Carbohydrate Polymers 83 (2011) 1507–1515

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Carboxymethylation of *kappa*-carrageenan for intestinal-targeted delivery of bioactive macromolecules

Kok Hoong Leong[a], Lip Yong Chung[a,*], Mohamed Ibrahim Noordin[a], Khalit Mohamad[a], Masato Nishikawa[b], Yoshinori Onuki[b], Mariko Morishita[b], Kozo Takayama[b]

[a] Department of Pharmacy, Faculty of Medicine, University of Malaya, Kuala Lumpur 50603, Malaysia
[b] Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Hoshi University, 2-4-41 Ebara, Shinagawa, Tokyo 142-8501, Japan

ARTICLE INFO

Article history:
Received 16 July 2010
Received in revised form
28 September 2010
Accepted 30 September 2010
Available online 7 October 2010

Keywords:
Carboxymethylation
Carrageenan
Microencapsulation
Drug delivery
Response surface methodology

ABSTRACT

The work presented herein discusses the carboxymethylation of *kappa*-carrageenan, a natural linear polysaccharide, to afford a pH-dependent swelling property allowing for intestinal-targeted delivery of bioactive macromolecules. The carboxymethylation conditions with respect to the volume and concentration of sodium hydroxide (V_{NaOH}, C_{NaOH}), weight of monochloroacetic acid (W_{MCA}), and reaction temperature (T) were optimized using a response surface method incorporating a multivariate spline interpolation technique (RSM^S). Fluorescein isothiocyanate-labeled dextran (FD-4; 4.4 kDa) was used as a hydrophilic macromolecule model. Beads made from encapsulating FD-4 in the carboxymethylated *kappa*-carrageenan displayed pH-dependent swelling and encapsulation efficiency of 74%. The release of FD-4 was low (23 ± 2%) in simulated gastric fluid (SGF) and high (90 ± 3%) in simulated intestinal fluid in a 2 h dissolution study. An additional *lambda*-carrageenan coating on the surface of the beads further reduced the FD-4 release in SGF. These carboxymethylated *kappa*-carrageenan beads may provide an efficient alternative approach for the oral delivery of hydrophilic macromolecules to the intestinal tract.

1. Introduction

Recent advances in biotechnology have driven the need to design suitable carriers for the oral delivery of bioactive macromolecules. Some bioactive macromolecules that have captured great interest include peptide or protein-based drugs, such as insulin and cyclosporine (Tan, Choong, & Dass, 2010); hormones such as thyrotropin-releasing and human growth hormone (Amet, Wang, & Shen, 2010; Tan et al., 2010); genes and vaccines for oral immunization (Chadwick, Kriegel, & Amiji, 2010; Page & Cudmore, 2001). However, there are critical barriers in creating an effective system for oral delivery of bioactive macromolecules, including low oral bioavailability due to their degradation in the highly acidic stomach environment, the presence of digestive enzymes, and the low permeability of these macromolecules through the intestinal epithelium (Chadwick et al., 2010; Page & Cudmore, 2001; Tan et al., 2010). Encapsulation of bioactive macromolecules improves their protection against degradation by low gastric pH and enzymes, providing a controlled release of the entrapped macromolecules (Tan et al., 2010). This encapsulation can be performed using natural polysaccharides such as alginate, chitosan and pectin (Liu, Jiao, Wang, Zhou, & Zhang, 2008).

Carrageenans are naturally occurring linear polysaccharides extracted from red seaweed. They have high molecular weight and are composed of repeating galactose residues. There are three primary classes of carrageenans available depending on the number and position of the ester sulfate groups, termed *kappa*-, *iota*-, and *lambda*-carrageenan. They have long been used commercially in the food industry, and now increasingly in pharmaceutical formulation studies (Gupta, Hariharan, Wheatley, & Price, 2001). They are known to form microparticles by *in situ* ionic gelation, and have successfully encapsulated some model drugs such as ibuprofen and verapamil (Sipahigil & Dortunc, 2001). Therefore, it is plausible that they could be used to encapsulate macromolecular drugs for oral delivery.

Although carrageenans are capable of encapsulating drugs, an efficient carrier for macromolecular drugs is required to ensure the release of its encapsulated drug in the intestinal region in order to prevent premature release and/or degradation in the stomach. Site-specific targeted release can be implemented by attaching pH-sensitive groups, such as carboxylic acid groups (–COOH), to the carrageenan polymeric structure through a carboxymethylation process. This pH-dependent swelling property is well suited for site-specific drug delivery to the intestine due to the change in pH from acidic ($pH \sim 1.2$) in the stomach to slightly alkaline ($pH \sim 7.4$) in the intestine as the drug carrier transits through the gastrointestinal tract (Peppas, Bures, Leobandung, & Ichikawa, 2000).

* Corresponding author. Tel.: +60 3 79674971; fax: +60 3 79674964.
E-mail addresses: chungly@um.edu.my, chungly@hotmail.com (L.Y. Chung).

0144-8617/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2010.09.062

Carboxymethylation was selected as a chemical means of attaching pendant carboxylic acid groups (COOH) due to it technical simplicity, low cost of chemical reagents, and its prior wide application in the industry to produce carboxylmethylcellulose (CMC) (Aguir & M'Henni, 2005). In addition to cellulose, polysaccharides such as starch (Kittipongpatana, Chaichanasak, et al., 2006), inulin (Verraest, Peters, Batelaan, & van Bekkum, 1995), and xylan (Petzold, Schwikal, & Heinze, 2006) have also been successfully carboxymethylated. However, the reaction conditions for the carboxymethylation process vary among different types of polysaccharides, and thus it was pertinent to optimize the carboxymethylation process specifically for carrageenan. Mathematical modeling such as response surface methodology and artificial neuro-network has greatly simplified and expedited the optimization time required to determine the optimal formula in a complex system. Among the emerging mathematical modeling methods is the response surface methodology (RSM^S), which incorporates a multivariate spline interpolation technique that has been proven useful in the modeling and prediction of synthetic chemical processes (Onuki, Nishikawa, Morishita, & Takayama, 2008).

Herein, we report the optimized reaction conditions for the carboxymethylation process of *kappa*-carrageenan using the RSM^S based on its pH-dependent swelling property and its macromolecule drug release behavior in both simulated gastric fluid (SGF) (pH 1.2) and simulated intestinal fluid (SIF) (pH 7.4) to produce a modified *kappa*-carrageenan for possible oral delivery of a bioactive macromolecule. The beads prepared from the optimized carboxymethylated *kappa*-carrageenan were also characterized by particle size, encapsulation efficiency and release rates of encapsulated fluorescein isothiocyanate (FITC)-labeled dextran (FD-4; 4.4 kDa) as a model for hydrophilic macromolecular drugs.

2. Materials and methods

2.1. Materials

Kappa-carrageenan (batch no.: 405301) and *lambda*-carrageenan (batch no.: NLV) were purchased from Marine Science Co. Ltd. (Tokyo, Japan). Potassium chloride, disodium hydrogen phosphate, deuterium oxide, sodium hydroxide, hydrochloric acid, and ethanol were obtained from Wako Pure Chemicals Industries Co. Ltd. (Osaka, Japan). Fluorescein isothiocyanate (FITC)-labeled dextran (4.4 kDa) (FD-4) and monochloroacetic acid were supplied by Sigma-Aldrich Co. (St. Louis, MO, USA).

2.2. Carboxymethylation of kappa-carrageenan

To model the carboxymethylation process for *kappa*-carrageenan, a four-factor, three-level Box-Behnken experimental design was adopted to generate 33 sample sets labeled K01 to K33 (Table 1). The four selected process factors were volume and concentration of sodium hydroxide (V_{NaOH}, C_{NaOH}), weight of monochloroacetic acid powder (W_{MCA}), and reaction temperature (T). The Box-Behnken design was selected as it is suitable for the construction of appropriate response surfaces critical for the mathematical optimization process (Kooijman, Ganzeveld, Manurung, & Heeres, 2003). The 33 experimental sample sets were generated in a randomized manner to avoid any systematic biases.

For carboxymethylation, 5 g powdered *kappa*-carrageenan was suspended in 100 ml 2-propanol and stirred for 30 min at room temperature. Next, the appropriate volume and concentration of sodium hydroxide solution was added, as indicated in Table 1, at a rate of 1 ml per 15 min, with continuous stirring at room temperature. Monochloroacetic acid of specified weight (Table 1) was then added portionwise to the reaction mixture over a period of 20 min. The reaction mixture was heated to a specified temperature (Table 1) with continuous stirring for 4 h to drive the reaction process to completion. The product was recovered through vacuum filtration and washed three times with 50 ml ethanol–water (4:1) and 50 ml ethanol wash alterations. The modified carrageenan was oven-dried at 70 °C overnight and powdered in a glass mortar.

Table 1
The Box-Behnken experimental design to study the four process factors (volume and concentration of sodium hydroxide, weight of monochloroacetic acid, and reaction temperature) for carboxymethylation of *kappa*-carrageenan at three levels.

Sample	V_{NaOH} (ml)[a]	C_{NaOH} (N)[b]	W_{MCA} (g)[c]	T (°C)[d]
K01	6	8	4.5	50
K02	6	8	9.1	50
K03	6	8	6.8	40
K04	6	8	6.8	60
K05	6	24	13.6	50
K06	6	24	27.2	50
K07	6	24	20.4	40
K08	6	24	20.4	60
K09	6	16	9.1	40
K10	6	16	18.1	60
K11	6	16	9.1	60
K12	6	16	18.1	40
K13	4	8	4.5	50
K14	4	24	13.6	50
K15	4	16	6.1	50
K16	4	16	12.1	50
K17	4	16	9.1	40
K18	4	16	9.1	60
K19	8	8	9.1	50
K20	8	24	27.2	50
K21	8	16	12.1	50
K22	8	16	24.2	50
K23	8	16	18.1	40
K24	8	16	18.1	60
K25	6	16	13.6	50
K26	6	16	13.6	50
K27	6	16	13.6	50
K28	6	16	9.1	50
K29	6	16	18.1	50
K30	6	16	13.6	40
K31	6	16	13.6	60
K32	4	16	9.1	50
K33	8	16	18.1	50

[a] V_{NaOH} is the volume of sodium hydroxide solution.
[b] C_{NaOH} is the concentration of the sodium hydroxide solution.
[c] W_{MCA} is the amount of monochloroacetic acid.
[d] T is the reaction temperature.

2.3. Characterization of modified kappa-carrageenan

2.3.1. Degree of carboxymethylation

The degree of substitution by carboxymethyl groups was determined by a previously described NMR protocol (van de Velde & Rollema, 2006). Briefly, 100 mg *kappa*-carrageenan or modified *kappa*-carrageenan was dissolved in 10 ml of 20 mM Na_2HPO_4 solution at 80 °C for 30 min in a water bath and allowed to cool to room temperature. The solution was dialyzed twice against 20 mM Na_2HPO_4 solution (1 l each), once against water (1 l) using Spectra/Por cellulose ester dialysis membrane with a molecular weight cut off of 500 (Cole-Palmer, Illinois, USA), and subsequently lyophilized overnight (Maxi-Dry Lyo, Heto-Holten, Allerod, Denmark). Then, 50 mg lyophilized carrageenan was re-dissolved in 10 ml of 20 mM Na_2HPO_4 solution. The solution was sonicated six times with 60 min for each sonication (VCX400 sonicator, Sonics and Materials Incorporation, California, USA, 19 mm tip, power 400 W, frequency 20 kHz) in melting ice. After centrifugation at 15,000 rpm for 60 min at 15 °C to remove abraded metal particles residue from the sonicator tip, the supernatant was lyophilized overnight (Maxi-Dry Lyo).

The lyophilized *kappa*-carrageenan or modified *kappa*-carrageenan (10 mg) was dissolved in 1 ml D_2O containing 20 mM Na_2HPO_4 and 10 mM 2,2-dimethyl-2-silapentane-3,3,4,4,5,5-d_6-5-sulfonate (DSS; as an internal standard). The sample was loaded into a NMR sample tube (Grade 5U-P, Kontes Glass Company, New Jersey, USA). The ^{1}H NMR spectrum was recorded at 25 °C on a JEOL Lambda-500 NMR spectrometer (Tokyo, Japan) operating at 500 MHz. Sixty-four scans were accumulated at a pulse angle of 90° with an interpulse delay of 3.7 s and an acquisition time of 3.2 s. The ^{13}C NMR spectrum was measured at 125.65 MHz accumulated over 20,000 scans with an interpulse delay of 2.0 s and an acquisition time of 0.9 s. ^{1}H/^{1}H COSY spectrum was acquired using a 512 × 512 resolution matrix with 64 scans per block and an acquisition time of 0.198 s. While ^{1}H/^{13}C HMQC measurement was performed using a 512 × 512 data matrix with 64 scans per block and an acquisition time of 0.164 s.

Chemical shifts (δ) were corrected relative to the internal standard, DSS (δ = 0.000 ppm for both ^{1}H and ^{13}C NMR according to the IUPAC recommendations) (van de Velde & Rollema, 2006). The chemical shifts of the proton and carbon peaks in the NMR spectra were consistent with published values (van de Velde & Rollema, 2006) and confirmed by ^{1}H/^{1}H COSY and ^{1}H/^{13}C HMQC spectra. The degree of carboxymethylation was calculated from the ^{1}H NMR spectrum using the integral of the proton peaks between the chemical shifts of 3.4–5.2 ppm, according to Eq. (1) (Heinze, Pfeiffer, & Lazik, 2001). This is based on 2-O- and 6-O-substitutions of the β-D-galactopyranose-4-sulfate unit (G) and 2-O-substitution of the 3,6-anhydro-D-galactopyranose moiety (DA) usually induce their respective low-field shifts (Fig. 1D and Scheme 1).

$$x_i = \frac{A\ (\text{proton(s) of the carboxymethylated } \kappa\text{-carrageenan at position O-}i)}{A\ (\text{proton(s) of the carboxymethylated } \kappa\text{-carrageenan at position O-}i) + A\ (\text{proton(s) of the non-carboxymethylated original at position O-}i)} \quad (1)$$

$$\text{Degree of substitution} = \sum x_i$$

where A represents the peak area; O is the oxygen atom at position i (i = positions 2 and 6 of the β-D-galactopyranose-4-sulfate unit (G), and position 2 of the 3,6-anhydro-D-galactopyranose moiety (DA)); x_i is the partial degree of substitution.

2.3.2. Swelling and gel fraction studies

Swelling and gel fraction studies were carried out based on a previously reported protocol (Onuki et al., 2008). In short, 0.1 g powdered modified *kappa*-carrageenan was weighed in a small dish, and the dish was carefully inserted into a 60 ml glass bottle. A total of 60 ml simulated gastric fluid (SGF) (pH 1.2) or simulated intestinal fluid (SIF) (pH 7.4), prepared according to USP XXII (USP, 1990), was slowly poured into the glass bottle. The sample was allowed to soak in SGF or SIF for 2 h at room temperature. Next, the excess solution was carefully removed, and the remaining gelled sample in the glass bottle was weighed. The gelled sample was lyophilized for three days and weighed again. The swelling ratio and percentage of gel fraction were calculated using Eq. (2) and (3) (Onuki et al., 2008).

$$\text{Swelling ratio} = \frac{W_{\text{water}}}{W_{\text{gel}}} \quad (2)$$

$$\text{Percentage gel fraction} = \frac{W_{\text{gel}}}{W_{\text{solid}}} \times 100 \quad (3)$$

where W_{water} is the weight of the sample after 2 h of soaking; W_{gel} is the weight of the sample after lyophilization; and W_{solid} is the initial weight of the sample.

2.4. Preparation of encapsulated FD-4 beads

Encapsulation of FD-4 was carried out based on a modified version of a previously reported protocol (Müller, Leuenberger, & Kissel, 1996; Sajeesh & Sharma, 2006). In short, 2 ml FD-4 solution (2 mg/ml) was added to 200 mg modified *kappa*-carrageenan and mixed thoroughly to form a viscous dispersion. The resulting dispersion was extruded dropwise through a disposable needle with an internal diameter of 0.5 mm into 20 ml of 1.5 M KCl–HCl solution (pH 1.2) with constant stirring (50 rpm). The encapsulated beads were retained in the KCl–HCl solution for 5 min to complete the curing reaction. The encapsulated beads were then collected by decantation, and allowed to soak in 5 ml ethanol for 2 min to remove surface water. The beads were then air-dried overnight at room temperature.

The same procedure was followed for coating of the encapsulated beads with *lambda*-carrageenan, except that 500 mg *lambda*-carrageenan was dissolved in the 20 ml of 1.5 M KCl–HCl solution (pH 1.2). The encapsulation efficiency (EE) was calculated as the difference between the total amount of FD-4 used to prepare the beads and the amount of FD-4 remaining in the hardening solution (1.5 M KCl–HCl solution) after removal of the beads, as shown in Eq. (4) (Sajeesh & Sharma, 2006).

$$\text{EE}\ (\%) = \frac{\text{total amount of FD-4} - \text{free FD-4 in supernatant}}{\text{total amount of FD-4}} \times 100 \quad (4)$$

2.5. Characterization of encapsulated FD-4 beads

The size and surface characteristics of the dried FD-4 encapsulated beads placed on a double-sided carbon adhesive tape mounted on an aluminum stab, were assessed using a field emission scanning electron microscope (Quanta 200 FESEM, FEI, Oregon, USA) in low-vacuum mode with 50, 2000, 8000, and 50,000× magnifications.

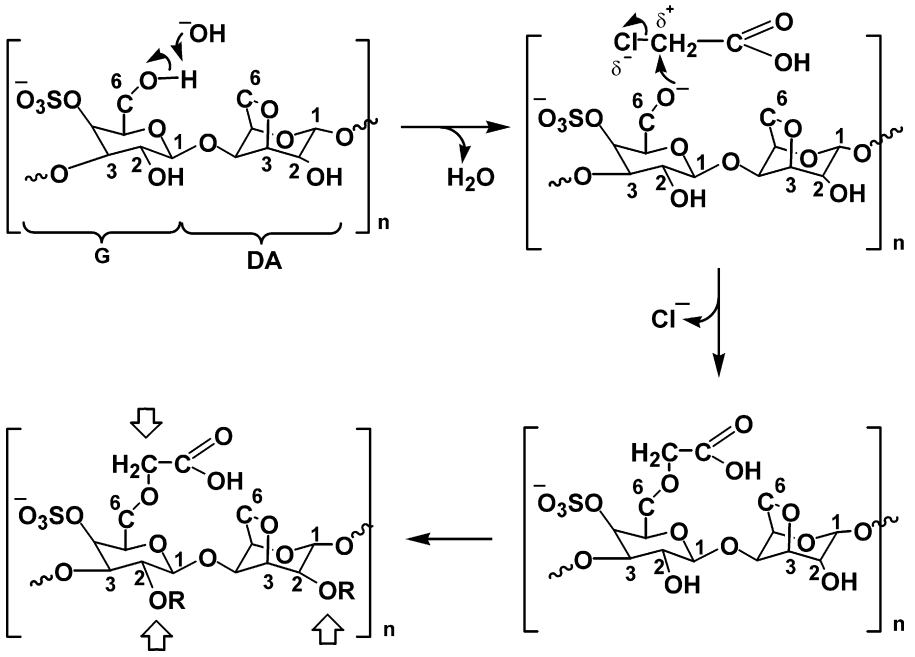
2.6. FD-4 release from encapsulated beads

The study was performed based a modified version of a previously reported protocol (Chadwick et al., 2010; Sajeesh & Sharma, 2006). In short, dried FD-4 encapsulated beads (200 mg) were placed in 20 ml SGF (pH 1.2) or SIF (pH 7.4) with stirring (150 rpm) at 37 °C for 2 h. One-ml aliquots of the solution were removed at time intervals and replaced with fresh medium, and the dilution effect was normalized mathematically. The amount of FD-4 released from the beads into the withdrawn solution was measured using a microplate luminometer (Mithras LB940, Berthold Japan, Tokyo, Japan) at excitation and emission wavelengths of 485 and 535 nm, respectively. The *in vitro* release study was conducted in triplicate.

2.7. Data analysis

The raw data on the process factors and responses for the 33 experimental sample sets were analyzed using the response surface method incorporated with the multivariate spline interpolation technique (RSM^S) within the dataNESIA® Version 3.0 software package (Yamatake Corp., Tokyo, Japan) to generate the response surfaces and simultaneously optimize the carboxymethylation process. The responses selected included the degree of carboxymethylation, the swelling ratio, the gel fraction in SGF and SIF, and the release of encapsulated FD-4 from the beads in SGF and SIF. The selected process factors were the volume and concentration of sodium hydroxide solution, the weight of monochloroacetic acid, and the reaction temperature.

The FD-4 release profiles from the encapsulated beads were first fitted into the Power law equation as shown in Eq. (5) (Lin & Metters,



Scheme 1. Reaction scheme for the carboxymethylation process on *kappa*-carrageenan. DA is 3,6-anhydro-α-D-galactopyranose; G is β-D-galactopyranose-4-sulfate. Numbers 1–6 show the carbon numberings. R = CH_2COOH or H; n = number of repeating units; arrow = possible positions for carboxymethylation.

2006; Siepmann & Peppas, 2001), and the calculated parameters n and k from the equation were used as part of the responses in the RSM^S modeling.

$$\frac{M_t}{M_\infty} = kt^n \tag{5}$$

where M_t is the amount of FD-4 released up to a specified time, t; M_∞ is the final amount of FD-4 released; k is the structural/geometric constant for a particular system; t is the sampling time; and n represents the release exponent of the release mechanism. Statistical analysis was carried out using Student's paired t-test, where $p < 0.05$ was selected as the criterion of significance.

3. Results and discussion

3.1. Carboxymethylation of kappa-carrageenan

The carboxymethylation of carrageenan employed the Williamson's ether synthesis process, a consecutive two-step reaction (Lawal, Lechner, Hartmann, & Kulicke, 2007). The reaction proceeds with a strong base such as sodium hydroxide that deprotonates the hydroxyl groups in *kappa*-carrageenan to form alkoxides, thereby increasing their nucleophilicity. The carboxymethyl groups are then formed in a reaction between the carrageenan alkoxides and monochloroacetic acid. The overall reaction is shown in Scheme 1.

A side reaction, shown below, takes place simultaneously resulting in the formation of sodium glycolate from sodium monochloroacetate and sodium hydroxide. Thus, the optimization study on the carboxymethylation process was carried out to improve substitution of the carboxylmethyl groups in *kappa*-carrageenan while minimizing the undesired side reaction.

$$NaOH + ClCH_2COONa \rightarrow HOCH_2COONa + NaCl$$

It has been shown that process factors such as the volume and concentration of sodium hydroxide solution (V_{NaOH}, C_{NaOH}), the weight of monochloroacetate acid powder (W_{MCA}), and the reaction temperature (T) strongly influence the efficiency of the carboxymethylation process (Lawal et al., 2007; Sangseethong, Ketsilp, & Sriroth, 2005). Using the Box-Behnken design, the influences of these process factors towards the dependent variables of the carboxymethylated carrageenan were studied. Similar experimental design has been adopted in the carboxymethylation of starch to determine the relationship between the process factors and dependent variables (Kooijman et al., 2003). In this study, the dependent variables, or the responses, included the degree of substitution (DS) (carboxymethylation), the swelling ratio, and the percentage of gel fraction in SGF and SIF solutions, as well as the parameters n and k obtained from the Power law equation after fitting the FD-4 release profiles in SGF and SIF solutions, respectively. The process factors and results of the responses for all the 33 experimental sample sets are summarized in Tables 1–3.

3.2. Degree of substitution in modified kappa-carrageenan

The ^{13}C NMR spectra of an ultrasonically degraded original *kappa*-carrageenan and a representative carboxymethylated carrageenan sample K22 (DS = 0.3204) are shown in Fig. 1A and B respectively (chemical shift ranging between δ = 48–200 ppm). The number of occurrences of carboxymethyl substitution in sample K22 is supported by the presence of additional resonances at δ = 182.61 and 180.68 ppm in its ^{13}C NMR spectrum (Fig. 1B), which were assigned to the carbonyl of the carboxylate group (COO^-), as reported in other carboxymethylated polysaccharides (Aguir & M'Henni, 2005; Jin, Zhang, Yin, & Nishinari, 2006). Further evidence of carboxymethylation having occurred on the C-6 hydroxyl group of the β-D-galactopyranose-4-sulfate unit (G-unit) in sample K22 comes from the appearance of a shoulder peak (δ = 63.94 ppm) on the G-C6 peak (δ = 63.54 ppm). This suggests two different environments for C-6, depending on whether or not carboxymethylation has occurred on the C-6 hydroxyl group. The signals at δ = 67.87 and 66.15 ppm were assigned to the methylene carbon atoms in the carboxymethoxy substituents. The appearance of only two peaks is probably due to the weak intensity of the signals in the ^{13}C NMR.

Fig. 1C and D shows the comparison between the ^1H NMR spectra for the original *kappa*-carrageenan and the representative carboxymethylated carrageenan sample, K22. The spectrum for K22 revealed the appearance of new proton peaks at δ = 3.70,

Table 2
The responses (swelling ratio and gel fraction) of carboxymethylated *kappa*-carrageenan and the Power law parameters (n and k) obtained from release profiles of encapsulated FD-4 from carboxymethylated *kappa*-carrageenan beads in simulated gastric (pH 1.2) and simulated intestinal fluids (pH 7.4).

Sample	Simulated gastric fluid (SGF) (pH 1.2)				Simulated intestinal fluid (SIF) (pH 7.4)			
	Swelling ratio[a,b]	Gel fraction (%)[a,b]	Power law's parameter, k[a,c]	Power law's parameter, n[a,c]	Swelling ratio[a,b]	Gel fraction (%)[a,b]	Power law's parameter, k[a,c]	Power law's parameter, n[a,c]
K01	1.15	91.07	0.10	1.04	1.22	95.29	0.36	0.61
K02	1.15	90.51	0.09	1.06	1.26	94.46	0.31	0.60
K03	1.23	91.85	0.25	1.06	1.27	95.77	0.31	0.61
K04	1.12	93.23	0.08	0.96	1.19	97.23	0.34	0.63
K05	1.12	91.95	0.50	1.60	1.11	95.63	0.51	0.95
K06	1.03	99.91	0.36	1.35	1.32	100.21	0.61	1.09
K07	1.07	96.74	0.32	1.30	1.31	98.87	0.30	0.57
K08	1.13	96.81	0.29	1.12	1.26	99.04	0.56	1.00
K09	1.30	91.26	0.05	0.98	1.33	95.98	0.50	0.76
K10	1.05	99.91	0.09	1.05	1.07	100.16	0.65	1.02
K11	1.12	93.09	0.06	0.99	1.24	97.71	0.45	0.75
K12	1.02	91.22	0.09	0.90	1.17	95.63	0.45	0.68
K13	1.10	96.34	0.64	0.69	1.15	98.97	0.26	0.46
K14	1.07	96.27	0.05	0.85	1.06	98.90	0.50	0.90
K15	1.11	94.83	0.04	0.78	1.22	97.28	0.35	0.61
K16	1.14	92.51	0.11	0.95	1.18	96.34	0.36	0.65
K17	1.09	96.32	0.05	0.67	1.23	99.46	0.34	0.59
K18	1.11	94.72	0.04	0.86	1.25	97.95	0.35	0.62
K19	1.04	99.91	0.65	0.82	1.16	99.92	0.40	0.69
K20	1.08	94.94	0.11	1.17	1.20	98.02	0.54	0.94
K21	1.27	93.25	0.05	1.00	1.19	97.21	0.80	1.14
K22	1.23	96.31	0.09	1.00	1.13	99.58	0.46	0.80
K23	1.18	92.23	0.07	0.56	1.33	96.52	0.48	0.84
K24	1.19	94.15	0.06	0.52	1.21	97.89	0.51	0.88
K25	1.11	94.43	0.04	0.65	1.26	97.72	0.37	0.61
K26	1.11	97.77	0.05	0.61	1.19	99.86	0.42	0.72
K27	1.12	92.60	0.06	0.92	1.09	96.10	0.51	0.95
K28	1.22	91.23	0.06	0.95	1.13	94.92	0.50	0.93
K29	1.24	91.38	0.07	0.94	1.11	95.14	0.46	0.86
K30	1.28	92.06	0.06	0.86	1.11	96.21	0.02	0.10
K31	1.24	92.37	0.58	0.92	1.16	95.67	0.68	1.02
K32	1.08	96.72	0.30	1.20	1.18	99.43	0.46	0.81
K33	1.02	96.93	0.05	0.92	1.17	99.63	0.61	1.04

[a] Each value represents the mean of three independent experiments.
[b] Swelling ratio and gel fraction were measured by Eqs. (2) and (3).
[c] k and n were determined using the Power law, Eq. (5).

3.93 and 4.31 ppm, attributed to the methylene protons in the carboxymethoxy substituents at positions C-2 and C-6 of the β-D-galactopyranose-4 sulfate unit (G-units) and position C-2 of the 3,6-anhydro-α-D-galactopyranose unit (DA-units) respectively. The partial degree of substitution (carboxymethylation) was determined using the ^{1}H NMR spectrum, as this is a more rapid method relative to ^{13}C NMR spectroscopy. Moreover, it was shown that ^{1}H NMR spectroscopy produces equally reliable data compared to other methods such as the high performance liquid chromatography (HPLC) or acid-base titration (Heinze, Liebert, Heinze, & Schwikal, 2004; Petzold et al., 2006). The partial degree of substitution of each modified *kappa*-carrageenan (K01–K33) was determined from the ^{1}H NMR spectra according to Eq. (1) (Table 3) (Heinze et al., 2001).

3.3. Swelling of modified kappa-carrageenan

The modified *kappa*-carrageenans (K01–K33) displayed pH-dependent swelling (Table 2). Generally, these samples had a lower swelling ratio relative to the unmodified *kappa*-carrageenan (1.5) in SGF solution (pH 1.2). However, in SIF solution (pH 7.4) most showed a higher swelling ratio relative to the unmodified *kappa*-carrageenan (1.1). This indicates that the introduction of the pendant carboxymethyl groups to the *kappa*-carrageenan confers pH-dependent swelling behavior. The pendant carboxymethyl groups ionize if the pH of the environment is above the pK_a of the carboxylic group (~3.4–3.7). Thus, at low pH (1.2; SGF), the carboxyl groups are protonated to promote the formation of hydrogen bonds with each other, thereby reducing the overall swelling ratio. At higher pH (7.4; SIF), the carboxyl groups are deprotonated, and a higher swelling ratio is conferred due to the collective electrostatic repulsion forces between the ionized acid groups. A similar pH-dependent swelling behavior has been observed in polymers containing pendant carboxylic acid groups such as alginate (Mohamadnia, Zohuriaan-Mehr, Kabiri, Jamshidi, & Mobedi, 2007), poly(acrylic acid) (Li, Wu, Wang, & Duan, 2006), and poly(methacrylic acid) (He, Li, & Lee, 2006).

3.4. RSM^S modeling

The data for the modified *kappa*-carrageenan (K01–K33) in Tables 1–3 were fitted into the dataNESIA® computer program to simulate the response surfaces using the multivariate spline interpolation (RSM^S) technique. The response surfaces indicated complex interrelationships between the four process factors and the release profile of encapsulated FD-4 from the beads in SGF (pH 1.2) (Fig. 2). Similar complex patterns were observed for beads in SIF (pH 7.4).

We performed a simultaneous optimization using the simulated annealing method to predict the conditions for the process factors that would provide a modified *kappa*-carrageenan with optimal macromolecule drug delivery characteristics (Table 4). To this end, the degree of substitution of *kappa*-carrageenan, the swelling ratio, and the Power law parameters k and n in SIF were maximized. Conversely, the swelling ratio, and the Power law parameters k and n in SGF were minimized, and the gel fraction in

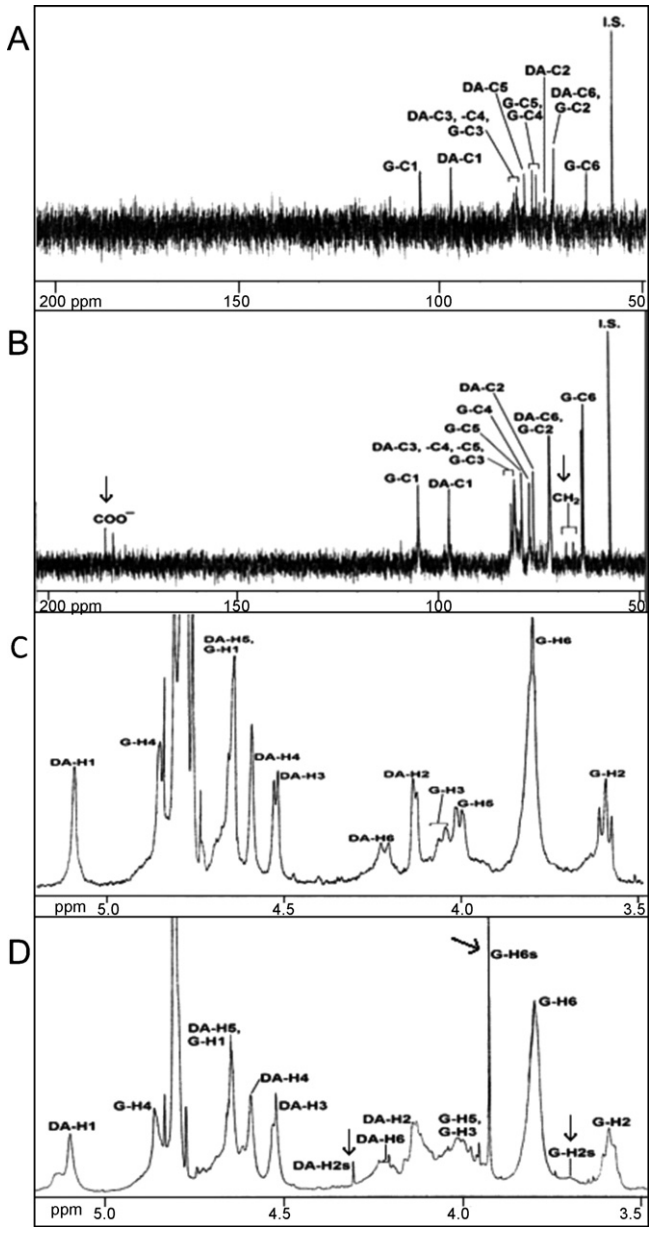


Fig. 1. (A) ^{13}C NMR spectrum of the original *kappa*-carrageenan. (B) ^{13}C NMR spectrum of a representative carboxymethylated *kappa*-carrageenan (sample K22). (C) ^{1}H NMR spectrum of the original *kappa*-carrageenan. (D) ^{1}H NMR spectrum of a representative carboxymethylated *kappa*-carrageenan (sample K22). DA is 3,6-anhydro-α-D-galactopyranose; G is β-D-galactopyranose-4 sulfate. C1–6 shows the carbon numberings. H1–6 shows the proton numberings. COO^- shows the carbonyl of the carboxylate group (arrow) and CH_2 shows the methylene group of the carboxymethoxy substituent (arrow). s denotes substituted (carboxymethylated) (arrow). I.S. denotes internal standard (2,2-dimethyl-2-silapentane-3,3,4,4,5,5-d_6-5-sulfonate: DSS).

both SGF and SIF were maintained above 95% using the dataNESIA® software.

Three independent batches of *kappa*-carrageenan were carboxymethylated using the optimized process factors (5 ml of 16 N sodium hydroxide solution, 5.3 g of monochloroacetic acid and temperature at 50 °C) predicted by the software. FD-4 was then encapsulated in beads prepared using the optimal modified *kappa*-carrageenan and the responses were determined experimentally. The results indicated that the experimental responses were similar to those predicted by the model with percentage errors below ±10% (Student's paired *t*-test, $p > 0.05$). This suggests that the spline technique provides reliable results, and is consistent with other studies, indicating that this approach is more accurate than normal polynomial response surface methodologies, particularly in complex systems (Schueremans & Gemert, 2005).

Table 3
The partial degree of substitution (DS) of samples (K01–K33) derived from ^{1}H NMR.

Sample	DS[a] (^{1}H NMR)			
	DA-O-2	G-O-2	G-O-6	Total DS
K01	0.0366	0.0123	0.0831	0.1320
K02	0.0282	0.0169	0.0860	0.1311
K03	0.0226	0.0329	0.0887	0.1442
K04	0.0266	0.0149	0.0853	0.1268
K05	0.0000	0.3171	0.2806	0.5977
K06	0.0000	0.1047	0.1729	0.2776
K07	0.2213	0.1327	0.2751	0.6291
K08	0.0645	0.1093	0.2782	0.4520
K09	0.1261	0.0272	0.2769	0.4302
K10	0.0146	0.0490	0.1353	0.1989
K11	0.1523	0.0386	0.3755	0.5664
K12	0.5269	0.0199	0.4541	1.0009
K13	0.0585	0.0214	0.0786	0.1585
K14	0.0382	0.0467	0.0781	0.1630
K15	0.0933	0.0384	0.2446	0.3763
K16	0.0481	0.0334	0.0767	0.1582
K17	0.1283	0.0372	0.2028	0.3683
K18	0.0244	0.0490	0.1469	0.2203
K19	0.0216	0.0150	0.0840	0.1206
K20	0.2414	0.0468	0.6709	0.9591
K21	0.2857	0.1181	0.3311	0.7349
K22	0.0838	0.0456	0.1910	0.3204
K23	0.1240	0.0761	0.2496	0.4497
K24	0.0776	0.0803	0.2395	0.3974
K25	0.1429	0.0585	0.2066	0.4080
K26	0.1706	0.0483	0.2607	0.4796
K27	0.0000	0.3462	0.2486	0.5948
K28	0.2396	0.0754	0.3563	0.6713
K29	0.3230	0.0326	0.3420	0.6976
K30	0.3319	0.0389	0.3210	0.6918
K31	0.1925	0.0707	0.3150	0.5782
K32	0.1770	0.0000	0.2449	0.4219
K33	0.6056	0.1585	0.4700	1.2341

[a] DS is the degree of carboxymethylation of the hydroxyl groups at C-2 and C-6 of the β-D-galactopyranose-4-sulfate unit (G-units) and C-2 of the 3,6-anhydro-α-D-galactopyranose unit (DA-units) in the *kappa*-carrageenan samples as determined by ^{1}H NMR according to Eq. (1).

It is evident from the results in Tables 2 and 3 (samples K05, K12, K20, K27, K28 and K33) that the degree of substitution is not directly related to the swelling of the modified *kappa*-carrageenan, nor FD-4 release from the beads. This variation is likely influenced by the substitution of carboxymethyl groups at the three possible positions in the repeating dimeric *kappa*-carrageenan structures. Differences in the position of substitution may affect the ability to form hydrogen bonds between the carboxylic acid groups in the gelled polymer, which in turn influences the swelling behavior of the modified carrageenan. This also leads to differences in the release of the entrapped FD-4 from these samples.

Carboxymethylation of carbohydrates relies on maximizing the degree of substitution (Heinze, Rensing, & Koschella, 2007; Jin et al., 2006; Kooijman et al., 2003; Mohamadnia et al., 2007). However, water solubility of carboxymethylated carbohydrates increases with increasing substitution, and this increase in water solubility in turn reduces gelling properties (Kittipongpatana, Sirithunyalug, & Laenger, 2006; Lawal et al., 2007). Since the gelling property must be maintained for drug encapsulation by ionic gelation processes, the gel fraction results were incorporated in the optimization process. For these reasons, we incorporated several core responses into the modeling rather than solely relying on the overall degree of substitution. These core responses included the swelling ratio, the percentage of gel fraction of the modified *kappa*-carrageenan, and *in vitro* release of encapsulated FD-4 from the beads. Together,

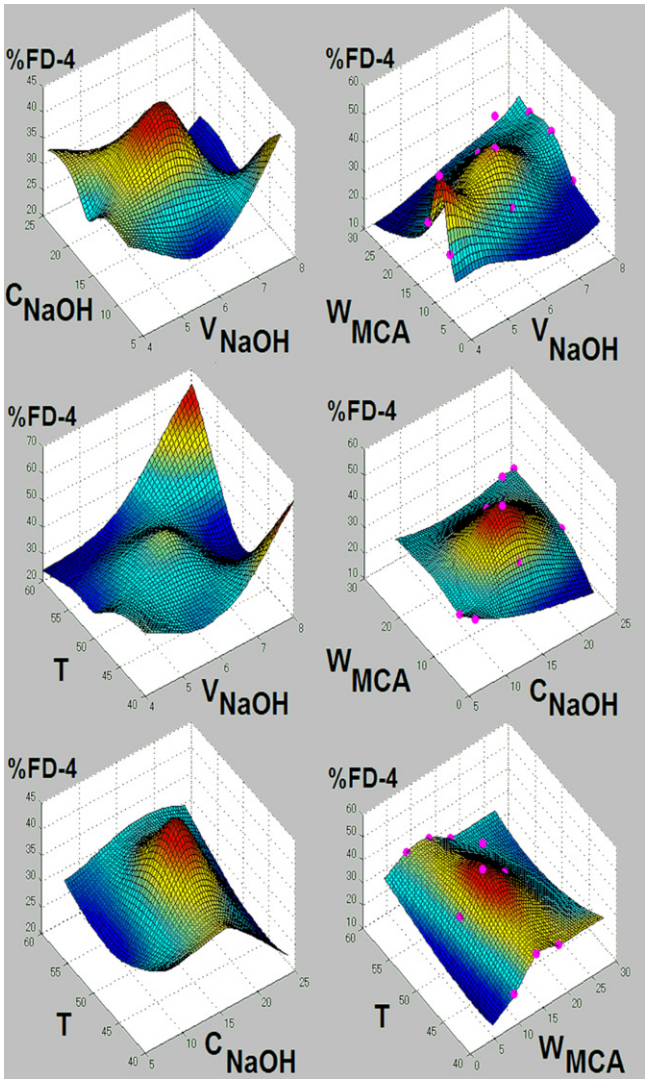


Fig. 2. Response surface plots of the percentage release of encapsulated FD-4 in simulated gastric fluid (SGF) at the end of 2 h dissolution against the process factors. V_{NaOH} is the volume of sodium hydroxide solution; C_{NaOH} is the concentration of the sodium hydroxide solution; W_{MCA} is the amount of monochloroacetic acid; T is the reaction temperature.

they provided a more accurate prediction in optimizing the process factors for the carboxymethylation of *kappa*-carrageenan as a bioactive macromolecule carrier.

3.5. Encapsulation of FD-4 beads

Carrageenan is an anionic polyelectrolyte hydrogel due to its sulfate groups. The presence of cations such as K^+, Na^+, or Ca^{2+} have been shown to induce conformational changes in the polymer, with initial coiling to helix transition, and subsequent aggregation of these helices to form gel networks (MacArtain, Jacquier, & Dawson, 2003; Naim, Samuel, Chauhan, & Paradkar, 2004). Due to this instantaneous ionic gelation property, encapsulation of drugs (Naim et al., 2004; Sipahigil & Dortunc, 2001) and enzymes (Kailasapathy & Lam, 2005) using carrageenan is possible. In our preliminary studies of the optimized carboxymethylated carrageenan, the final product failed to encapsulate FD-4 to form small beads when simply dropped into a 1.5 M KCl solution (~pH 4). This pH is above the pK_a of the carboxylic acid groups, and the electrostatic repulsion provided by any negatively charged carboxylic acid residues might prevent proper gelation. If the product was placed

Table 4
The responses (degree of substitution, swelling ratio, gel fraction) of carboxymethylated *kappa*-carrageenan and the Power law parameters (n and k) obtained from release profiles of encapsulated FD-4 from carboxymethylated *kappa*-carrageenan beads in simulated gastric (SGF) (pH 1.2) and simulated intestinal fluids (SIF) (pH 7.4) predicted by RSM[S] and from experimental data.

Responses	[a]Predicted value from RSM[S]	[b]Experimental value	[c]Percentage error (%)
Degree of substitution	1.1482	1.1413 ± 0.0283	−0.60
Parameter k in SGF	1.17	1.11 ± 0.07	−5.53
Parameter n in SGF	0.22	0.23 ± 0.02	8.81
Swelling ratio in SGF	1.00	1.00 ± 0.01	0.47
Percentage gel fraction in SGF	99.95	99.87 ± 0.01	−0.08
Parameter k in SIF	1.86	1.96 ± 0.05	5.20
Parameter n in SIF	0.93	0.94 ± 0.03	1.41
Swelling ratio in SIF	1.25	1.23 ± 0.01	−1.01
Percentage gel fraction in SIF	100.11	99.85 ± 0.01	−0.27

[a] From the RSM[S] model, the predicted optimum process factors for carboxymethylation of *kappa*-carrageenan with desirable FD-4 release characteristics were volume of sodium hydroxide solution (V_{NaOH}), 5.5 ml; concentration of the sodium hydroxide solution (C_{NaOH}), 16 N; amount of monochloroacetic acid (W_{MCA}), 5.3 g; and, reaction temperature (T), 50 °C.

[b] The experimental values are expressed in mean ± standard deviation of three independent experiments.

[c] No significant difference detected between the predicted responses and those obtained experimentally using student's paired t-test ($p > 0.05$).

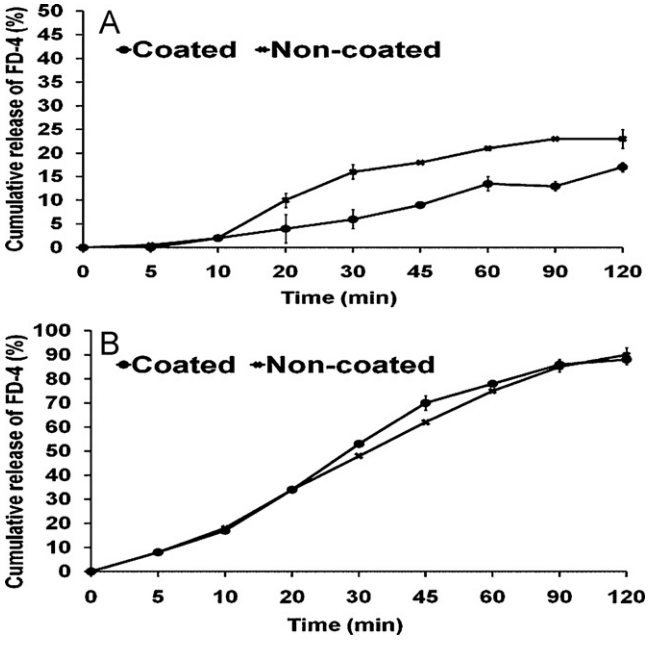


Fig. 3. (A) Release profile of FD-4 from non-coated and *lambda*-carrageenan coated carboxymethylated *kappa*-carrageenan (optimal formula) beads in simulated gastric fluid (SGF) over 2 h and (B) Release profile of FD-4 from non-coated and *lambda*-carrageenan coated carboxymethylated *kappa*-carrageenan (optimal formula) beads in simulated intestinal fluid (SIF) over 2 h.

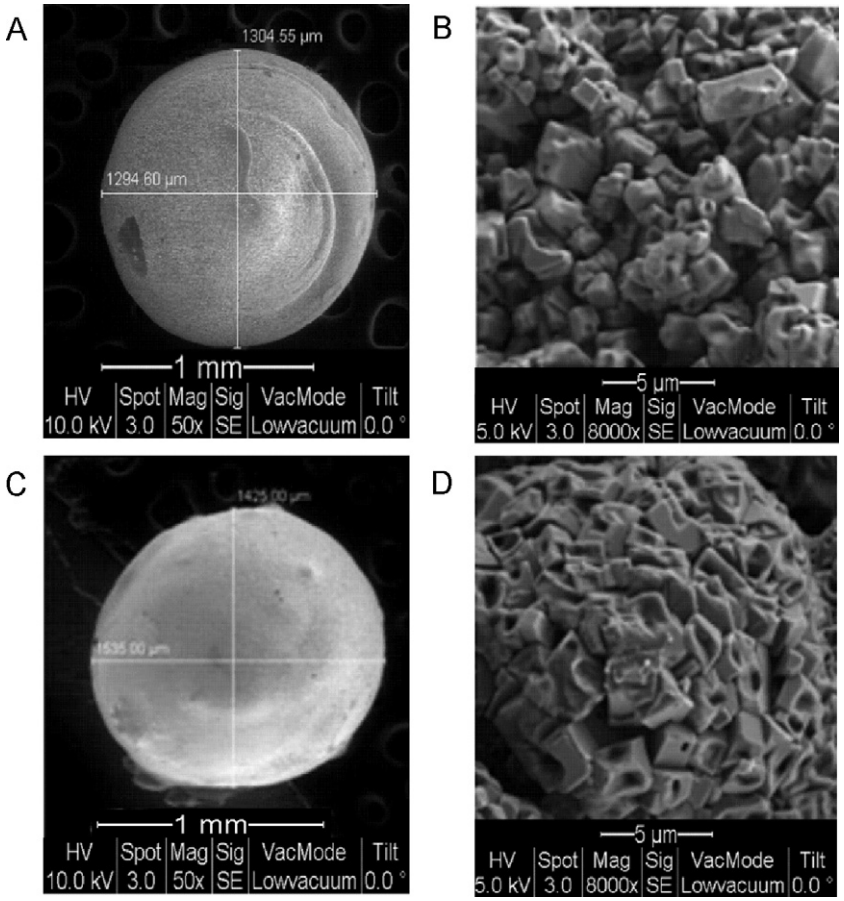


Fig. 4. Scanning electron micrographs (SEM) showing beads made from encapsulating FD-4 in carboxymethylated *kappa*-carrageenan at (A) 50× and (B) 8000× magnifications, and beads made from encapsulating FD-4 in carboxymethylated *kappa*-carrageenan and which have been coated with *lambda*-carrageenan at (C) 50× and (D) 8000× magnifications.

in 20 ml deionized water with a pH adjusted to 1.2, the product formed encapsulated beads in solution, but the beads collapsed, becoming flat sheets during the drying process. Therefore, the presence of both cations and the maintenance of low pH (pH < 3.5) are important factors for the proper gelation and encapsulation by the carboxymethylated *kappa*-carrageenan.

3.6. Release kinetics

The release of FD-4 from the optimal carboxymethylated *kappa*-carrageenan beads in SGF and SIF is shown in Fig. 3A and B. Based on the parameter n calculated from fitting the release data into the Power law, average n values for the optimal modified *kappa*-carrageenan are 0.23 ± 0.02 (mean ± SD, $n = 3$) in SGF and 0.94 ± 0.03 in SIF. For a spherical system when $n \leq 0.43$, the release mechanism is diffusion-controlled (Case I), while when $n \geq 0.85$, the release mechanism is swelling-controlled (Case II) (Lin & Metters, 2006; Siepmann & Peppas, 2001). Thus, the release mechanism of entrapped FD-4 from the optimal carboxymethylated *kappa*-carrageenan beads in SGF is through diffusion-control, while in SIF, it is through swelling-control. Since the modified *kappa*-carrageenan has a tightly enclosed network in an acidic environment, the entrapped FD-4 macromolecules escaped through diffusion. On the other hand, in a slightly basic environment (pH 7.4), the network structure opens up and swells due to the negatively charged carboxylate groups, leading to a release of FD-4. After 2 h, 23 ± 2% (mean ± SD, three independent experiments) of FD-4 was released from the beads in SGF, while 90 ± 3% was released in SIF (Fig. 3A and B). This is consistent with recent literature on the encapsulation of protein-based drugs using pH-sensitive polymers such as polymethylacrylic acid and chitosan, which have shown a cumulative release of 20% in pH 1.2 and 80% in pH 7.4 dissolution media (Lin et al., 2007; Sajeesh & Sharma, 2006).

To improve the performance of the system, the beads were coated with *lambda*-carrageenan, a non-gelling carrageenan frequently used as a thickener in food products. Coating of the beads reduced the release of FD-4 in SGF to 17 ± 1%; however, the release in SIF was not compromised, remaining at 88 ± 2%. Therefore, coating *lambda*-carrageenan on the surface of the beads slowed the release of FD-4 in SGF since it was also required to permeate the coated layer before reaching the surrounding dissolution medium.

The average encapsulation efficiency of FD-4 in the beads prepared with the optimal carboxymethylated *kappa*-carrageenan was 74 ± 2% (mean ± SD, $n = 3$), while that for *lambda*-carrageenan coated beads was 72 ± 2%. The beads were spherical in shape with an average diameter of 1304 ± 113 µm (mean ± SD, $n = 50$), while coated beads were slightly larger, with an average diameter of 1440 ± 131 µm (from SEM measurements; Fig. 4A and C). The surfaces of both uncoated and coated dried beads showed crystalline structures due to the organized helicity of the polymer chains during gelation (Fig. 4B and D).

4. Conclusion

This study has successfully incorporated a pH-dependent swelling property into *kappa*-carrageenan through a carboxymethylation process, using the RSM^S as a modeling tool to predict the optimal process factors to produce a bioactive

macromolecule carrier with desirable characteristics. The encapsulated macromolecular fluorescence marker (FD-4) was released from the beads at a low level (ca. 20%) in simulated gastric fluid (SGF), and at a high level (ca. 90%) in simulated intestinal fluid (SIF) over a 2-h period. This suggests that carboxymethylated *kappa*-carrageenan is able to protect encapsulated bioactive macromolecules such as peptides, hormones or gene fragments, from being degraded in the stomach and provides targeted release in the intestine.

Acknowledgements

This work was supported by research grants from Tacara Sdn. Bhd., Malaysia and University of Malaya, Malaysia (Grant No.: PS129/2007C). Mr. K. H. Leong acknowledges a National Science Fellowship and training grants from the Ministry of Science, Technology and Innovation, Malaysia (Ref. No: P/0089/01/2006/PHAR) and Tacara Sdn. Bhd., Malaysia. The funders had no role in data collection, data analysis, data interpretation or writing of the report. The authors thank Dr. H. Kasai and Miss N. Kobayashi of Hoshi University, Tokyo, Japan for the NMR measurements, and Dr. M. J. C. Buckle of University of Malaya, Malaysia for proof reading the manuscript.

References

Aguir, C., & M'Henni, M. F. (2005). Experimental study on carboxymethylation of cellulose extracted from *Posidonia oceanica*. *Journal of Applied Polymer Science*, *99*, 1808–1816.

Amet, N., Wang, W., & Shen, W. C. (2010). Human growth hormone-transferrin fusion protein for oral delivery in hypophysectomised rats. *Journal of Controlled Release*, *141*, 177–182.

Chadwick, S., Kriegel, C., & Amiji, M. (2010). Nanotechnology solutions for mucosal immunization. *Advanced Drug Delivery Reviews*, *62*, 394–407.

Gupta, V. K., Hariharan, M., Wheatley, T. A., & Price, J. C. (2001). Controlled-release tablets from carrageenans: effect of formulation, storage and dissolution factors. *European Journal of Pharmaceutics and Biopharmaceutics*, *51*, 241–248.

He, H., Li, L., & Lee, L. J. (2006). Photopolymerization and structure formation of methacrylic acid based hydrogels in water/ethanol mixture. *Polymer*, *47*, 1612–1619.

Heinze, T., Liebert, T., Heinze, U., & Schwikal, K. (2004). Starch derivatives of high degree of functionalization 9: carboxylmethyl starches. *Cellulose*, *11*, 239–245.

Heinze, T. H., Pfeiffer, K., & Lazik, W. (2001). Starch derivatives with high degree of functionalization. III. Influence of reaction conditions and starting materials on molecular structure of carboxymethyl starch. *Journal of Applied Polymer Science*, *81*, 2036–2044.

Heinze, T., Rensing, S., & Koschella, A. (2007). Starch derivatives of high degree of functionalization. 13. Novel amphiphilic starch products. *Starch*, *59*, 199–207.

Jin, Y., Zhang, H., Yin, Y., & Nishinari, K. (2006). Comparison of curdlan and its carboxymethylated derivative by means of Rheology, DSC and AFM. *Carbohydrate Research*, *341*, 90–99.

Kailasapathy, K., & Lam, S. H. (2005). Application of encapsulated enzymes to accelerate cheese ripening. *International Dairy Journal*, *15*, 929–939.

Kittipongpatana, O. S., Chaichanasak, N., Kanchongkittipoan, S., Panturat, A., Taekanmark, T., & Kittipongpatana, N. (2006). An aqueous film-coating formulation based on sodium carboxymethyl mungbean starch. *Starch*, *58*, 587–589.

Kittipongpatana, O. S., Sirithunyalug, J., & Laenger, R. (2006). Preparation and physicochemical properties of sodium carboxymethyl mungbean starches. *Carbohydrate Polymers*, *63*, 105–112.

Kooijman, L. M., Ganzeveld, K. J., Manurung, R. M., & Heeres, H. J. (2003). Experimental studies on the carboxymethylation of arrowroot starch in isopropanol–water media. *Starch*, *55*, 495–503.

Lawal, O. S., Lechner, M. D., Hartmann, B., & Kulicke, W.-M. (2007). Carboxymethyl cocoyam starch: synthesis, characterisation and influence of reaction parameters. *Starch*, *59*, 224–233.

Li, X., Wu, W., Wang, J., & Duan, Y. (2006). The swelling behavior and network parameters of guar gum/poly(acrylic acid) semi-interpenetrating polymer network hydrogels. *Carbohydrate Polymers*, *66*, 473–479.

Lin, C.-C., & Metters, A. T. (2006). Hydrogels in controlled release formulations: Network design and mathematical modeling. *Advanced Drug Delivery Reviews*, *58*, 1379–1408.

Lin, Y.-H., Mi, F.-L., Chen, C.-T., Chang, W.-C., Peng, S.-F., Liang, H.-F., et al. (2007). Preparation and characterization of nanoparticles shelled with chitosan for oral insulin delivery. *Biomacromolecules*, *8*, 146–152.

Liu, Z., Jiao, Y., Wang, Y., Zhou, C., & Zhang, Z. (2008). Polysaccharides-based nanoparticles as drug delivery systems. *Advanced Drug Delivery Reviews*, *60*, 1650–1662.

MacArtain, P., Jacquier, J. C., & Dawson, K. A. (2003). Physical characteristics of calcium induced κ-carrageenan networks. *Carbohydrate Polymers*, *53*, 395–400.

Mohamadnia, Z., Zohuriaan-Mehr, M. J., Kabiri, K., Jamshidi, A., & Mobedi, H. (2007). pH-sensitive IPN hydrogel beads of carrageenan-alginate for controlled drug delivery. *Journal of Bioactive and Compatible Polymers*, *22*, 342–356.

Müller, B. G., Leuenberger, H., & Kissel, T. (1996). Albumin nanospheres carriers for passive drug targeting: An optimized manufacturing technique. *Pharmaceutical Research*, *13*, 32–37.

Naim, S., Samuel, B., Chauhan, B., & Paradkar, A. (2004). Effect of potassium chloride and cationic drug on swelling, erosion and release from k-carrageenan matrices. *AAPS PharmSciTech*, *5*, 1–8.

Onuki, Y., Nishikawa, M., Morishita, M., & Takayama, K. (2008). Development of photocrosslinked polyacrylic acid hydrogel as an adhesive for dermatological patched: Involvement of formulation factors in physical properties and pharmacological effects. *International Journal of Pharmaceutics*, *349*, 47–52.

Page, D. T., & Cudmore, S. (2001). Innovations in oral gene delivery: Challenges and potentials. *Drug Discovery Today*, *6*, 92–101.

Peppas, N. A., Bures, P., Leobandung, W., & Ichikawa, H. (2000). Hydrogels in pharmaceutical formulations. *European Journal of Pharmaceutics and Biopharmaceutics*, *50*, 27–46.

Petzold, K., Schwikal, K., & Heinze, T. (2006). Carboxymethyl xylan-synthesis and detailed structure characterization. *Carbohydrate Polymers*, *64*, 292–298.

Sajeesh, S., & Sharma, C. P. (2006). Cyclodextrin–insulin complex encapsulated polymethacrylic acid based nanoparticles for oral insulin delivery. *International Journal of Pharmaceutics*, *325*, 147–154.

Sangseethong, K., Ketsilp, S., & Sriroth, K. (2005). The role of reaction parameters on the preparation and properties of carboxymethyl cassava starch. *Starch*, *57*, 84–93.

Schueremans, L., & Gemert, D. V. (2005). Benefits of splines and neural networks in simulation based structural reliability analysis. *Structural Safety*, *27*, 246–261.

Siepmann, J., & Peppas, N. A. (2001). Modeling of drug release from delivery systems based on hydroxypropyl methylcellulose (HPMC). *Advanced Drug Delivery Reviews*, *48*, 139–157.

Sipahigil, O., & Dortunc, B. (2001). Preparation and in vitro evaluation of verapamil HCl and ibuprofen containing carrageenan beads. *International Journal of Pharmaceutics*, *228*, 119–128.

Tan, M. L., Choong, P. F. M., & Dass, C. R. (2010). Recent developments in liposomes, microparticles and nanoparticles for protein and peptide drug delivery. *Peptides*, *31*, 184–193.

USP. (1990). *United States Pharmacopeia XXII*. USA: The United States Pharmacopeial Convention Incorp., pp. 1580–1581.

van de Velde, F., & Rollema, H. S. (2006). High resolution NMR of carrageenans. In G. A. Webb (Ed.), *Modern magnetic resonance* (pp. 1605–1610). Netherlands: Springer.

Verraest, D. L., Peters, J. A., Batelaan, J. G., & van Bekkum, H. (1995). Carboxymethylation of inulin. *Carbohydrate Research*, *271*, 101–112.