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. The objectives were, therefore, 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, of 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.

Keywords: Carrageenan; Insulin; Lectin; Microparticles; Oral delivery

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 & Panchangnula, 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 physiologically more desirable, because orally administrated insulin mimics the physiological pathway that undergoes the first hepatic bypass and produces a similar effect as 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 such an ionotropic gelation process 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* acidic environment. The enhanced protection is attributed to the ionic interaction between the sulfate groups in the dextran sulfate with the amino acid residues in the insulin molecules (Martins, Sarmento, Souto & Ferreira, 2007). Such a phenomenon was previously noted when protein-polyions complexation reduced the rate of protein escape due to enhanced electrostatic interactions (Kamiya & Klibanov, 2003). Both Tiyaboonchai, Woiszwillo, Sims & Middaugh (2003) and Sarmento, Ribeiro, Veiga, Ferreira & Neufeld (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 M-cells and regular intestinal absorptive cells in the intestine (Yin et al., 2007), and 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 the 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 x 100 mm) were from Merck KGaA (Darmstadt, Germany). Syringes (1 mL) and needles with diameters of 25G (0.50 x 16 mm), 26G (0.45 x 13 mm) and 27G (0.40 x 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

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136 *2.2.1. Synthesis and characterization of carboxymethylated kappa-carrageenan*

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138 Carboxymethylated *kappa*-carrageenan was prepared by a previously described
139 method (Leong et al., 2011). In brief, 5 g of powdered *kappa*-carrageenan was suspended in
140 100 mL of 2-propanol and stirred for 30 min at room temperature. Next, 5 mL of 16 N
141 sodium hydroxide solution was added at a rate of 1 mL per 15 min with continuous stirring at
142 room temperature. Monochloroacetic acid (5.3 g) was then added portionwise to the reaction
143 mixture over a period of 20 min. The reaction mixture was heated to 50 °C with continuous
144 stirring for 4 h to drive the reaction process to completion. The product was recovered
145 through vacuum filtration and washed alternately with 50 mL of ethanol-water (4:1) and 50
146 mL of ethanol three times. The modified carrageenan was oven dried at 70 °C overnight and
147 powdered in a glass mortar.

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

153 Molecular weight was measured using size-exclusion liquid chromatography
154 (Spichtig & Austin, 2008). Briefly, the system consisted of a Waters 2690 solvent delivery
155 module, a Waters 2410 refractive index detector (Waters Co., Milford, MA, USA), two
156 coupled Waters Ultrahydrogel Linear columns (7.8 mm x 300 mm) and a Waters millennium
157 v3.02 workstation. The mobile phase was 0.1 M lithium nitrate with a flow rate of 0.6

158 mL/min. A sample volume of 100 µL was injected into the system at 10 mg/mL. Standard
159 solutions of polyethylene oxide (24.2–932 kDa) (Showa Denko, Kanagawa, Japan) at 10
160 mg/mL were analyzed, and the logarithm of the molecular weight of the standard versus
161 retention time was used to construct a standard curve for the estimation of molecular weight
162 (linearity, $R^2 = 0.993$).
163 Sulfate content was determined using ion chromatography. Briefly, a 1 mg sample
164 was hydrolyzed in 1 mL of 2 M trifluroacetic acid (TFA) for 1 h in a 120 ºC oil bath. The
165 hydrolyzed sample was allowed to cool, and 2 mL of deionized water was added and mixed.
166 It was then centrifuged at 5,000 rpm, and 50 µL of the sample was analyzed using an ICS
167 1600 ion-chromatography system equipped with a conductivity detector (Dionex
168 Corporation, Sunnyvale, CA, USA). The anions were separated on a Waters IC-Pak Anion
169 (4.6 x 150 mm) column (Waters Co., USA) with a mobile phase of borate-gluconate buffer
170 (pH 8.5) and flow rate of 2 mL/min. The sulfate content was expressed as a weight
171 percentage of the analyzed sample (% w/w).

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173 *2.2.2. Preparation of insulin-loaded microparticles*

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175 Insulin-loaded microparticles were prepared using an ionotropic gelation process
176 (Sipahigil & Dortunç, 2001). For this purpose, 125–175 mg powdered carboxymethylated
177 *kappa*-carrageenan was dissolved in 1 mL of pH-adjusted (pH 4.0–7.0) deionized water with
178 0.3 M sodium acetate buffer (pH 4.0). To this solution, 1 mL of human insulin (10–30
179 mg/mL) was added and mixed thoroughly to form a viscous dispersion. The resulting
180 dispersion was then loaded into a 1 mL syringe and extruded dropwise through a needle of

varying 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 dessicator overnight at 4 ºC.

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

204 microparticles were washed with 5 mL of 1.5 M potassium chloride-HCl (pH 1.2) and
205 deionized water (5 mL). The beads were then dried in a dessicator overnight at 4 ºC.

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207 *2.2.4. Determination of insulin encapsulation efficiency and insulin load*

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209 To determine the encapsulation efficiency and the total insulin load of the obtained
210 microparticles from Section 2.2.2, an indirect method to measure the insulin content in the
211 1.5 M potassium chloride-HCl solution (hardening solution) using an established HPLC
212 protocol was performed (Deeb, Preu & Wätzig, 2007). The system consisted of a Waters
213 2690 solvent delivery module, a Waters 996 PDA (Waters Co., Milford, MA, USA), two
214 coupled Chromolith Performance RP-18e columns (4.6 mm x 100 mm, Merck, Darmstadt,
215 Germany) and a Waters millennium v3.02 workstation. The composition of the mobile phase
216 was 0.2 M sodium sulfate adjusted to pH 2.3 with orthophosphoric acid and acetonitrile
217 (76.5:23.5) with a flow rate of 1 mL/min. The sample volume was 50 μL and it was
218 monitored at 214 nm. Standard solutions of human insulin (0.01–1.00 mg/mL) (Fig. 1) were
219 analyzed and the AUC values were used to construct the standard curve for the estimation of
220 insulin content in the hardening solution. The encapsulation efficiency and drug-loading
221 capacity were calculated as follows:

222

223 Encapsulation efficiency (EE) = {[Insulin added (mg) – Free insulin in supernatant (mg)] /
224 Insulin added (mg)} x 100%

225

Drug-loading capacity (DLC) = {[Insulin added (mg) – Free insulin in supernatant (mg)] / Polymer used (mg)} x 100%

The HPLC method was validated for limit of detection (5 µg insulin/mL), linearity (R^2 = 0.992 ± 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 intraday and interday 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%. There were no matrix effects of the different media (SGF and SIF) employed.

2.2.5. Determination of 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 the determination of 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 (0.005–1.00 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:

Surface conjugated lectin = {[Lectin added (mg) – Free lectin in supernatant (mg)] / Lectin added (mg)} x 100%

This HPLC method was validated for the limit of detection (1 µg lectin/mL), linearity (R^2 = 0.994 ± 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 intraday and interday 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%. There were no matrix effects of the different media (SGF and SIF) employed.

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 buffer 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 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, 2,000, 8,000 and 50,000x 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 was 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 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 was 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 into the Power law equation (Lin & Metters, 2006; Siepmann & Peppas, 2001) to calculate *n* and determine the insulin release kinetics:

$M_t / M_\infty = kt^n$

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. Statistical analyses were carried out using Student's paired *t*-test, where $p < 0.05$ was selected as the criterion of significance.

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) Dulbecco's modified Eagle's medium (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 ºC 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 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:

$$\text{Cell viability (\%)} = A_{sample} / A_{control} \times 100\%$$

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).

$$\text{Cell death (\%)} = (A_{sample} - A_{spontaneous} / A_{Triton\text{-}X} - A_{spontaneous}) \times 100\%$$

where A_{sample} is the absorbance measured after treatment with sample, $A_{spontaneous}$ is the absorbance measured without treatment and $A_{Triton\text{-}X}$ 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 et al., 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 ºC in a humid atmosphere of 5% CO_2 for various time intervals (0.5, 1, 2, 4, 8 and 16 h). After the incubation periods, 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-hour 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 percentage 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 ºC 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:

$$\text{Relative bioavailability (BA)} = [AUC_{(oral)} \times Dose_{(sc)} / AUC_{(sc)} \times Dose_{(oral)}] \times 100\%$$

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, California, 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 in 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 the microparticles could not be formed. 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.

Insulin is composed of 51 amino acid residues with both amino and carboxylic acid side groups. These groups 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

442 negatively charged, whereas a net positive charge of 0.93 to 0.19 occurs at pH 4.8–5.2. At a
443 pH lower than 4.8, insulin has a net positive charge of more than 1 (Wintersteiner &
444 Abramson, 1932). The effect of pH on the encapsulation of insulin by carboxymethylated
445 *kappa*-carrageenan microparticles was studied by adding different volumes of a weak acid
446 buffer (0.3 M sodium acetate buffer) into the carrageenan-insulin mixture. The addition of 0.1
447 mL of acetate buffer shifted the pH to 5.0 ± 0.1, giving the insulin molecule a net positive
448 charge of less than 1. When 0.5 mL and 1.0 mL of acetate buffer were added, the pH was
449 reduced to 4.4 ± 0.2 and 4.0 ± 0.2, respectively. In these environments, the insulin molecules
450 have a net positive charge of more than 1 (Wintersteiner & Abramson, 1932). The findings
451 showed a significant encapsulation improvement (97.7 ± 2.2%) when insulin has a net
452 positive charge of less than 1 (Fig. 2C), which could be due to the favorable ionic interaction
453 between the positively charged amino groups of insulin and the permanently negatively
454 charged sulfate groups of carrageenan. If the insulin molecule assumed a net positive charge
455 higher than 1, then the encapsulation efficiency decreased (78.9 ± 5.2% and 82.2 ± 3.4%)
456 (Fig. 2C).

457 The drug-loading capacity of the microparticles was investigated by increasing the
458 amount of insulin (10–35 mg) in the insulin-carrageenan mixture. The encapsulation
459 efficiency showed a significant drop when 30 mg of insulin was loaded into the system.
460 Therefore, 25 mg was selected for the optimal drug load with an encapsulation efficiency of
461 94.2 ± 2.6%, and a drug-loading capacity of 13.5 ± 0.4% was achieved (Fig. 2D). These high
462 insulin-loaded microparticles possess several advantages, such as a smaller dosage form and
463 the ability to create a high drug-concentration gradient. This high drug-concentration gradient
464 serves as a driving force to assist the absorption of the drug across the intestinal barrier. The
465 findings showed an improved drug load compared to earlier reports on similar needle-based

466 microparticle encapsulation techniques that had insulin-loading capacities of 2.48–3.00%
467 (Martins et. al., 2007), 2.00–2.86% (Ramkissoon-Ganorkar, Liu, Baudys & Kim, 1999) and
468 1.49–1.68% (Rekha & Sharma, 2009). Notably, the drug-loading capacity showed an increase
469 of 0.2–0.4% upon the incorporation of 0.5% (w/v) dextran sulfate into its formulation
470 (Martins, Sarmento, Souto & Ferreira, 2007). Such improvements were attributed to the ionic
471 interactions of the negatively charged sulfate and the amino acids of the insulin. The
472 prepared carboxymethylated *kappa*-carrageenan polymer had permanent negatively charged
473 sulfate groups and also showed an improvement on drug-loading capacity. This result further
474 suggests that the presence of sulfate groups may prevent the premature leakage of insulin
475 from this microparticle system.

476 The lectin surface functionalization of insulin-loaded microparticles was performed
477 using polyglutaraldehyde as the crosslinker (Tanriseven & Ölçer, 2008). During the
478 activation step, polyglutaraldehyde is induced to selectively react with hydroxyl groups
479 present on the surface of the carrageenan microparticles (Machado, Lopes, Sousa & Airoldi,
480 2009). Between 0.1 to 0.6% (v/v), polyglutaraldehyde showed no significant difference
481 compared to the control in its ability to release insulin (Fig. 2E). However, at concentrations
482 greater than 0.6% (v/v) polyglutaraldehyde, the insulin release decreased. Thus, 0.6% (v/v)
483 polyglutaraldehyde was selected as the optimum concentration for surface activation of the
484 carrageenan microparticles. As the concentrations of lectin increased from 0.25 to 0.75
485 mg/mL, the percentage weight of the lectin surface-functionalized microparticles bound to rat
486 intestine increased from 54.6 to 82.0% (Fig. 2F). At higher concentrations of lectin, the
487 mucoadhesiveness remained at around 80–82%, whereas non-lectin-functionalized
488 microparticles showed a mucoadhesiveness of 49.6%. Thus, the optimized concentration of
489 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 ± 113 μm (mean ± SD, n = 50) and a smooth surface at 50x magnification, but they appeared crystalline-like under 8000x magnification (Fig. 3A & B). Lectin-functionalized microparticles were less spherical, with a similar size (1,273 ± 201 μm) but with a fibrous surface (Fig 3C & 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 rapidly 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 mechanism (anomalous transport) (Lin & Metters, 2006; Siepmann & Peppas, 2001). Thus, the release mechanism of insulin from non-functionalized microparticles is of a mixed mode but is predominantly diffusion-controlled, whereas for lectin-functionalized microparticles, it is diffusion-controlled.

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 (Figs. 5A & 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 of 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 a selective absorption of nutrients and acts as a protective barrier against harmful foreign materials such as antigens, bacteria, viruses and toxins. The intestine 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, Morishita, Onuki & Takayama, 2007), it is pertinent to

559 determine the effect of the drug carrier system against the integrity of tight junctions. Neither
560 lectin nor non-functionalized microparticles caused the opening of the tight junction, as
561 shown in Fig. 5C. There were no significant differences in the transepithelial electric
562 resistance (TEER) values of the samples, as compared to control (ANOVA, $p > 0.05$).

563

564 *3.4. In vivo studies*

565

566 Insulin entrapped in lectin-functionalized and non-functionalized carboxymethylated
567 *kappa*-carrageenan microparticles induced significant hypoglycemic effects on diabetic rats
568 (Figs. 6A & B). In contrast, the oral administration of human insulin (100 IU/mL), treatment
569 with capsules containing "empty" microparticles and lack of treatment did not induce a
570 significant hypoglycemic effect, and no detectable human insulin was found in the serum.
571 The observed level of the hypoglycemic response of insulin entrapped in microparticles is
572 related to the serum concentration of human insulin absorbed through the intestinal tract
573 (Figs. 6A & B), and is in turn correlated to the oral dose of insulin entrapped (25, 50 and 100
574 IU/mL) in the microparticles administered.
575 Insulin entrapped in lectin surface-functionalized carboxymethylated *kappa*-
576 carrageenan microparticles further increased and prolonged the hypoglycemic effect
577 compared to non-functionalized microparticles containing an equivalent amount of insulin.
578 This finding suggests that the grafting of lectin (wheat germ agglutinin; WGA) on the surface
579 of the microparticles improves the adhesive interactions of these microparticles with the
580 glycoconjugates present on the surface of the intestinal lining (Zhang, Ping, Huang & Xu,
581 2005; Zhang et al., 2006). The intimate contact of the microparticles creates a localized high

gradient of insulin at the intestinal wall, which assists 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 plasma 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%). The highest bioavailability obtained was 14.8 ± 0.7% for lectin-functionalized microparticles administered orally at 100 IU/kg, whereas non-functionalized microparticles achieved 8.3 ± 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, Ping, Huang & Xu, 2005) and lipid-based carriers (7.1%) (Zhang et al., 2006).

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 compared 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 a better basal and prolonged hypoglycemic control.

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739
740

741

742 **Table Caption**

743

744 **Table 1**

745 Pharmacokinetics of various insulin formulations administered orally or subcutaneously to

746 diabetic Sprague-Dawley rats[a].

747

748

749 **Figure Captions**

750

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

752

753 **Fig. 2.** Preparation and characterization of insulin entrapped in lectin-functionalized

754 carboxymethylated *kappa*-carrageenan microparticles. (**A**) Encapsulation efficiency of insulin

755 using various polymer weights (125–175 mg) and needle sizes (0.4–0.5 mm). (**B**) Size of

756 microparticles[a] using various polymer weights (125–175 mg) and needle sizes (0.4–0.5 mm).

757 (**C**) The effect of pH on the encapsulation efficiency of insulin by the addition of 0.3 M

758 sodium acetate buffer into the insulin-polymer mixture. (**D**) Encapsulation efficiency of

759 insulin with increasing amounts of drug (10–35 mg). (**E**) Percentage of insulin release in

760 simulated intestinal fluid (SIF) (pH 7.4) after crosslinking with polyglutaraldehyde (0.1–1.0%

761 v/v). (**F**) Percentage of microparticles by weight adhered to short segments (6 cm) of rat

762 intestine after lectin functionalization with increasing amount of lectin (0.25–1.25 mg). The

763 results are expressed as the mean ± SD (n = 6) except [a](n = 50). [b]Parameters selected for the

764 fabrication of lectin-functionalized and non-functionalized microparticles for *in vitro* and *in*
765 *vivo* studies.
766
767 **Fig. 3.** Scanning electron micrographs (SEM) showing microparticles made from
768 encapsulating insulin in carboxymethylated *kappa*-carrageenan at (**A**) 50x and (**B**) 8000x
769 magnifications and lectin-functionalized microparticles made from insulin entrapped in
770 carboxymethylated *kappa*-carrageenan at (**C**) 50x and (**D**) 8000x magnifications.
771
772 **Fig. 4.** Dissolution profile of non-functionalized and lectin-functionalized carboxymethylated
773 *kappa*-carrageenan microparticles in simulated gastric fluid (SGF) at 37 ºC for 2 h, followed
774 by simulated intestinal fluid (SIF) at 37 ºC for 8 h. The results were expressed as the mean ±
775 SD (n = 6).
776
777 **Fig. 5.** (**A**) Cell viability (%) of human colon cells (Caco-2) measured using the MTS assay
778 after exposure to non-functionalized and lectin-functionalized carboxymethylated *kappa*-
779 carrageenan microparticles (0.5–20 mg/mL) over 1, 2 or 3 days. (**B**) Cell death (%) of Caco-2
780 cells measured using the LDH assay after exposure to non-functionalized and lectin-
781 functionalized carboxymethylated *kappa*-carrageenan microparticles (0.5–20 mg/mL) over 1,
782 2 or 3 days. The results were expressed as the mean ± SD (n = 6).
783
784 **Fig. 6.** (**A**) Hypoglycemia effect induced by various formulations after oral or subcutaneous
785 administration to diabetic Sprague-Dawley rats. The values were calculated as the percent
786 depression of blood glucose compared to the value before the start of the experiment. (**B**)
787 Serum human insulin levels of diabetic Sprague-Dawley rats after oral or subcutaneous
788 administration of various formulations. The results were 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]	$AUC_{0\text{-}36h}$ (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	2	376.5 ± 65.5	14.3 ± 1.1
Lectin-funtionalized microparticles (50 IU/kg)[b]	81.5 ± 6.7	2	666.9 ± 178.0	12.8 ± 1.5
Lectin-funtionalized microparticles (100 IU/kg)[b]	175.3 ± 25.4	4	1559.0 ± 174.6	14.8 ± 0.7
Non-funtionalized microparticles (50 IU/kg)[b]	70.6 ± 13.4	2	446.2 ± 26.0	8.5 ± 0.2
Non-funtionalized microparticles (100 IU/kg)[b]	114.1 ± 12.4	4	869.4 ± 22.8	8.3 ± 0.1

[a] The data were obtained from Fig. 6B.

[b] Oral administration.

[c] Each value represents the mean (± SD); $n = 6$.

Figure 1
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Fig. 1

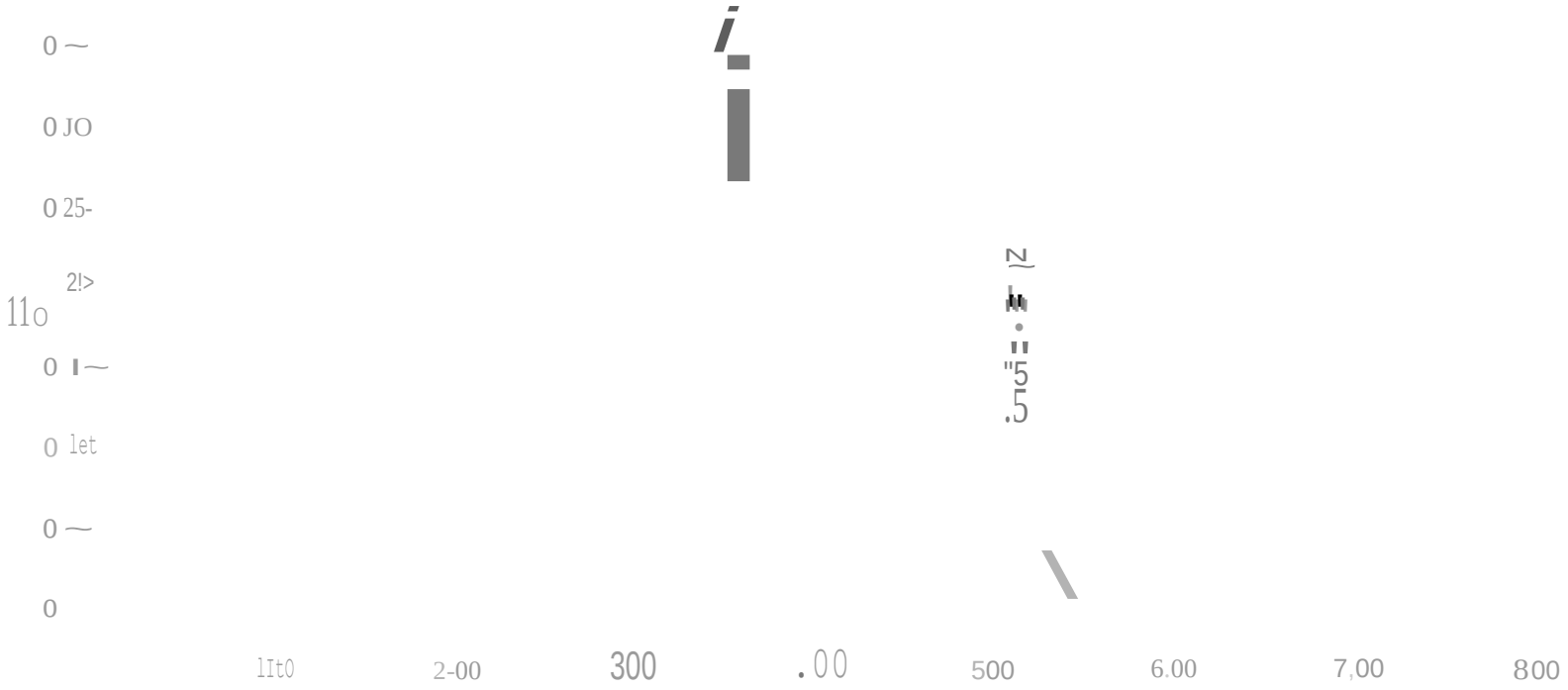

Figure 2A-F
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Fig. 2

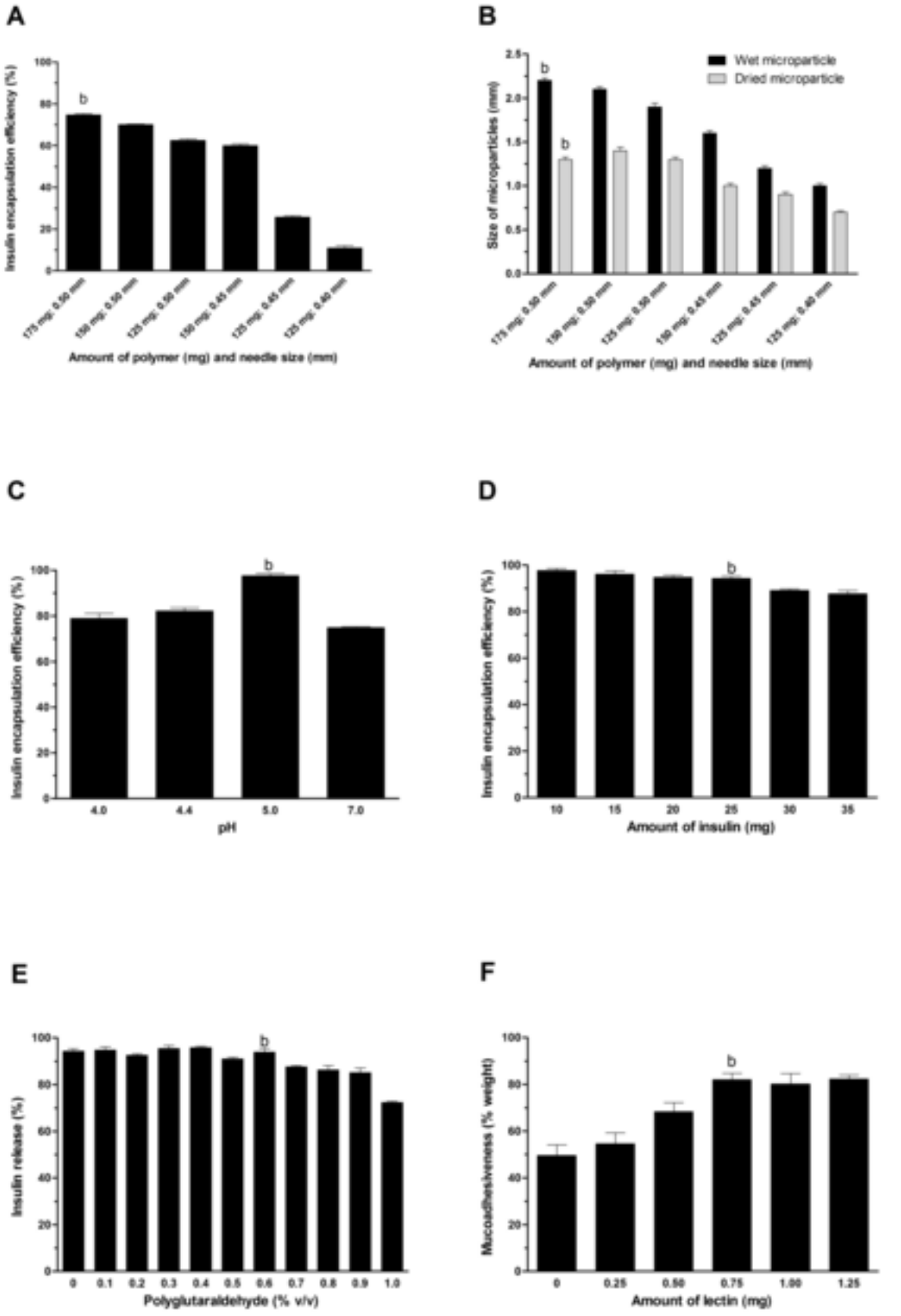

Figure 3A-D
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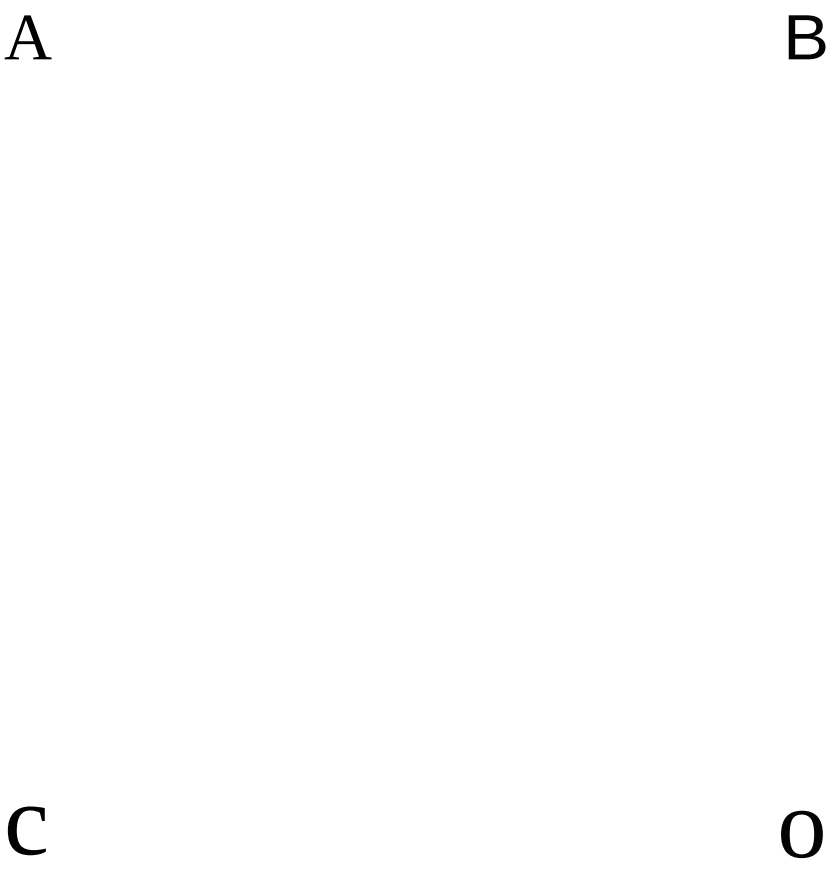

Fig. 3

Figure 4
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Fig.4

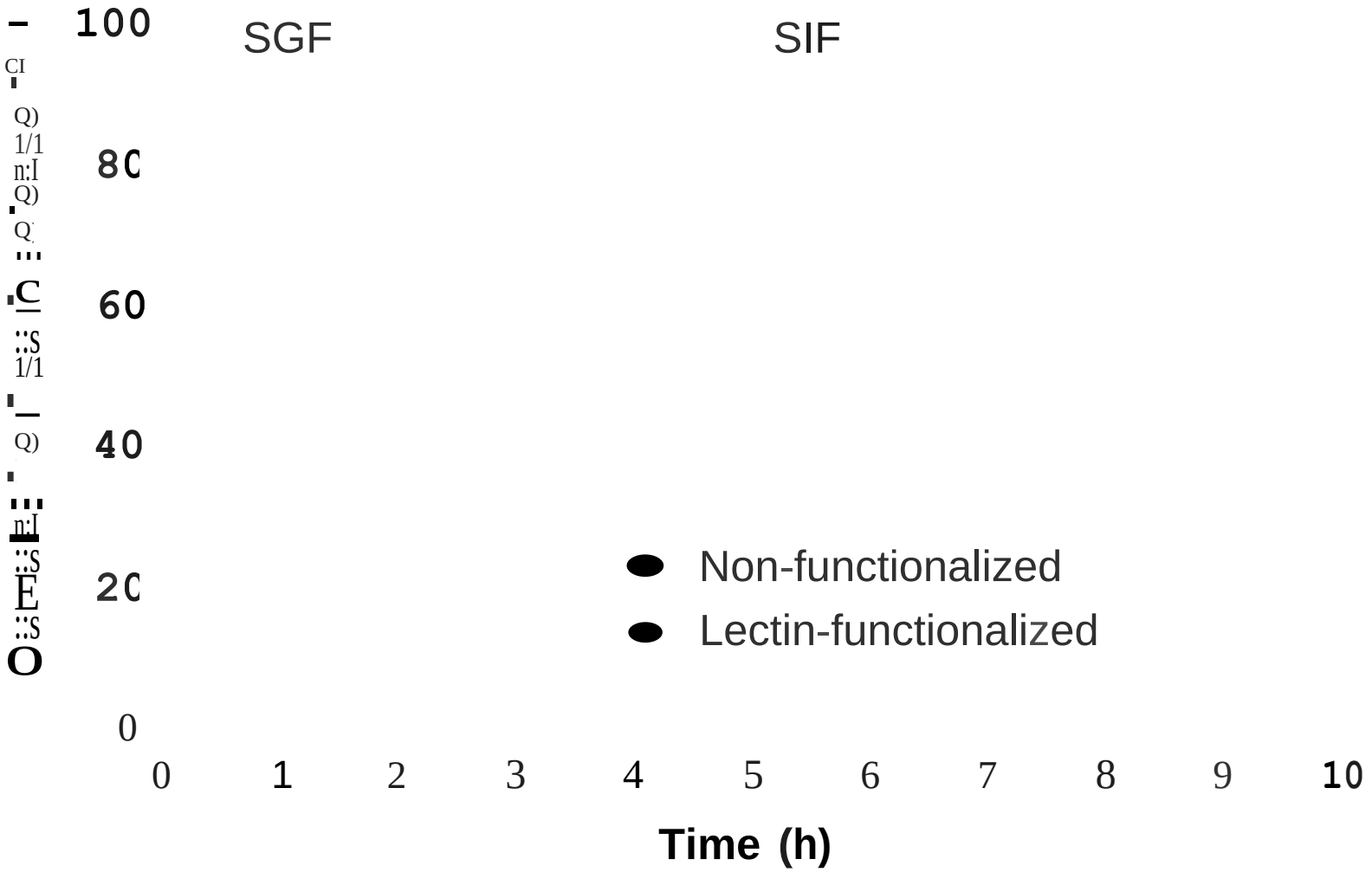

Figure 5A-C
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Fig. 5

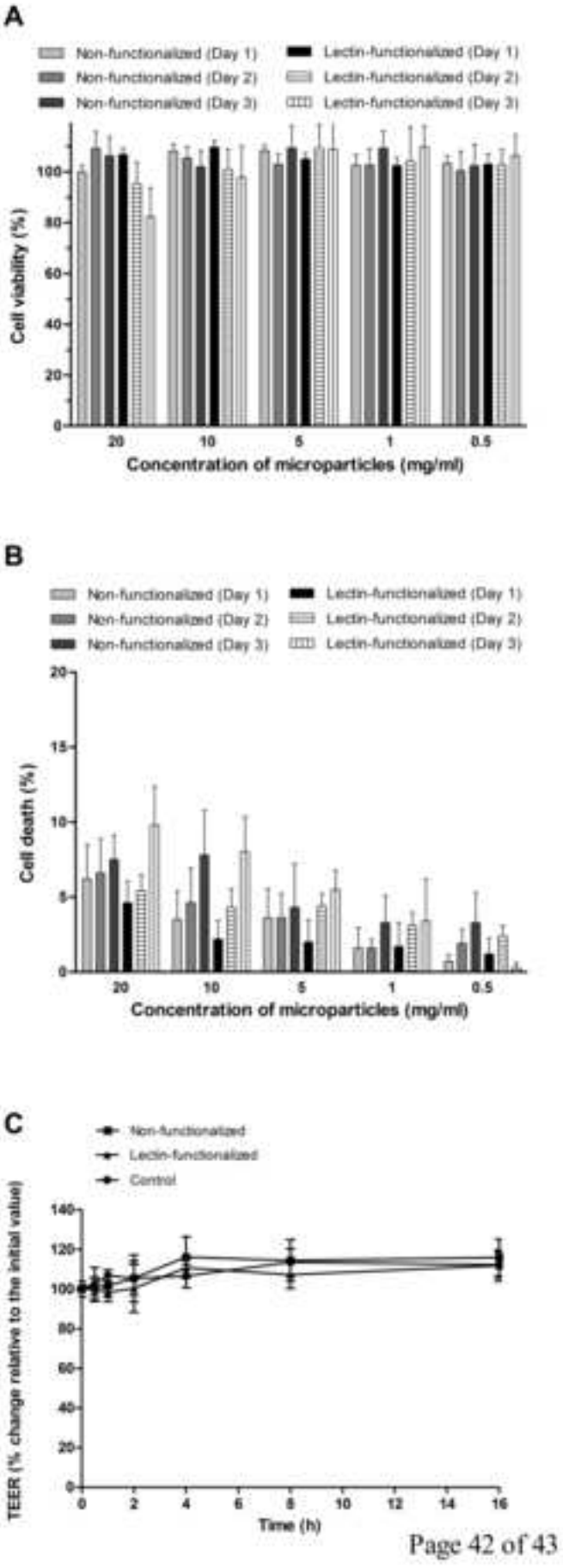

Figure 6A & B
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Fig. 6