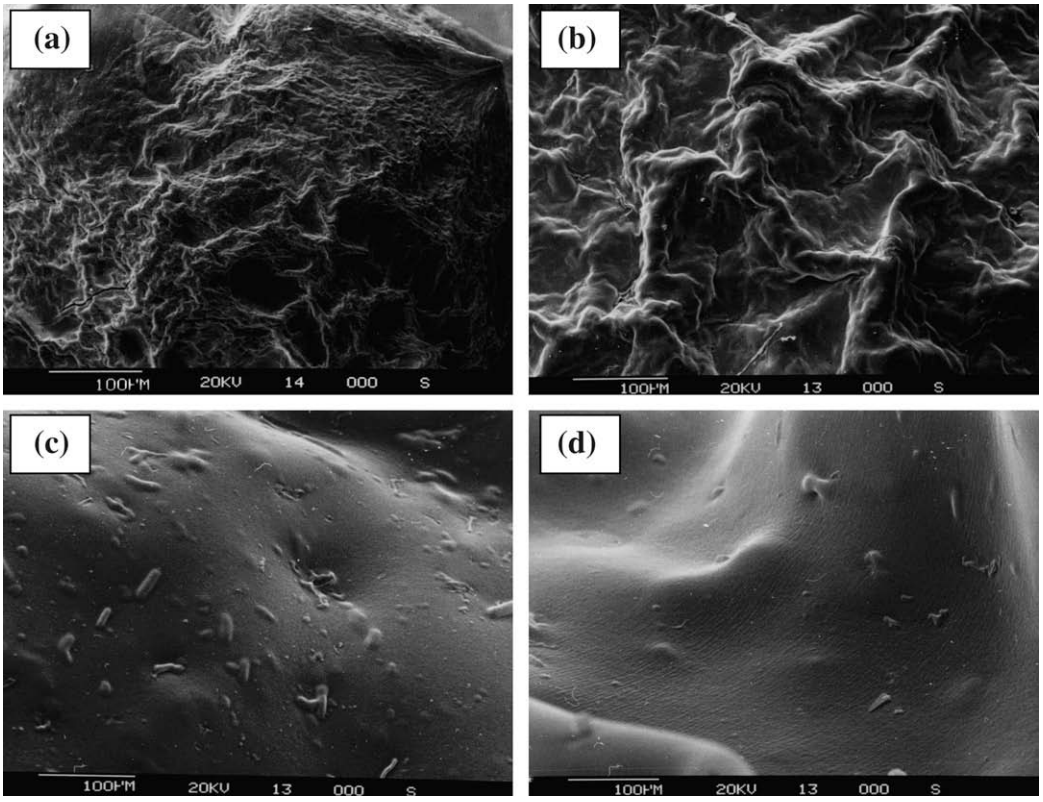


Fig. 3. Surface morphology of (a) alginate microspheres, PF0 (b) non-coated CMCs-g-AAs/alginate microspheres, PF4 and coated CMCs-g-AAs/alginate microspheres (c) one step, PF7 and (d) two steps, PF8.

two steps crosslinking/coating procedure of microspheres none significantly increases the size as compared to the one step process.

3.6. Swelling characteristics

The swelling behavior represents one of the most significant characteristics that control rate of drug release from hydrogels. The equilibrium swelling of the developed hydrogel microspheres was measured in pH 2.1 (SGF) for 2 h followed by 8 h in pH 7.4 (SIF) at 37 °C (Table 1). It was found that the swelling values attained at equilibrium by the formulations, PF1-PF8, are significantly different (P .0069 and <.001 in SGF and SIF, respectively) from the values attained by the control formulation, PF0. The statistical analysis of the obtained results (Table 1) showed also a significant effect ($P < .05$) for the $CaCl_2$ concentration on equilibrium swelling of the microspheres formulations based on 60% alginate:40% CMCs-g-AAs in both SGF and SIF. This effect of $CaCl_2$, however, was found to be non significant, in both SGF and SIF, for the microspheres based on equal percents of alginate and CMCs-g-AAs. In SGF, the alginate percent has a non significant effect on the equilibrium swelling whatever the concentration of $CaCl_2$ (0.05 or 0.1 M), whereas, in SIF, increasing the percent of alginate in the microspheres developed using 0.1 M $CaCl_2$ significantly decreases their equilibrium swelling. Coating of microspheres with Cs-g-PEG copolymer either through one or two steps procedure has a non significant effect ($P > .05$) on equilibrium swelling except in SIF for the microspheres prepared using 60% alginate:40% CMCs-g-AAs in presence of 0.1 M $CaCl_2$. The coated and non-coated microspheres formulations prepared from 60% alginate:40% CMCs-g-AAs and using 0.1 M $CaCl_2$ (PF4, PF7 and PF8) in addition to the control formulation (PF0) were selected, as representative formulations, for further investigation of the factors affecting their equilibrium swelling and swelling patterns. The statistical analysis of the equilibrium swelling data of PF4, PF7 and PF8 (Table 1) revealed that these microspheres formulations have significantly lower swelling values at equilibrium in SGF (P .0007) than the control, PF0. In SIF, however, they showed significantly ($P < .001$) higher equilibrium swelling values than PF0.

The swelling patterns of PF4, PF7 and PF8 microspheres were also investigated in comparison with PF0 as shown in Fig. 5. From the figure, the hydrogel microspheres prepared from alginate alone (control, PF0) attained relatively higher swelling values in SGF as compared to the microspheres based on a combination of alginate with CMCs-g-AAs (PF4, PF7 and PF8). Moreover, the Ca-crosslinked alginate microspheres (PF0) showed a limited swelling in SIF which consequently limits drug release from them in intestine. Therefore, it seems that, the microspheres based on alginate/CMCs-g-AAs, developed in this study, are likely more appropriate for the specific delivery of protein drugs to intestine than the hydrogel microspheres prepared from alginate alone. The hydrogel microspheres based on alginate/CMCs-g-AAs (PF4, PF7 and PF8) showed various swelling behaviors depending on the method and extent of crosslinking. For instance, PF4 (crosslinked with Ca^{2+} alone) showed higher swelling values at both SGF and SIF than PF7 and PF8 (crosslinked with both Ca^{2+} and Cs-g-PEG). Also, as appeared from the figure, PF7 (one step crosslinking) showed, in general, lower swelling than PF8 (two steps crosslinking). In the one step process, a strong crosslinking and consequently low swelling occurred due to the competition between two cationic crosslinkers (Ca^{2+} and Cs-g-PEG) to interact with the anionic mixture of alginate and CMCs-g-AAs. Whereas, in the two steps gelation process, Ca^{2+} plays the essential role in crosslinking of the polymeric matrix in the first step followed by formation of a thin hydrogel layer in the second step via the polyelectrolyte complexation between Cs-g-PEG and the negative charges remaining on the surface of hydrogel core

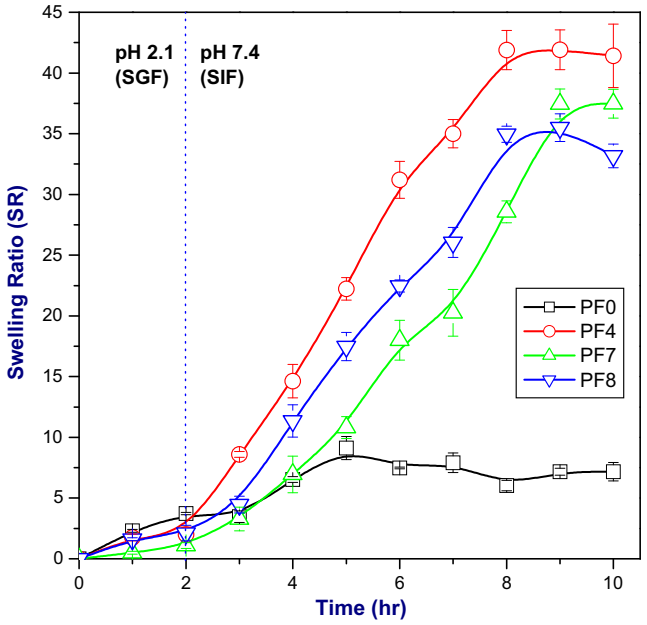


Fig. 5. Swelling patterns of some developed hydrogel microspheres in SGF for 2 h, followed by 8 h in SIF.

produced in first step. This tends to explain why the crosslinking of the microspheres prepared using two steps processes (PF8) is lower and consequently of higher swelling than microspheres prepared through one step process (PF7).

In a trial to investigate consistency of swelling behavior of the developed microspheres in SGF and to examine their ability to minimize loss of protein drugs in SGF and also their stability towards the harsh high acidity of stomach, a cyclic swelling study was carried out for PF4, PF7, PF8 and PF0 in SGF at 37 °C (Fig. 6). From this figure, the control formulation (alginate microspheres, PF0) attained SR of about 2.86 at equilibrium in the first cycle. Little change was noticed in the other two cycles except a slight decrease in weight of the dried hydrogel. In case of the microspheres based on alginate in a combination with CMCs-g-AAs (PF4, PF7 and PF8), the swelling ratios attained at equilibrium in the first cycle were 1.82, 1.14 and 2.31, respectively. Deswelling

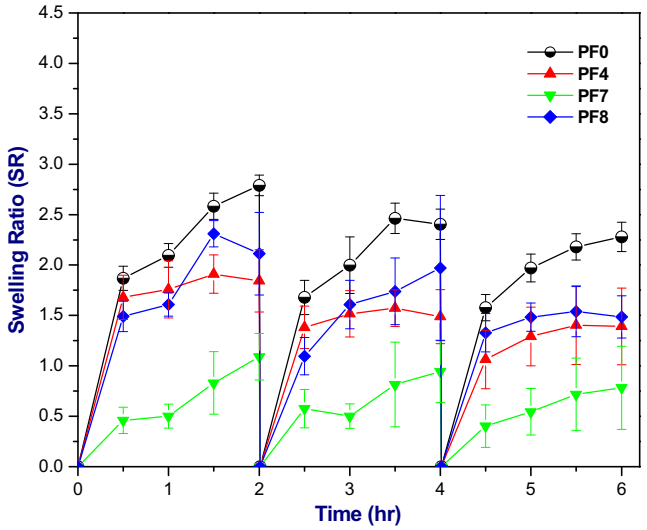


Fig. 6. Cyclic swelling of the microspheres PF0, PF4, PF7 and PF8 in SGF at 37 °C.

of samples and repeating the experiment shows that the equilibrium swelling decreases. The third cycle shows almost a small change in the maximum equilibrium swelling with a slight decrease in the time of achieving this equilibrium. The lack of change seen in the last two cycles implies a kind of consistency in swelling patterns of the developed microspheres and also tends to indicate that little or no readily soluble fraction remains in the developed hydrogel matrix after three consecutive swelling times in SGF. This tends to reflect a reasonable stability of the developed hydrogel carrier in the harsh high acidity environment of stomach.

3.7. In-vitro biodegradation study

A preliminary *in-vitro* enzymatic degradation study of the developed hydrogel microspheres was carried out in presence of lysozyme. Lysozyme is an enzyme abundantly present in many human secretions, such as tears, saliva and mucus. The percent of remaining weight of hydrogel microspheres was determined as a function of time and taken as a measure of the enzymatic degradation. Fig. 7 shows the enzymatic degradation patterns of the microspheres PF4, PF7 and PF8 in addition to the control formulation, PF0 in presence of 0.2% lysozyme. From the figure, almost all the microspheres start to degrade in the first two hours. The control microspheres (PF0) showed a significantly ($P < .0025$) lower degradation extent (higher % of remaining weights) than the microspheres based on combination of alginate with CMCs-g-AAs copolymer (PF4, PF7 and PF8). This effect of CMCs-g-AAs onto biodegradation rate of microspheres can be attributed to its role in increasing the swelling extent of microspheres in pH 7.4 (Scheme 3). This increase in swelling accelerates the diffusion of enzyme through the hydrogel matrix and consequently increases the rate of degradation. The statistical analysis of the biodegradation data revealed also that coating of microspheres with Cs-g-PEG copolymer (PF7 and PF8) significantly reduces their tendency to degrade as compared to non-coated microspheres (PF4). This may be attributed to presence of the PEG side chains with its limited biodegradation. Moreover, from the obtained preliminary results (Fig. 7) and within the range of studied formulations, the microspheres prepared through one step crosslinking/coating process (PF7) tend to offer slower enzymatic degradation up to 5 h than microspheres prepared through two steps (PF8). Then, after the first 5 h, the degradation of PF7 was much faster than PF8. This effect of the number of crosslinking/coating steps onto the degradation extent was found to be statistically non significant (P .0593).

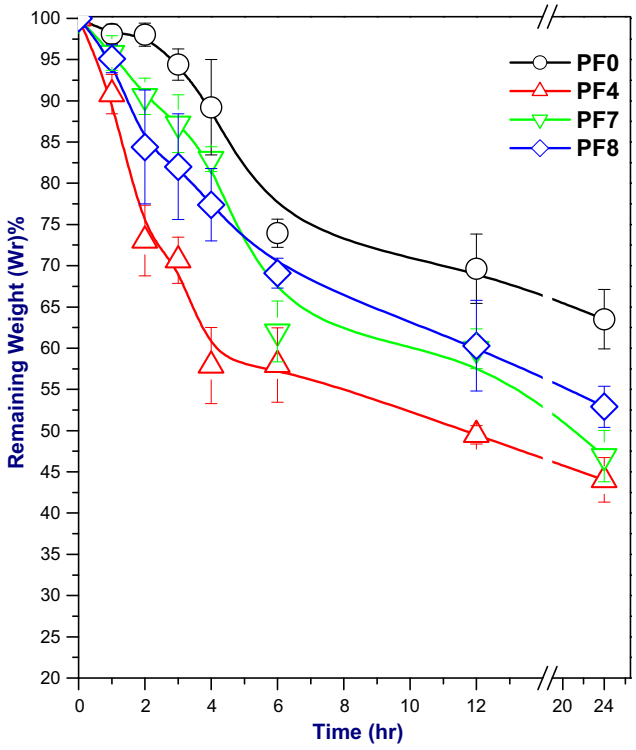


Fig. 7. A preliminary *in-vitro* enzymatic degradation of some of the developed hydrogel microspheres.

3.8. Entrapment capacity and in-vitro release studies

From the statistical analysis results of drug entrapment capacity (Ec%) data (Table 1), it was found that amount of BSA entrapped in the formulations PF0–PF8 are significantly different ($P < .0001$) from the control formulation, PF0. It was found also that Ec% of BSA increases significantly with increasing the concentration of $CaCl_2$. For instance, PF3 (0.05 M $CaCl_2$) and PF4 (0.1 M $CaCl_2$) showed Ec% of 32.0 ± 4 and 47.9 ± 2, respectively. With $CaCl_2$ concentration kept constant, increasing the alginate percent in the developed microspheres increased (non significantly) their Ec%. For example, PF2 (50% alginate) has Ec% of 43.2 ± 3 while PF4 (60% alginate) has Ec% of 47.9 ± 2. Moreover, it was found that, using of Cs-g-PEG copolymer in addition to the Ca^{2+} in crosslinking/coating of alginate/CMCs-g-AAs mixture enhances significantly the microspheres ability to entrap more amount of drug. For instance, the Ec% of PF7 and PF8 is much higher than PF4. It appeared also from the results that, the one step process is relatively more efficient in drug entrapment than the two steps process. This may be attributed to the more loss of drug upon transferring the microspheres from $CaCl_2$ solution to the Cs-g-PEG solution in the two steps process. This effect of coating method (one or two steps) on Ec% found to be more statistically significant in the formulations based on 50% alginate:50% CMCs-g-AAs than other formulations.

Fig. 8 shows the cumulative release profiles of BSA from some of the developed hydrogel microspheres at 37 °C for 2 h in SGF followed by 8 h in SIF. The percent of drug released was much higher in SIF than in SGF because the release rate from a hydrogel is particularly a reflection of its swelling behavior (diffusion mechanism). As discussed earlier, the swelling of the developed micro-

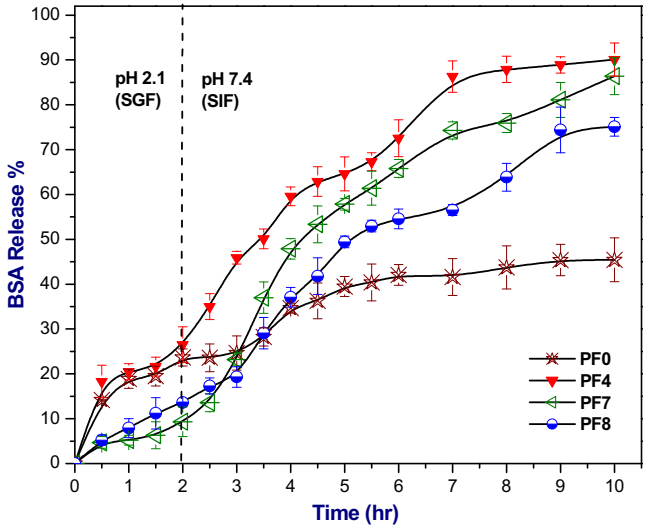


Fig. 8. Cumulative release profiles of a model protein drug, BSA from the developed hydrogel microspheres at 37 °C for 2 h in SGF followed by 8 h in SIF.

spheres in SIF was much higher than in SGF. From the figure, various amounts of BSA were released (lost) at 37 °C within 2 h in SGF depending on the hydrogel composition. Although the difference in BSA release (loss) in SGF from formulations of different compositions (PF4, PF7, PF8 in addition to the control, PF0) was found statistically non significant, it still shows a numeric difference. For instance, the microspheres crosslinked with Ca^{2+} alone (PF0 and PF4) lost a relatively high amount (23.6 ± 2% and 26.5 ± 4%, respectively) of the uploaded amount of BSA at 37°C after 2 h in SGF. Microspheres crosslinked by a combination of Ca^{2+} and Cs-g-PEG (PF7 and PF8), however, showed a limited loss of drug in SGF (only 9.2 ± 3% and 13.6 ± 0.1%, respectively).

In SIF, the microspheres developed in this study showed promising sustained release patterns with significantly high percents of drug released as compared to the microspheres based on alginate alone (the control, PF0). After 8 h in SIF, for instance, PF4, PF7 and PF8 released 90.1 ± 4%, 86.4 ± 4% and 75.1 ± 2% of BSA, respectively, as compared to 45.4 ± 5% only in case of the control microspheres, PF0. The statistical analysis of the drug released percents after 8 h in SIF showed a non significant difference (P 0.2716) between the non-coated formulation (PF4) and the coated formulation (PF7). Also, after 8 h in SIF, the microspheres formulation obtained through one step crosslinking/coating procedure (PF7) attained a significantly (P .0069) higher percents of drug release as compared to PF8 (obtained through two steps procedure).

In conclusion, the values and profiles of the drug released from the microspheres developed in this investigation showed that these enhanced microspheres can achieve, in general, very promising sustained release patterns in intestine with a very limited loss of protein drugs in stomach as compared to the hydrogel beads developed in our previous study (El-Sherbiny et al., 2010). Also, from the obtained data and statistical analysis of biodegradation and release profiles, it seems that the microspheres formulations prepared from 60% alginate:40% CMCs-g-AAs using 0.1 M $CaCl_2$ (PF4 and PF7) showed the most desired profiles. These two formulations (PF4 and PF7) achieved almost the same (non significant difference) promising biodegradation extent at 24 h (Fig. 7). Moreover, both formulations accomplished almost same (non significant difference) drug release percents at 8 h in SIF (Fig. 8) which are significantly higher than other investigated formulations. However, the microspheres formulations coated with Cs-g-PEG through one step procedure (PF7) can be considered the optimum formulation within the limit of this study as compared to PF4. This is because, PF7 showed a very limited loss of the model protein drug in SGF as compared to PF4 in addition to the very promising sustained release of PF7 beyond 8 h in SIF and its favorable biodegradation extent. Moreover, PF7 achieved the highest entrapment capacity of the model drug (83.1 ± 5%).

4. Conclusion

The swelling measurements of the developed hydrogel microspheres at 37 °C in both SGF and SIF clearly showed their pH-responsive nature. The *in-vitro* biodegradation study of the microspheres was carried out in SIF at 37 °C in presence of lysozyme and showed promising degradation rates. Morphology and size of the microspheres were also investigated. Bovine serum albumin (BSA) was entrapped in microspheres as a model for protein drugs and the *in-vitro* release profiles were established at 37 °C in both SGF and SIF. The preliminary investigation of the hydrogel microspheres developed in this study showed a consistent swelling pattern, high entrapment efficiency and promising sustained release profiles of the model protein drug.

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References

Ameye, D., Voorspoels, J., Foreman, P., Tsai, J., Richardson, P., Geresh, S., et al. (2001). Trypsin inhibition, calcium and zinc ion binding of starch-g-poly(acrylic acid) copolymers and starch/poly(acrylic acid) mixtures for peroral peptide drug delivery. *Journal of Controlled Release, 75*, 357–364.

Anal, A. K., Bhopatkar, D., Tokura, S., Tamura, H., & Stevens, W. F. (2003). Chitosan-alginate multilayer beads for gastric passage and controlled intestinal release of protein. *Drug Development and Industrial Pharmacy, 29*, 713–724.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry, 72*, 248–254.

Decho, A. W. (1999). Imaging an alginate polymer gel matrix using atomic force microscopy. *Carbohydrate Research, 315*, 330–333.

Dolatabadi-Farahani, T., Vasheghani-Farahani, E., & Mirzadeh, H. (2006). Swelling behaviour of alginate-N,O-carboxymethyl chitosan gel beads coated by chitosan. *Iranian Polymer Journal, 15*(5), 405–415.

El-Sherbiny, I. M. (2009). Synthesis, characterization and metal uptake capacity of a new carboxymethyl chitosan derivative. *European Polymer Journal, 45*, 199–210.

El-Sherbiny, I. M., Abdel-Bary, E. M., & Harding, D. R. K. (2010). Preparation and in vitro evaluation of new pH-sensitive hydrogel beads for oral delivery of protein drugs. *Journal of Applied Polymer Science, 115*(5), 2828–2837.

El-Sherbiny, I. M., & Elmahdy, M. M. (in press). Preparation, characterization, structure and dynamics of carboxymethyl chitosan grafted with acrylic acid sodium salt. *Journal of Applied Polymer Science*.

Ge, H. C., & Luo, D. K. (2005). Preparation of carboxymethyl chitosan in aqueous solution under microwave irradiation. *Carbohydrate Research, 340*, 1351–1356.

Hari, P. R., Chandy, T., & Sharma, C. P. (1996). Chitosan/calcium alginate microcapsules for intestinal delivery of nitrofurantoin. *Journal of Microencapsulation, 13*, 319–329.

Harris, J. M., Struck, E. C., Case, M. G., Paley, M. S., Yalpani, M., Van Alstine, J. M., et al. (1984). Synthesis and characterization of poly(ethylene glycol) derivatives. *Journal of Polymer Science; Polymer Chemistry Edition, 22*, 341–352.

Heller, J., Barr, J., Ng, S., Shen, H., Gurny, R., Schwach-Abdelaoui, K., et al. (2002). Development of poly(ortho esters) and their application for bovine serum albumin and bupivacaine delivery. *Journal of Controlled Release, 78*, 133–141.

Imren, D., Gumusderelioglu, M., & Guner, A. (2010). In vitro release kinetics of bovine serum albumin from highly swellable dextran hydrogels. *Journal of Applied Polymer Science, 115*, 740–747.

Ju, H. K., Kim, S. Y., Kim, S. J., & Lee, Y. M. (2002). pH/temperature-responsive semi-IPN hydrogels composed of alginate and poly(N-isopropylacrylamide). *Journal of Applied Polymer Science, 83*, 1128–1139.

Lin, Y. H., Liang, H. F., Chung, C. K., Chen, M. C., & Sung, H. W. (2005). Physically crosslinked alginate/N,O-carboxymethyl chitosan hydrogels with calcium for oral delivery of protein drugs. *Biomaterials, 26*, 2105–2113.

Liu, Z., Jiao, Y., & Zhang, Z. (2007). Calcium-carboxymethyl chitosan hydrogel beads for protein drug delivery system. *Journal of Applied Polymer Science, 103*(5), 3164–3168.

Luessen, H. L., Bochard, G., Verhoef, J. C., Lehr, C. M., de Boer, G. A., & Junginger, H. E. (1995). Mucoadhesive polymers in peroral peptide drug delivery. II. Carbomer and polycarbophil are potent inhibitors of the intestinal proteolytic enzyme trypsin. *Pharmaceutical Research, 12*, 1293–1298.

Mi, F. L., Liang, H. F., Wu, Y. C., Lin, Y. S., Yang, T. F., & Sung, H. W. (2005). pH-sensitive behavior of two-component hydrogels composed of N,O-carboxymethyl chitosan and alginate. *Journal of Biomaterial Science, Polymer Edition, 16*(11), 1333–1345.

Murata, Y., Kodama, Y., Isobe, T., Kofuji, K., & Kawashima, S. (2009). Drug release profile from calcium-induced alginate–phosphate composite gel beads. *International Journal of Polymer Science, 2009*, 1–4.

Nsereko, S., & Amiji, M. (2002). Localized delivery of paclitaxel in solid tumors from biodegradable chitin microparticle formulations. *Biomaterials, 23*, 2723–2731.

Ortega-Rivas, E. (2005). Handling and processing of food powders and particulates. In C. Onwulata (Ed.), *Encapsulated and powdered foods* (pp. 78–81). Boca Raton: CRC Press, Taylor & Francis G.

Peppas, N. A., & Sahlin, J. J. (1996). Hydrogels as mucoadhesive and bioadhesive materials: a review. *Biomaterials, 17*, 1553–1561.

Ramadas, M., Paul, W., Dileep, K. J., Anitha, Y., & Sharma, C. P. (2000). Lipoinsulin encapsulated alginate-chitosan capsules: intestinal delivery in diabetic rats. *Journal of Microencapsulation, 17*, 405–411.

Rekha, M. R., & Sharma, C. P. (2008). Phthalyl chitosan–poly(ethylene oxide) semi-interpenetrating polymer network microparticles for oral protein delivery: An in vitro characterization. *Journal of Applied Polymer Science, 110*, 2787–2795.

Roberts, G. A. F., & Domszy, J. G. (1982). Determination of the viscometric constants for chitosan. *International Journal of Biological Macromolecules, 4*, 374–377.

Shu, X. Z., & Zhu, K. J. (2002). The release behavior of brilliant blue from calcium-alginate gel beads coated by chitosan: The preparation method effect. *European Journal of Pharmaceutics and Biopharmaceutics, 53*, 193–201.

Vandenberg, G. W., Drolet, C., Scott, S. L., & de la Noue, J. (2001). Factors affecting protein release from alginate-chitosan coacervate microcapsules during production and gastric/intestinal simulation. *Journal of Controlled Release, 77*, 297–307.

Wittaya-areekul, S., Kruenate, J., & Prahsarn, C. (2006). Preparation and in vitro evaluation of mucoadhesive properties of alginate/chitosan microparticles containing prednisolone. *International Journal of Pharmaceutics, 312*, 113–118.

Zhang, X., Zhang, H., Wu, Z., Wang, Z., Niu, H., & Li, C. (2008). Nasal absorption enhancement of insulin using PEG-grafted chitosan nanoparticles. *European Journal of Pharmaceutics and Biopharmaceutics, 68*, 526–534.