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Lectin-functionalized carboxymethylated *kappa*-carrageenan microparticles for oral insulin delivery

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ABSTRACT

We hypothesized that pH-responsive carboxymethylated *kappa*-carrageenan microparticles could protect entrapped oral insulin from acidic and proteolytic degradation in the gastrointestinal tract. Therefore, the objectives of this study were to prepare and characterize insulin entrapped in lectin-functionalized carboxymethylated *kappa*-carrageenan microparticles and to evaluate their therapeutic efficacy *in vitro* and *in vivo*. The encapsulation of insulin was performed using an ionic gelation technique and was optimized to give an encapsulation efficiency of 94.2 ± 2.6% and a drug-loading capacity of 13.5 ± 0.4%. The microparticles were further surface-lectin-functionalized for improved intestinal mucoadhesiveness. The oral administration of insulin entrapped in the microparticles led to a prolonged duration of the hypoglycemic effect, up to 12–24 h, in diabetic rats. From the release profile and the low toxicity of the microparticles, it can be concluded that these lectin-functionalized carboxymethylated *kappa*-carrageenan microparticles have the potential to be developed into an oral insulin delivery system.

1. Introduction

The current administration of peptide-based drugs, such as insulin, is predominately via the parenteral route, which has a number of disadvantages. These include discomfort due to repeated and prolonged dosage regimes, high variation in bioavailability and a non-physiological delivery pattern (Takei & Kasatani, 2004). These issues have brought about an increased effort to develop alternative delivery systems (Pillai & Panchagnula, 2001). The recent introduction of an inhaled delivery system for insulin was short-lived and resulted in the withdrawal of the product from the market by pharmaceutical companies (Opar, 2008). Recently, a pre-clinical study of an oral insulin formulation for type-2 diabetic patients showed promising results (Kapitza et al., 2010). Therefore, the oral delivery of insulin still remains an attractive alternative delivery route. Some advantages of the oral delivery system include the elimination of the risk of needle infection, increased patient compliance and a lower cost of therapy (Heller, Kozlovski, & Kurtzhals, 2007; Russell-Jones, 2004). It is also more physiologically relevant because orally administrated insulin undergoes first hepatic bypass and produces a similar effect to pancreas-secreted insulin (Sarmento, Ribeiro, Veiga, Ferreira, & Neufeld, 2007). However, peptide-based drugs, such as insulin, are difficult to deliver orally due to enzymatic degradation and their inability to transverse the biological barriers of the gastrointestinal tract. Therefore, recent research has focused on protecting the drug from degradation using drug carriers that include enzyme inhibitors and improving absorption via the incorporation of permeability enhancers (Khafagy, Morishita, Onuki, & Takayama, 2007).

Among the drug carriers investigated, carriers derived from natural polysaccharides have commanded particular interest due to their biodegradability, biocompatibility, hydrophilicity and protective properties (Liu, Jiao, Wang, Zhou, & Zhang, 2008). Natural polysaccharides, such as alginates and chitosan, were extensively used because of their favorable characteristics for drug entrapment (Sarmento, Ferreira, Jorgensen, & van de Weert, 2007). The advantage of using such hydrogels is the ease of performing water-based ionotropic gelation during the process of drug encapsulation. Moreover, it has been shown that ionotropic gelation preserves the bioactive conformation of the insulin drug (Martins, Sarmento, Souto, & Ferreira, 2007).

A recent report shows that the incorporation of dextran sulfate in the encapsulation of insulin with alginate and chitosan polymer mixtures improved the protection of insulin in an acidic *in vitro* environment. The enhanced protection is attributed to the

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ionic interaction between the sulfate groups in the dextran sulfate with the amino acid residues in the insulin molecules (Martins et al., 2007). Such a phenomenon was previously noted when protein–polyion complexation reduced the rate of protein escape due to enhanced electrostatic interactions (Kamiya & Klibanov, 2003). Both Tiyaboonchai, Woiszwillo, Sims, and Middaugh (2003) and Sarmento, Ribeiro et al. (2007) highlighted the prolonged glycemic effect and the promotion of sustained insulin availability *in vivo* with the inclusion of dextran sulfate as a physical mixture in their carrier systems.

To assist drug absorption in the intestinal region, mucoadhesive polymers have been adopted (Chowdary & Rao, 2004; Andrews, Laverty, & Jones, 2009). These mucoadhesive particles are able to prolong the residence time at the site of release, initiate contact with the intestinal barrier and create a drug concentration gradient that promotes the penetration of the drug through the intestinal membrane (Smart, 2005). Naturally derived mucoadhesive polymers, such as lectin, show promising mucoadhesive properties, particularly at the intestinal site (Clark, Hirst, & Jepson, 2000; Bies, Lehr, & Woodley, 2004), and they may be exploited for an intestinal-targeted delivery system (Lehr, 2000; Peppas & Kavimandan, 2006). Among the different types of lectin, wheat germ agglutinin (WGA), a glycoprotein from *Triticum vulgare*, binds to N-acetyl-D-glucosamine and sialic acid moieties, which are mainly found on both intestinal M-cells and regular intestinal absorptive cells (Yin, Chen, Qiao, Wei, & Hu, 2007), and it improves drug absorption for oral insulin delivery (Zhang et al., 2006).

Recently, we reported a new pH-responsive carboxymethylated *kappa*-carrageenan developed using a modeling technique for the intestinal-targeted delivery of macromolecules. The *in vitro* dissolution study indicated that the model molecule, fluorescein isothiocyanate-labeled dextran entrapped in carboxymethylated *kappa*-carrageenan microparticles, showed minimal release in an acidic environment (simulated gastric fluid (SGF)) but showed favorable release in simulated intestinal fluid (SIF), suggesting its potential as a carrier for the oral delivery of hydrophilic macromolecules to the intestinal tract (Leong et al., 2011).

Unlike earlier studies that used dextran sulfate in the form of a physical mixture incorporated into the delivery systems, the carboxymethylated *kappa*-carrageenan used here contains naturally occurring sulfate groups in the polymer chain. It is conceivable that these covalently linked sulfate groups improve the encapsulation efficiency, drug-loading capacity and stability of insulin entrapped in the carrageenan microparticles via ionic interactions between the carrageenan sulfate groups and the amino groups of the amino acid residues in insulin. This phenomenon is also likely to inhibit the release of insulin from the microparticles and, together with lectin functionalization of the microparticles, is likely to make it behave as a prolonged-sustained release system in the intestinal region. Herein, we report our findings on the use of lectin-functionalized carboxymethylated *kappa*-carrageenan microparticles as an alternative and improved carrier for the oral delivery of insulin.

2. Materials and methods

2.1. Materials

kappa-Carrageenan (batch no.: 405301) was supplied by the Marine Science Co., Ltd. (Tokyo, Japan). Human recombinant insulin, lectin from *Triticum vulgaris* (WGA), 200 mM L-glutamine, fetal bovine serum (FBS), 0.25% trypsin-EDTA and phosphate-buffered saline (pH 7.4) tablets were purchased from Sigma–Aldrich (St. Louis, MO, USA). Potassium chloride, sodium hydroxide, potassium dihydrogen orthophosphate, 37% fuming hydrochloric acid and acetonitrile were supplied by Fisher Scientific UK, Ltd. (Loughborough, Leicestershire, UK). Glutaraldehyde 25% (v/v), orthophosphoric acid, sodium acetate salt and Chromolith Performance RP-18e HPLC columns (4.6 mm × 100 mm) were from Merck KGaA (Darmstadt, Germany). Syringes (1 mL) and needles with diameters of 25G (0.50 mm × 16 mm), 26G (0.45 mm × 13 mm) and 27G (0.40 mm × 13 mm) were supplied by Terumo (Laguna, Philippines). Dulbecco's modified Eagle's medium (DMEM), 100 mM non-essential amino acid, Hank's Balanced Salt Solution (HBSS), 50 μg/mL gentamycin and 2.5 μg/mL amphotericin B were purchased from the Invitrogen Corporation (Carlsbad, CA, USA). The MTS assay kit [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] was supplied by Promega (Madison, WI, USA), and the LDH (lactate dehydrogenase) assay kit was from Roche (Mannheim, Germany).

2.2. Methods

2.2.1. Synthesis and characterization of carboxymethylated kappa-carrageenan

Carboxymethylated *kappa*-carrageenan was prepared by a previously described method (Leong et al., 2011). In brief, 5 g of powdered *kappa*-carrageenan was suspended in 100 mL of 2-propanol and stirred for 30 min at room temperature. Next, 5 mL of 16 N sodium hydroxide was added at a rate of 1 mL per 15 min with continuous stirring at room temperature. Monochloroacetic acid (5.3 g) was then added portionwise to the reaction mixture over a period of 20 min. The reaction mixture was heated to 50 °C with continuous stirring for 4 h to drive the reaction process to completion. The product was recovered through vacuum filtration and washed alternately with 50 mL of ethanol–water (4:1) and 50 mL of ethanol three times. The modified carrageenan was oven dried at 70 °C overnight and powdered in a glass mortar.

The degree of carboxymethylation on the modified *kappa*-carrageenan was determined using a NMR protocol as described previously (Leong et al., 2011). The swelling and gelling properties of the modified carrageenan in simulated gastric fluid (SGF) (pH 1.2) and simulated intestinal fluid (SIF) (pH 7.4) were measured using a previously described method (Leong et al., 2011).

Molecular weight was measured using size-exclusion liquid chromatography (Spichtig & Austin, 2008). Briefly, the system consisted of a Waters 2690 solvent delivery module, a Waters 2410 refractive index detector (Waters Co., Milford, MA, USA), two coupled Waters Ultrahydrogel Linear columns (7.8 mm × 300 mm) and a Waters millennium v3.02 workstation. The mobile phase was 0.1 M lithium nitrate with a flow rate of 0.6 mL/min. A sample volume of 100 μL was injected into the system at 10 mg/mL. Standard solutions of polyethylene oxide (24.2–932 kDa) (Showa Denko, Kanagawa, Japan) at 10 mg/mL were analyzed, and the logarithm of the molecular weight of the standard versus retention time was used to construct a standard curve for the estimation of molecular weight (linearity, $R^2 = 0.993$).

Sulfate content was determined using ion chromatography. Briefly, a 1 mg sample was hydrolyzed in 1 mL of 2 M trifluoroacetic acid (TFA) for 1 h in a 120 °C oil bath. The hydrolyzed sample was allowed to cool, and 2 mL of deionized water was added and mixed. It was then centrifuged at 5000 rpm, and 50 μL of the sample was analyzed using an ICS 1600 ion-chromatography system equipped with a conductivity detector (Dionex Corporation, Sunnyvale, CA, USA). The anions were separated on a Waters IC-Pak Anion (4.6 mm × 150 mm) column (Waters Co., USA) with a mobile phase of borate-gluconate buffer (pH 8.5) and flow rate of 2 mL/min. The sulfate content was expressed as a weight percentage of the analyzed sample (% w/w).

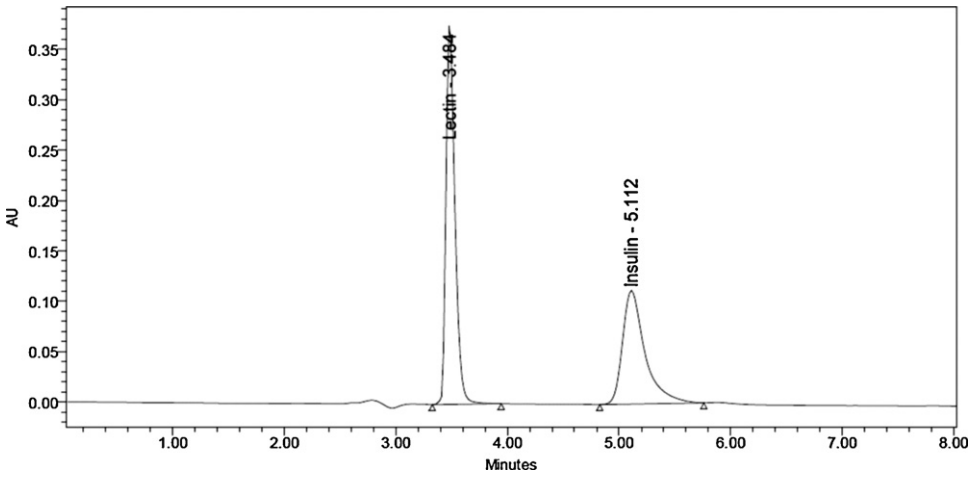

Fig. 1. HPLC profile of lectin (100 μg/mL) and human insulin (100 μg/mL).

2.2.2. Preparation of insulin-loaded microparticles

Insulin-loaded microparticles were prepared using an ionotropic gelation process (Sipahigil & Dortunç, 2001). For this purpose, 125–175 mg powdered carboxymethylated *kappa*-carrageenan was dissolved in 1 mL of pH-adjusted (pH 4.0–7.0) deionized water with 0.3 M sodium acetate buffer (pH 4.0). To this solution, 1 mL of human insulin (10–30 mg/mL) was added and mixed thoroughly to form a viscous dispersion. The resulting dispersion was then loaded into a 1 mL syringe and extruded dropwise through needles of varying sizes (internal diameter, 0.4–0.5 mm) into 20 mL of 1.5 M potassium chloride–HCl solution (pH 1.2) with constant stirring (50 rpm) under a constant stream of air blown perpendicular to the tip of the needle. The insulin-loaded microparticles were then collected by decantation and dried in a desiccator overnight at 4 °C.

2.2.3. Preparation of insulin-loaded lectin surface-conjugated microparticles

To functionalize the surface of the insulin microparticles, the microparticles were first surface activated with polyglutaraldehyde (Tanriseven & Ölçer, 2008), followed by conjugation to lectin (Montisci, Giovannuci, Duchêne, & Ponchel, 2001). Briefly, 0.15 mL of 1 N sodium hydroxide was added to 5 mL of 25% (v/v) glutaraldehyde solution to give a pH of 10.5, and the mixture was shaken at 200 rpm for 30 min. Then 0.15 mL of 1 N hydrochloric acid was added to the reaction mixture to neutralize and stop the polymerization reaction to give a polyglutaraldehyde solution. Then 0.53 mL of the polyglutaraldehyde solution was acidified with 0.25 mL of 1 M sulfuric acid, brought up to 1 mL with deionized water to give 12.5% (v/v) and then further diluted to 0.1–5.0% (v/v) polyglutaraldehyde using deionized water as needed.

The freshly collected insulin-loaded microparticles from Section 2.2.2 were soaked in 10 mL of polyglutaraldehyde solution (0.1–5.0%, v/v) for 1 h. Then the microparticles were rinsed four successive times with 5 mL of 1.5 M potassium chloride–HCl solution (pH 1.2) to remove excess crosslinking reagent, followed by washing with deionized water (5 mL).

The surface-activated insulin-loaded microparticles were immersed in a lectin solution (0.25–1.25 mg/mL PBS, pH 7.4) for 30 min. The lectin surface-conjugated microparticles were washed with 5 mL of 1.5 M potassium chloride–HCl solution (pH 1.2) and deionized water (5 mL). The beads were then dried in a desiccator overnight at 4 °C.

2.2.4. Determination of insulin encapsulation efficiency and insulin load

To determine the encapsulation efficiency and the total insulin load of the obtained microparticles from Section 2.2.2, an indirect method to measure the insulin content in the 1.5 M potassium chloride–HCl solution (hardening solution) using an established HPLC protocol was performed (Deeb, Preu, & Wätzig, 2007). The system consisted of a Waters 2690 solvent delivery module, a Waters 996 PDA (Waters Co., Milford, MA, USA), two coupled Chromolith Performance RP-18e columns (4.6 mm × 100 mm, Merck, Darmstadt, Germany) and a Waters millennium v3.02 workstation. The composition of the mobile phase was 0.2 M sodium sulfate adjusted to pH 2.3 with orthophosphoric acid and acetonitrile (76.5:23.5) with a flow rate of 1 mL/min. The sample volume was 50 μL and it was monitored at 214 nm. Standard solutions of human insulin (0.01–1.00 mg/mL) (Fig. 1) were analyzed, and the AUC values were used to construct the standard curve for the estimation of insulin content in the hardening solution. The encapsulation efficiency and drug-loading capacity were calculated as follows:

$$\text{Encapsulation efficiency (EE)} = \frac{\text{insulin added (mg)} - \text{free insulin in supernatant (mg)}}{\text{insulin added (mg)}} \times 100\%$$

$$\text{Drug-loading capacity (DLC)} = \frac{\text{insulin added (mg)} - \text{free insulin in supernatant (mg)}}{\text{polymer used (mg)}} \times 100\%$$

The HPLC method was validated for the limit of detection (5 μg insulin/mL), linearity ($R^2 = 0.992 \pm 0.007$ (mean ± SD; $n = 6$)) and repeatability at 7.5 μg/mL (low), 75 μg/mL (medium) and 750 μg/mL (high concentration) ($n = 6$) for both intra-day and inter-day runs. The precision of the analysis, as measured by the coefficient of variation (CV) and accuracy, was within an acceptable range of less than ±10%. No matrix effects were observed for the two different media (SGF and SIF).

2.2.5. Determination of the degree of surface lectin conjugation

To determine the amount of lectin conjugated to the surface of the microparticles, an indirect HPLC method to measure the unreacted lectin in Section 2.2.3 was adopted. This procedure was performed in parallel to determine the insulin encapsulation efficiency and insulin load described in Section 2.2.4. The HPLC system

and conditions described in Section 2.2.4 were used. Standard solutions of lectin (0.001–1.00 mg/mL) (Fig. 1) were analyzed, and the AUC values were used to construct the standard curve for the estimation of unreacted lectin in the hardening solution (Section 2.2.3). The amount of lectin conjugated to the insulin-loaded microparticles was calculated as follows:

$$\text{Surface-conjugated lectin} = \frac{\text{lectin added (mg)} - \text{free lectin in supernatant (mg)}}{\text{lectin added (mg)}} \times 100\%$$

This HPLC method was validated for the limit of detection (1 μg lectin/mL), linearity ($R^2 = 0.994 \pm 0.003$ (mean ± SD; $n = 6$)) and repeatability at 7.5 μg/mL (low), 75 μg/mL (medium) and 750 μg/mL (high concentration) ($n = 6$) for both intra-day and inter-day runs. The precision of the analysis, as measured by the CV and accuracy, was within an acceptable range of less than ±10%. No matrix effects were observed for the two different media (SGF and SIF).

2.2.6. Mucoadhesive determination

Mucoadhesive determination of surface lectin-conjugated microparticles was performed using the everted sac method (Santos et al., 1999). Male Sprague–Dawley rats (200–270 g) were sacrificed under ether, and intestinal segments were obtained. The intestinal segments were washed with 10 mL of ice-cold phosphate-buffered saline (pH 7.2) containing 200 mg/dL glucose (PBSG). The intestines were cut into 6-cm lengths, everted using a stainless steel rod and lightly washed with PBSG to remove remaining impurities. One end of the intestine was sealed, and 1.5 mL of PBSG was added and finally sealed to form an intestinal sac. The intestinal sac was incubated in 5 mL of PBSG containing 60 mg lectin-functionalized microparticles in a 37 °C water bath and agitated at 100 rpm for 30 min. After incubation, the intestinal sac with bound microparticles on the outer surface was carefully removed, and the PBSG with unbound microparticles was centrifuged at 5000 rpm for 30 min. The supernatant was discarded, and the remaining unbound microparticles were freeze-dried until they reached a constant weight (three days). The mucoadhesiveness of the microparticles was determined by subtracting the initial weight of the microparticles from the weight of the unbound microparticles and expressed as the percent binding.

2.2.7. Microparticle size and surface characteristics determination

The diameters of freshly prepared (wet) and dried microparticles were estimated using a microscope (CX31, Olympus Optical Co., Ltd., Tokyo, Japan) with an eyepiece linear graticule. For the determination of the size and surface characteristics of the lectin-functionalized and non-functionalized microparticles fabricated with the optimized parameters, the dried microparticles were placed on double-sided carbon adhesive tape mounted on an aluminum stab, and they were assessed using a field emission scanning electron microscope (Quanta 200 FESEM, FEI, Oregon, USA) in a low-vacuum mode with 50×, 2000×, 8000× and 50000× magnifications.

2.3. In vitro studies

2.3.1. In vitro insulin release kinetics

The study was performed based on a modified version of a previously reported protocol (Leong et al., 2011). In short, dried insulin-loaded microparticles (200 mg) were placed in 20 mL of simulated gastric fluid (SGF) (pH 1.2) with stirring (100 rpm) at 37 °C for 2 h. Then 1-mL aliquots of the solution were removed at set time intervals and replaced with fresh medium, and the dilution effect was normalized mathematically. After 2 h, the SGF was carefully removed, replaced and incubated with 20 mL of SIF (pH 7.4) with stirring at 37 °C for 8 h. One-milliliter aliquots of the solution were removed at set time intervals and replaced with fresh medium. The level of insulin released from the microparticles into the SGF (pH 1.2) and SIF (pH 7.4) was determined by HPLC, as described in Section 2.2.4. The biological activity of the released insulin at the final 10-h time point was measured using a commercially available human insulin ELISA kit (Mercodia AB, Uppsala, Sweden). Briefly, 25 μL of the samples and insulin standards were added to the appropriate wells of a 96-well microplate in triplicate, followed by the addition of 100 μL of enzyme conjugate and incubation at room temperature for 60 min. The reaction mixtures were then removed from the wells and the microplate was washed six times with washing buffer; 200 μL of tetramethylbenzidine (TMB) substrate was then added and incubated for 15 min. Fifty-microliters of stop solution were added, and the absorbance was measured at 450 nm using a microplate reader (Infinity M200, Tecan, Mannedorf, Switzerland). The insulin concentrations of the samples were obtained from the absorbance readings of the standard concentration curve.

Insulin profiles from the encapsulated microparticles were fitted to the power law equation (Lin & Metters, 2006; Siepmann & Peppas, 2001) to calculate n and determine the insulin release kinetics:

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

where M_t is the amount of insulin released up to a specified time, t; M_∞ is the final amount of insulin 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.

2.3.2. Cell culture

Human colorectal carcinoma cells (Caco-2) from American Type Culture Collection (ATCC) were growth as monolayers in high glucose (4.5 g/L) DMEM supplemented with 1% non-essential amino acids, 2 mM L-glutamine, 10% fetal bovine serum (FBS), 50 μg/mL gentamycin and 2.5 μg/mL amphotericin B. Cells were cultured at 37 °C in a humid atmosphere of 5% CO_2.

2.3.3. Cell viability assays

Caco-2 cells (passage no.: 51–59; 50,000 (MTS assay) or 25,000 (LDH assay) cells per well in 96-well microplates seeded 24 h prior to treatment) were incubated for 1–3 days with microparticles at final concentrations of 0.5–20 mg/mL. Positive controls consisted of 5-fluorouracil (0.005–500 μg/mL) and Triton-X (1%, v/v). Cytotoxicity was evaluated by measurements of the cell viability (growth inhibition) and cell death (cellular membrane damage), using standard MTS and LDH release assays, respectively (Jos et al., 2009). The percent of cell viability and cell death were calculated as follows:

$$\text{cell viability}\,(\%) = \frac{A_{\text{sample}}}{A_{\text{control}}} \times 100\%$$

where A_{sample} is the absorbance measured after treatment with the sample, and A_{control} is the absorbance measured for the untreated cells (negative control).

$$\text{cell death}\,(\%) = \left(\frac{A_{\text{sample}} - A_{\text{spontaneous}}}{A_{\text{Triton-X}} - A_{\text{spontaneous}}}\right) \times 100\%$$

where A_{sample} is the absorbance measured after treatment with sample, $A_{\text{spontaneous}}$ is the absorbance measured without treatment

and $A_{\text{Triton-X}}$ is the absorbance measured after treatment with 1% (v/v) Triton-X.

Investigation of the cellular tight junction integrity was conducted by seeding cells at 280,000 cells per well on a 24-well polycarbonate Transwell filter insert microplate (Costar, Corning Inc., New York, USA), as described previously (Simon, Behrens, Dailey, Wittmar, & Kissel, 2007). Cells were grown on the membrane inserts (0.4 μm pore size) for 21 days. After 21 days, the initial transepithelial electric resistance (TEER) was measured at room temperature with an EVOM™ voltammeter (World Precision Instruments, Berlin, Germany) equipped with Endohm™ electrodes. Microparticles at concentrations of 0.5–20 mg/mL and control (culture medium) were introduced and incubated at 37 °C in a humid atmosphere of 5% CO_2 for various time intervals (0.5, 1, 2, 4, 8 and 16 h). After the incubation period, TEER measurements were taken and changes in the cellular tight junctions were calculated as the percent change relative to the initial resistance value.

2.4. In vivo study

2.4.1. Animals

Male Sprague–Dawley rats (220–270 g) were housed at 20–25 °C and 55 ± 5% relative humidity with a 12-h light–dark cycle. A standard pellet diet and water were provided *ad libitum* during acclimatization. Experimental work was carried out at the Center for Animal Studies, University Malaya Medical Center, Kuala Lumpur, Malaysia, in accordance with institutional guidelines (animal ethics approval reference number: FAR/008/12/2008/CLP(R)).

2.4.2. Glucose-lowering effect and in vivo bioavailability

The procedure described earlier was adopted with minor modifications (Morishita et al., 2006). Diabetes was induced in male Sprague–Dawley rats by intraperitoneal administration of 45 mg/kg of streptozocin in 0.1 M sodium citrate buffer (pH 4.0). After two weeks, rats with fasting blood glucose levels above 300 mg/dL were randomly allocated into nine groups of 6–8 rats in each. The rats were fasted 12 h before and during the experimental period, but water was provided *ad libitum*.

The microparticles were pre-packed in hard gelatin capsules (size 9, Qualicaps® capsule, Shionogi Qualicaps Co., Ltd., Nara, Japan) and administered orally at 25, 50 and 100 IU insulin/kg using a bulb-tipped gavage needle. Non-lectin surface-functionalized microparticles were administered at 50 and 100 IU insulin/kg. Positive controls received subcutaneous injection of 2 IU insulin/kg and the oral administration of insulin solution at 100 IU/kg. Negative controls received oral administration of capsules containing "empty" microparticles or were untreated. Blood was collected from the tail vein immediately before treatment and 0.5, 1, 1.5, 2, 4, 6, 8, 12, 24 and 36 h after administration. Blood glucose levels were measured using an Accu-Check Active blood glucose meter (Roche, Mannheim, Germany). The post-treatment blood glucose levels were expressed as the percent of pre-treatment blood glucose. For quantitative serum insulin determination, the blood samples were centrifuged at 5000 rpm at 4 °C for 10 min, and the resulting serum was stored at −80 °C before analysis. The level of insulin in the serum was then measured using the ELISA method described in Section 2.3. The relative bioavailability of the oral formulations against the subcutaneous administration of insulin was calculated as follows:

$$\text{relative bioavailability (BA)} = \left[\frac{\text{AUC}_{\text{oral}} \times \text{dose}_{\text{sc}}}{\text{AUC}_{\text{sc}} \times \text{dose}_{\text{oral}}}\right] \times 100\%$$

where AUC is the total area under the curve of the serum insulin concentration at time intervals; oral represents oral formulation and sc represents subcutaneous administration.

2.5. Statistical analysis

Results were expressed as the mean ± SD ($n = 6$). Statistical significance was determined by one-way ANOVA followed by the Bonferroni *post hoc* test (GraphPad Prism, version 5.00, San Diego, CA, USA). Differences were considered significant when $p < 0.05$.

3. Results and discussion

3.1. Synthesis and characterization of carboxymethylated kappa-carrageenan

Carboxymethylated *kappa*-carrageenan was synthesized from *kappa*-carrageenan and characterized. Briefly, ^{1}H NMR and ^{13}C NMR spectra of carboxymethylated *kappa*-carrageenan were consistent with our earlier report (Leong et al., 2011). The degree of carboxymethylation was 1.1413 ± 0.0283, the swelling ratios in SGF and SIF were 1.00 ± 0.01 and 1.23 ± 0.01, respectively, which were consistent with the parameters for the optimum formulation (Leong et al., 2011). Size-exclusion liquid chromatography and ion chromatography analyses showed that the molecular weights of native *kappa*-carrageenan and carboxymethylated *kappa*-carrageenan were 840 ± 15 and 762 ± 13 kDa (mean ± SD; $n = 5$), whereas the sulfate contents were 22.0 ± 0.8 and 18.4 ± 0.6% (w/w) (mean ± SD; $n = 3$), respectively. These data suggest that the carboxymethylation conditions adopted caused only minimal scissoring of the carrageenan backbone and reduction of the sulfate groups.

3.2. Preparation of insulin-loaded microparticles

The encapsulation of insulin in microparticles was performed with different quantities of carboxymethylated *kappa*-carrageenan (125–175 mg) and needle sizes (0.4–0.5 mm), using 10 mg of insulin. A carboxymethylated *kappa*-carrageenan weight of 175 mg caused blockage of both the 0.45- and 0.4-mm needles, whereas 150 mg blocked the needle size of 0.4 mm and prevented the formation of microparticles. The highest percentage of insulin encapsulated in microparticles (74.8 ± 1.2%) was obtained from a polymer weight of 175 mg using a 0.5-mm needle (Fig. 2A). At the fixed needle size (0.5 mm), the encapsulation efficiency of insulin decreased with lower amounts of carboxymethylated *kappa*-carrageenan even though the size of the microparticles remained unchanged. However, decreasing the needle size from 0.5 to 0.4 mm for 125 mg of carboxymethylated *kappa*-carrageenan reduced both the encapsulation efficiency and the microparticle size from 1.3 ± 0.2 mm to 0.7 ± 0.1 mm in diameter (Fig. 2B). Therefore, 175 mg of carboxymethylated *kappa*-carrageenan and a needle size of 0.5 mm were used to prepare insulin-loaded microparticles.

A constant stream of air, blowing perpendicular to the tip of the needle during the encapsulation process, produced insulin-loaded microparticles of smaller diameter. For instance, the 0.4-mm needle with a perpendicular air stream in this study produced microparticles of 1.0 ± 0.2 mm diameter. While in studies without the use of a perpendicular air stream across the needle, 2.0 mm (Ramkissoon-Ganorkar, Liu, Baudys, & Kim, 1999) and 2.1 ± 0.1 mm diameter (Martins et al., 2007) microparticles were produced using a smaller 0.2-mm needle. Microparticles of smaller diameter have a larger surface area to volume ratio, and this inevitably increases the contact surface of the microparticles with the intestinal wall, improving the absorption of encapsulated insulin into the systemic circulation. The use of sonication to reduce particle size in other studies gave low encapsulation efficiencies, 69.3 ± 4.5% (Zhang, Ping, Huang, & Xu, 2005) and 40.2 ± 6.3% (Zhang et al.,

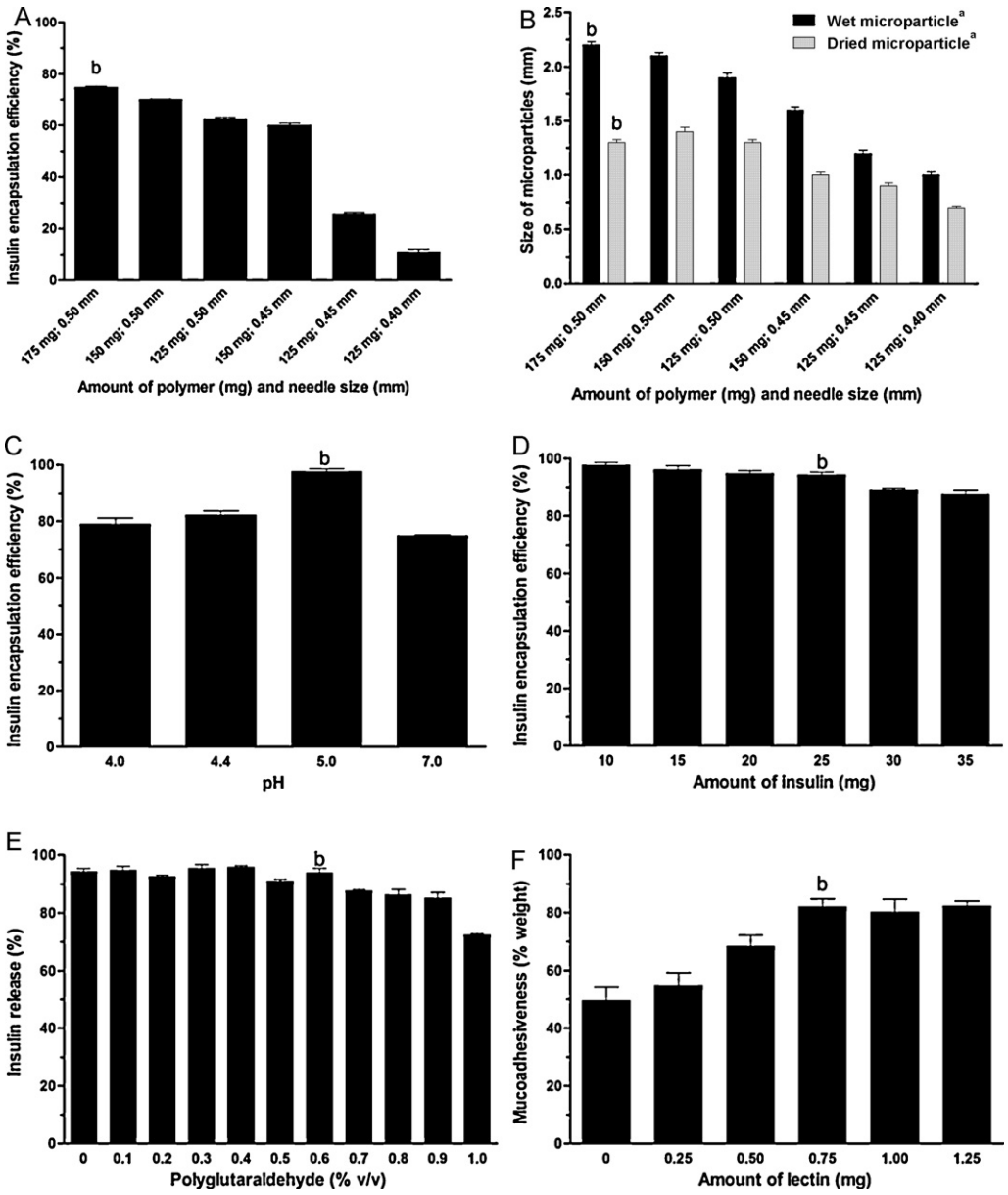

Fig. 2. Preparation and characterization of insulin entrapped in lectin-functionalized carboxymethylated *kappa*-carrageenan microparticles. (A) Encapsulation efficiency of insulin using various polymer weights (125–175 mg) and needle sizes (0.4–0.5 mm). (B) Size of microparticles[a] using various polymer weights (125–175 mg) and needle sizes (0.4–0.5 mm). (C) The effect of pH on the encapsulation efficiency of insulin by the addition of 0.3 M sodium acetate buffer into the insulin–polymer mixture. (D) Encapsulation efficiency of insulin with increasing amounts of drug (10–35 mg). (E) Percentage of insulin release in simulated intestinal fluid (SIF) (pH 7.4) after crosslinking with polyglutaraldehyde (0.1–1.0% v/v). (F) Percentage of microparticles by weight adhered to short segments (6 cm) of rat intestine after lectin functionalization with increasing amount of lectin (0.25–1.25 mg). The results are expressed as the mean ± SD ($n = 6$) except [a]($n = 50$). [b]Parameters selected for the fabrication of lectin-functionalized and non-functionalized microparticles for *in vitro* and *in vivo* studies.

2006). Moreover, the use of harsh encapsulation processes, such as sonication and organic solvents, may degrade the entrapped protein drug. Thus, the ionotropic gelation technique with mild encapsulation conditions performed fully in an aqueous medium was adopted to preserve the biological activity of the insulin drug.

Insulin is composed of 51 amino acid residues with both amino and carboxylic acid side-chains. These side-chains form a net negative or positive charge (isoelectric balance) for the insulin molecule, depending on the pH of the environment. Above pH 5.4, insulin is negatively charged, whereas a net positive charge of 0.93–0.19 occurs at pH 4.8–5.2. At a pH lower than 4.8, insulin has a net positive charge of more than 1 (Wintersteiner & Abramson, 1932). The effect of pH on the encapsulation of insulin by carboxymethylated *kappa*-carrageenan microparticles was studied by adding different volumes of a weak acid buffer (0.3 M sodium acetate buffer) into the carrageenan–insulin mixture. The addition of 0.1 mL of acetate buffer shifted the pH to 5.0 ± 0.1, giving the insulin molecule a net positive charge of less than 1. When 0.5 mL and 1.0 mL of acetate buffer were added, the pH was reduced to 4.4 ± 0.2 and 4.0 ± 0.2, respectively. In these environments, the insulin molecules have a net positive charge of more than 1 (Wintersteiner & Abramson, 1932). The findings showed a significant encapsulation improvement (97.7 ± 2.2%) when insulin has a net positive charge of less than 1 (Fig. 2C), which could be due to the favorable ionic interaction between the positively charged amino groups of insulin and the permanently negatively charged sulfate groups of carrageenan. If the insulin molecule assumed a net positive charge higher than

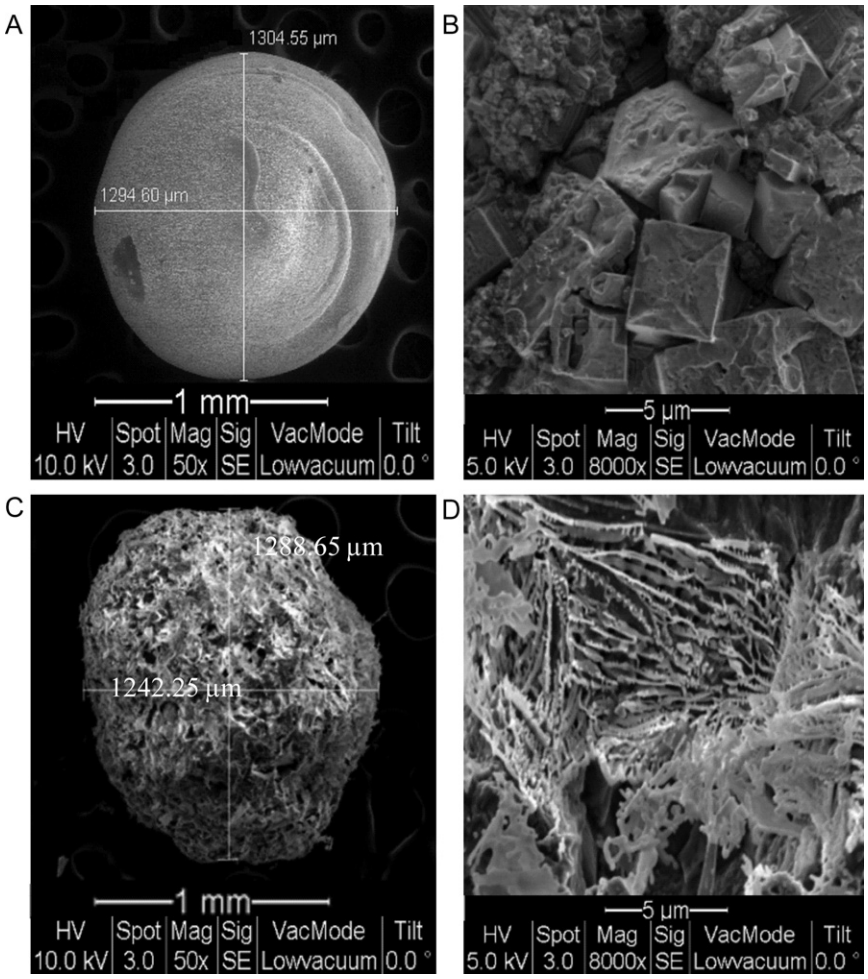

Fig. 3. Scanning electron micrographs (SEM) showing microparticles made from encapsulating insulin in carboxymethylated *kappa*-carrageenan at (A) 50× and (B) 8000× magnifications and lectin-functionalized microparticles made from insulin entrapped in carboxymethylated *kappa*-carrageenan at (C) 50× and (D) 8000× magnifications.

1, then the encapsulation efficiency decreased (78.9 ± 5.2% and 82.2 ± 3.4%) (Fig. 2C).

The drug-loading capacity of the microparticles was investigated by increasing the amount of insulin (10–35 mg) in the insulin–carrageenan mixture. The encapsulation efficiency showed a significant drop when 30 mg of insulin was loaded into the system.

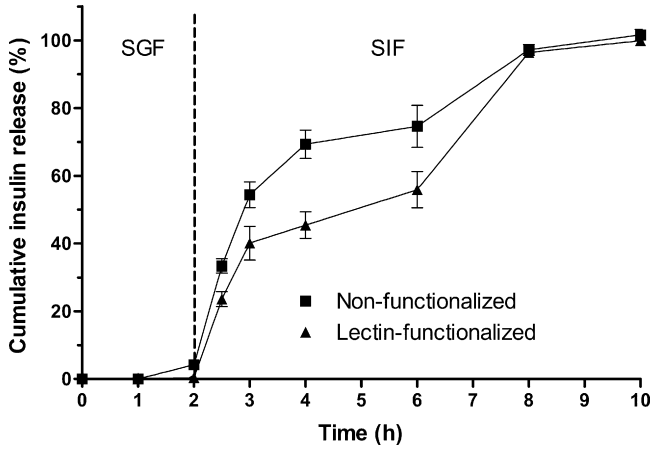

Fig. 4. Dissolution profile of non-functionalized and lectin-functionalized carboxymethylated *kappa*-carrageenan microparticles in simulated gastric fluid (SGF) at 37 °C for 2 h, followed by simulated intestinal fluid (SIF) at 37 °C for 8 h. The results are expressed as the mean ± SD ($n = 6$).

Therefore, 25 mg was selected for the optimal drug load with an encapsulation efficiency of 94.2 ± 2.6%, and a drug-loading capacity of 13.5 ± 0.4% was achieved (Fig. 2D). These high insulin-loaded microparticles possess several advantages, such as a smaller dosage form and the ability to create a high drug-concentration gradient. This high drug-concentration gradient serves as a driving force to assist the absorption of the drug across the intestinal barrier. The findings showed an improved drug load compared to earlier reports on similar needle-based microparticle encapsulation techniques that had insulin-loading capacities of 2.48–3.00% (Martins et al., 2007), 2.00–2.86% (Ramkissoon-Ganorkar et al., 1999) and 1.49–1.68% (Rekha & Sharma, 2009). The smaller microparticles with higher insulin-loading capacity produced in this study showed it is important to adjust the pH of the insulin–carrageenan mixture to create a more compact drug–polymer matrix that can accommodate a higher drug content. Furthermore, the drug-loading capacity showed an increase of 0.2–0.4% upon the incorporation of 0.5% (w/v) dextran sulfate into its formulation (Martins et al., 2007). Such improvements were attributed to the ionic interactions of the negatively charged sulfate and the amino acids of the insulin. The prepared carboxymethylated *kappa*-carrageenan polymer had permanent negatively charged sulfate groups and also showed an improvement in drug-loading capacity. This result further suggests that the presence of sulfate groups may prevent the premature leakage of insulin from this microparticle system.

The lectin surface functionalization of insulin-loaded microparticles was performed using polyglutaraldehyde as the crosslinker

(Tanriseven & Ölçer, 2008). During the activation step, polyglutaraldehyde is induced to selectively react with the hydroxyl groups present on the surface of the carrageenan microparticles (Machado, Lopes, Sousa, & Airoldi, 2009). Between 0.1 and 0.6% (v/v), polyglutaraldehyde showed no significant difference compared to the control in its ability to release insulin (Fig. 2E). However, at concentrations greater than 0.6% (v/v) polyglutaraldehyde, the insulin release decreased. Thus, 0.6% (v/v) polyglutaraldehyde was selected as the optimum concentration for surface activation of the carrageenan microparticles. As the concentrations of lectin increased from 0.25 to 0.75 mg/mL, the percentage weight of the lectin surface-functionalized microparticles bound to rat intestine increased from 54.6 to 82.0% (Fig. 2F). At higher concentrations of lectin, the mucoadhesiveness remained at around 80–82%, whereas non-lectin-functionalized microparticles showed a mucoadhesiveness of 49.6%. Thus, the optimized concentration of lectin for surface functionalization was 0.75 mg/mL.

Non-lectin-functionalized carrageenan microparticles were spherical in shape with an average diameter of $1{,}304 \pm 113\ \mu m$ (mean ± SD; $n = 50$) and a smooth surface at 50× magnification, but they appeared crystalline-like under 8000× magnification (Fig. 3A and B). Lectin-functionalized microparticles were less spherical, with a similar size ($1273 \pm 201\ \mu m$) but with a fibrous surface (Fig. 3C and D). This clearly showed that lectin had been successfully conjugated to the surface of the microparticles and assumed a strain-like fibrous structure, which accounts for the improved adhesion to the intestinal wall.

3.3. *In vitro* studies

3.3.1. *In vitro* insulin release

The *in vitro* release of insulin from non-surface-functionalized and lectin surface-functionalized carboxymethylated *kappa*-carrageenan microparticles was investigated to simulate the transition of microparticles from the stomach to the intestinal region after oral ingestion with 2 h in simulated gastric fluid (SGF) followed by 8 h in simulated intestinal fluid (SIF) (Fig. 4). The release of insulin from non-lectin surface-functionalized microparticles in SGF was minimal (4.2 ± 0.4%) during the first 2 h. Upon transferring to SIF, the insulin was gradually released within 10 h of the studied period. Complete insulin release was observed at 10 h when analyzed using the HPLC method. With lectin surface-functionalized microparticles, the release of insulin was further inhibited in SGF and SIF, and full release was observed at 10 h.

To ascertain the biological activity of the released insulin in SIF (pH 7.4), the samples collected at 10 h were analyzed using ELISA. The results were in good accordance with those measured using HPLC (Fig. 4), giving values of 11.1 ± 5.0% higher for non-lectin surface-functionalized microparticles and 13.6 ± 1.9% higher for lectin surface-functionalized microparticles. These data clearly suggest that the microparticles preserve the biological activity of insulin in the systems tested.

Based on the parameter n calculated by fitting the release data into the power law, the average n value for the non-functionalized microparticles is 0.45 ± 0.06 (mean ± SD; $n = 5$) and 0.36 ± 0.10 for lectin-functionalized microparticles in SIF. For a spherical system, when $n \leq 0.43$, the release mechanism is diffusion-controlled (Case I), whereas when $n \geq 0.85$, the release mechanism is swelling-controlled (Case II) and values between 0.43 and 0.89 present a mixed mode of a both diffusion- and swelling-controlled mechanisms (anomalous transport) (Lin & Metters, 2006; Siepmann & Peppas, 2001). Thus, the release mechanism of insulin from non-functionalized microparticles is a mixed mode but is predominantly diffusion-controlled, whereas for lectin-functionalized microparticles, it is diffusion-controlled.

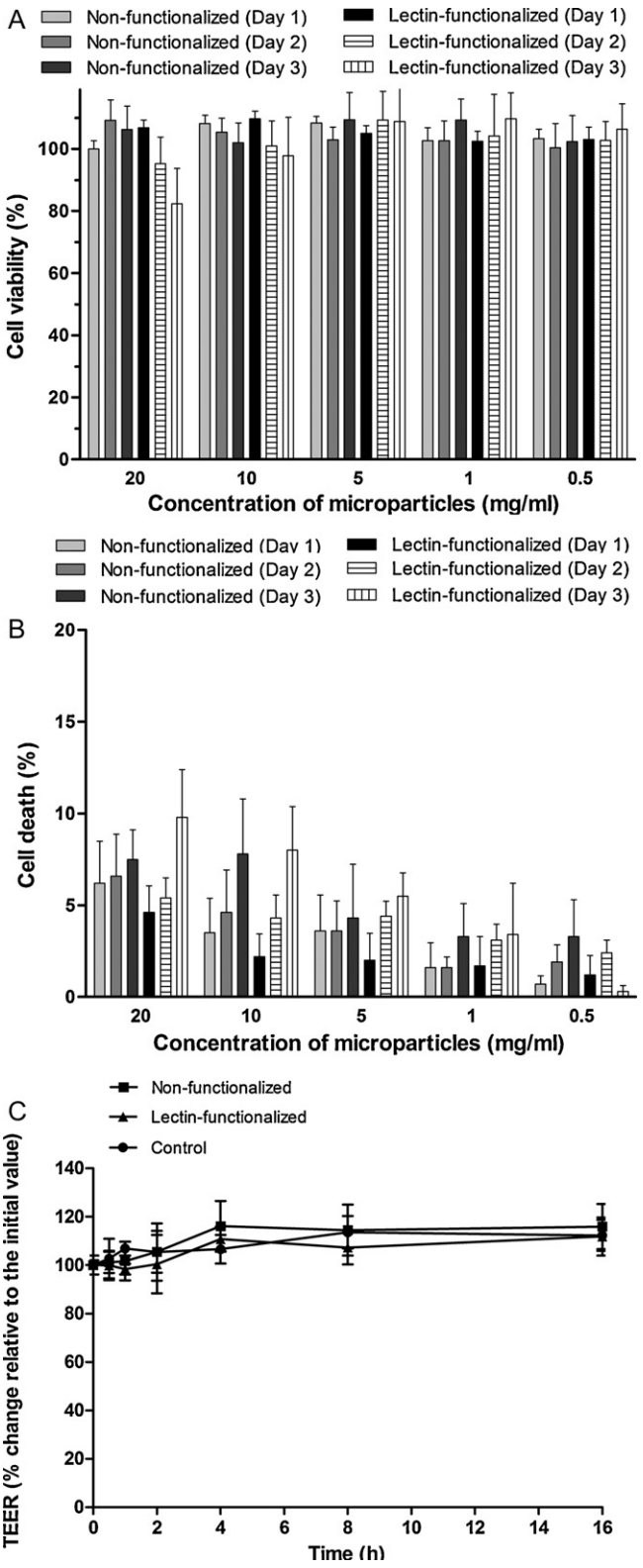

Fig. 5. (A) Cell viability (%) of human colon cells (Caco-2) measured using the MTS assay after exposure to non-functionalized and lectin-functionalized carboxymethylated *kappa*-carrageenan microparticles (0.5–20 mg/mL) over 1, 2 or 3 days. (B) Cell death (%) of Caco-2 cells measured using the LDH assay after exposure to non-functionalized and lectin-functionalized carboxymethylated *kappa*-carrageenan microparticles (0.5–20 mg/mL) over 1, 2 or 3 days. The results are expressed as the mean ± SD ($n = 6$).

Table 1
Pharmacokinetics of various insulin formulations administered orally or subcutaneously to diabetic Sprague–Dawley rats.[a]

Formulations (dose administered)	C_{max} (mIU/L)[c]	T_{max} (h)[c]	$AUC_{0-36\,h}$ (mIU h/L)[c]	Relative bioavailability (%)[c]
Subcutaneous injection (2 IU/kg)	125.5 ± 6.1	1	210.9 ± 4.7	100.0
Lectin-funtionalized microparticles (25 IU/kg)[b]	56.3 ± 3.8[d]	2	376.5 ± 65.5	14.3 ± 1.1[d]
Lectin-funtionalized microparticles (50 IU/kg)[b]	81.5 ± 6.7[d]	2	666.9 ± 178.0[d]	12.8 ± 1.5[d]
Lectin-funtionalized microparticles (100 IU/kg)[b]	175.3 ± 25.4[d]	4	1559.0 ± 174.6[d]	14.8 ± 0.7[d]
Non-funtionalized microparticles (50 IU/kg)[b]	70.6 ± 13.4[d]	2	446.2 ± 26.0[d]	8.5 ± 0.2[d]
Non-funtionalized microparticles (100 IU/kg)[b]	114.1 ± 12.4	4	869.4 ± 22.8[d]	8.3 ± 0.1[d]

[a] The data were obtained from Fig. 6B.
[b] Oral administration.
[c] Each value represents the mean (± SD); $n = 6$.
[d] $p < 0.05$ compared to subcutaneous injection (2 IU/kg).

Notably, the *n* value of fluorescein isothiocyanate (FITC)-labeled dextran (4.4 kDa) (FD-4) encapsulated in the same carboxymethylated *kappa*-carrageenan microspheres in SIF reported in our earlier study was 0.94 ± 0.03 (Leong et al., 2011). This result clearly suggests that the release of FD-4 was purely swelling-controlled, whereas the release of insulin from these microparticles in this study is predominantly under diffusion control. Unlike FD-4, there are probably ionic interactions between the amino groups in the insulin molecule and the sulfate groups of carboxymethylated *kappa*-carrageenan to impart diffusion-controlled insulin release. This phenomenon also explains why the complete release of FD-4 in SIF took 2 h (Leong et al., 2011), whereas for the entrapped insulin in our study, the complete release was extended to 6–8 h. Hence, the presence of sulfate groups in carboxymethylated *kappa*-carrageenan imparts a sustained-release property to entrapped insulin.

3.3.2. Cell viability studies

To investigate the suitability of lectin-functionalized and non-functionalized carboxymethylated *kappa*-carrageenan microparticles for insulin delivery purposes, both MTS and LDH assays were carried out. The assays revealed that the microparticles at 0.5–10 mg/mL did not reduce the viability of Caco-2 cells to a significant level compared to the untreated control ($p > 0.05$) (Fig. 5A and B). Microparticles at 20 mg/mL showed a reduction of cell viability upon exposure to lectin-functionalized microparticles, from 106.8 ± 2.5 at day 1 to 95.3 ± 8.5 at day 2 and finally to 82.3 ± 11.5 at day 3. Other types of lectins showed a similar reduction in cell viability upon longer exposure periods at such high concentrations (Petrossian, Banner, & Oppenheimer, 2007). However, lectins for drug targeting are normally below the microgram range and are unlikely to provoke such effects (Gabor, Bogner, Weissenboeck, & Wirth, 2004). Moreover, the higher drug-loading capacity of our system suggests that less carrier is required to deliver an equivalent amount of drug.

The intestinal membrane in humans provides selective absorption of nutrients and acts as a protective barrier against harmful foreign materials, such as antigens, bacteria, viruses and toxins. The intestinal lining constitutes sheets of cells closely bound together, and a tight junction is located in the intercellular space between cells (González-Mariscal, Nava, & Hernández, 2005). Because the opening of these tight junctions permits the invasion of harmful substances (Khafagy et al., 2007), it is pertinent to determine the effect of the drug carrier system against the integrity of tight junctions. Neither lectin nor non-functionalized microparticles caused the opening of the tight junction, as shown in Fig. 5C. There were no significant differences in the transepithelial electric resistance (TEER) values of the samples compared to the control ($p > 0.05$).

3.4. In vivo studies

Insulin entrapped in lectin-functionalized and non-functionalized carboxymethylated *kappa*-carrageenan microparticles induced significant hypoglycemic effects in diabetic rats (Fig. 6A and B). In contrast, the oral administration of human insulin solution (100 IU/kg), treatment with capsules containing "empty" microparticles and lack of treatment did not induce a significant hypoglycemic effect, and no detectable human insulin was found in the serum. The observed level of the hypoglycemic response of insulin entrapped in microparticles is related to the serum concentration of human insulin absorbed through the intestinal tract (Fig. 6A and B), and it is in turn correlated to the oral dose of insulin entrapped (25, 50 and 100 IU/kg) in the microparticles administered.

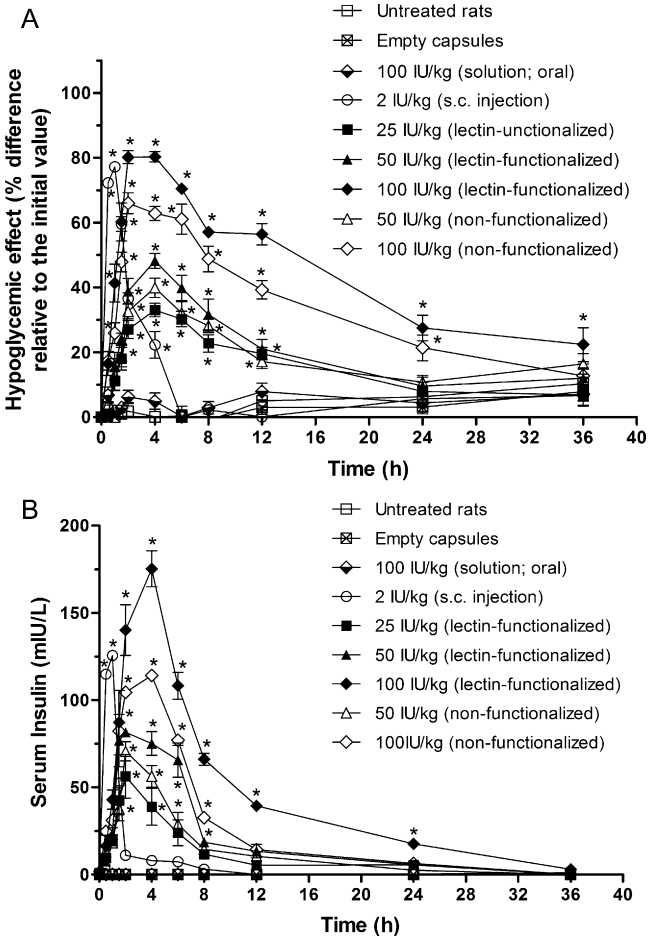

Fig. 6. (A) Hypoglycemic effect induced by various formulations after oral or subcutaneous administration to diabetic Sprague–Dawley rats. The values were calculated as the percent depression of blood glucose compared to the value before the start of the experiment. (B) Serum human insulin levels of diabetic Sprague–Dawley rats after oral or subcutaneous administration of various formulations. The results are expressed as the mean ± SD ($n = 6$). *$p < 0.05$ compared to untreated rats, empty capsules or 100 IU/kg (solution; oral).

Insulin entrapped in lectin surface-functionalized carboxymethylated *kappa*-carrageenan microparticles further increased and prolonged the hypoglycemic effect compared to non-functionalized microparticles containing an equivalent amount of insulin. This finding suggests that the grafting of lectin (wheat germ agglutinin; WGA) on the surface of the microparticles improves the adhesive interactions of these microparticles with the glycoconjugates present on the surface of the intestinal lining (Zhang et al., 2005, 2006). The intimate contact of the microparticles creates a localized high gradient of insulin at the intestinal wall, which assists in the absorption of insulin across the intestinal wall into the systemic circulation.

Table 1 summarizes the pharmacokinetic parameters of orally administered insulin-loaded microparticles and the subcutaneous injection of 2 IU insulin/kg. The area under the serum insulin concentration–time curve (AUC) for all of the formulations was calculated over the experimental period of 36 h. The bioavailability was calculated relative to the subcutaneous injection of 2 IU insulin/kg. The overall bioavailability of the lectin-functionalized microparticles (12.8–14.8%) clearly outperformed that of the non-functionalized microparticles (8.3–8.5%) ($p < 0.05$). The highest bioavailability obtained was $14.8 \pm 0.7\%$ for lectin-functionalized microparticles administered orally at 100 IU/kg, whereas non-functionalized microparticles achieved $8.3 \pm 0.1\%$. The serum insulin bioavailability of lectin-functionalized microparticles at 50 IU/kg in this study (12.8%) is clearly higher than similar lectin-functionalized oral carriers such as liposome-based (9.1%) (Zhang et al., 2005) and lipid-based carriers (7.1%) (Zhang et al., 2006). Currently, oral insulin formulations such as Insugen (HIM2; available commercially), and 4-CNAB and Capsulin (in clinical trials) show rapid insulin action that last no longer than 6–8 h (Clement, Dandona, Still, & Kosutic, 2004; Kapitza et al., 2010; Luzio et al., 2010). While the insulin-loaded microparticles in this study showed prolonged glycemic control that lasted 12–24 h, and hence complement existing insulin formulations to provide for the basal insulin needs of the patient.

4. Conclusion

This study clearly shows that insulin entrapped in lectin-functionalized carboxymethylated *kappa*-carrageenan microparticles was protected from hydrolysis and proteolysis by stomach acids and enzymes. Grafting of lectin (WGA) on the surface of the microparticles improves the interactions of these microparticles with the intestinal wall and enhances the absorption of insulin compared to the non-functionalized microparticles. The covalently bound, negatively charged sulfate groups (18.4%, w/w) in carboxymethylated *kappa*-carrageenan interact with the amino groups of the amino acid residues in insulin via ionic interactions that prevented the bulk release of insulin in the intestine, and these interactions imparted a sustained release of up to 12–24 h for the insulin entrapped in the microparticles in contrast to the rapid dissipation of the hypoglycemic effect of insulin via the parenteral route. Therefore, this lectin-functionalized oral formulation might serve as a promising alternative or as a complementary therapy to parenteral administration to provide better basal and prolonged hypoglycemic control.

Acknowledgements

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