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Systematic investigation of fabrication conditions of nanocarrier based on carboxymethyl chitosan for sustained release of insulin

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ABSTRACT

pH-responsive nanoparticles (NPs) comprised of degradable carboxymethyl chitosan (CMCS) crosslinked with $CaCl_2$ were simply prepared *via* ionic gelation. Fabrication conditions including insulin dosage, CMCS concentration, and crosslinking density were systematically investigated for insulin loading and release *in vitro*. The encapsulation efficiencies (EE), loading capacity (LC) and average size of the NPs decreased with the increasing insulin concentrations (<0.192 mg/mL), while they notably increased as the insulin dosage was above 0.192 mg/mL. When the concentration of CMCS increased from 0.5 to 2.0 mg/mL, the EE of the NPs reduced while the size of the NPs increased. We further demonstrated that crosslinking density offered a simple method for tuning the properties of the NPs towards various insulin concentrations. The mass ratio 10:5 of CMCS to $CaCl_2$ exhibited the optimal performance at higher insulin concentration, whereas a higher crosslinking density of 10:7 (m:m) gave the optimal performance at low insulin concentration. The cumulative release of insulin from insulin loaded NPs decreased with the elevating crosslinking density. These findings not only provided a better understanding of the synthesis of CMCS NPs but also contributed to the practical applications of insulin loading and release.

1. Introduction

Insulin is the most commonly used and effective drug for controlling plasma glucose levels. Insulin therapy is generally delayed despite the dire consequences, and partly due to the inconvenience and complications associated with insulin administration by injection [1]. A recent study showed that over half of users reported intentionally skipping insulin injections at some point, and 20% reported regularly skipping injections, leading to a dangerous lapse in treatment [2]. Hence, in the last decade, the research focus has shifted from the development of insulin alternatives to the pursuing of alternative delivery methods [3–6]. The use of biocompatible and biodegradable nanoparticles has been described as a promising strategy towards oral administration of proteins and peptides[7].

Chitosan is a linear polysaccharide composed of randomly distributed β-(1–4)-linked D-glucosamine and N-acetyl-D-glucosamine and produced by the alkaline deacetylation of chitin [8–10]. Chitosan and its derivatives possess excellent biocompatibility, biodegradability, non-toxic and hydrophility, which render them promising candidates of oral insulin delivery [11,12]. Carboxymethyl chitosan (CMCS) has carboxymethyl groups on hydroxyl sites of the glucosamine units of the chitosan structure, which has desirable water-solubility besides the advantageous biological properties of chitosan. CMCS based nanoparticles (NPs) have been extensively studied for delivery of anti-cancer agents, therapeutic proteins, genes and antigens [13–16]. It has been reported that carboxymethyl chitin NPs could be easily formed with Ca^{2+} through simple ionic gelification [17], and the carboxyl groups of CMCS make its own similar capacity of binding Ca^{2+} [18]. Moreover, many studies have emphasized the significance of concentration of chitosan in the NPs loading capacity [8,19,20].

We have previously demonstrated that CMCS NPs with negative surface charges could improve insulin delivery capability, which exhibited a higher mucoadhesion in small intestine *in ex vivo* studies, and could regulate the blood glucose level more effectively, compared to the chitosan NPs with positive surface charges

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Table 1
Formulation of synthetic CMCS NPs sat different conditions.

Sample	CMCS (mg/mL)	CMCS:$CaCl_2$ (m:m)
1	2	10:5
2	1	10:5
3	0.75	10:5
4	0.5	10:5
5	0.5	10:1
6	0.5	10:3
7	0.5	10:7

[21,22]. In this work, we extend the previous work to systematically study the effects of fabrication conditions of CMCS NPs, including insulin dosage, CMCS concentration, and crosslinking density, on the insulin loading capacity. The encapsulation efficiencies (EE), loading capacity (LC), size, surface charge and release profile of these NPs were evaluated. Furthermore, the effect of preparation conditions on insulin release under different pH conditions was also comprehensively studied.

2. Materials and methods

2.1. Materials

CMCS (weight-average molecular weight (MW): 190 kDa, degree of deacetylation (DD): 85.31%, degree of substitution (DS) of carboxymethyl: 0.95) was obtained from our laboratory using the method described by Chen [23,24] previously. Insulin was purchased from Sigma (St. Louis, USA). All other reagents and solvents were of analytical grade.

2.2. Preparation of CMCS nanoparticles (NPs)

CMCS NPs were synthesized by a simple ionic gelation method with calcium chloride ($CaCl_2$) solution [25]. In brief, CMCS was dissolved in triple distilled water at predetermined concentration, and insulin was dissolved in hydrochloric acid solution (pH 2). 1 mL of insulin solution was added into 8 mL of CMCS solution dropwise under constant stirring. Subsequently, 1 mL $CaCl_2$ solution was added into the mixture dropwise under constant stirring for 1 h until NPs formation. The same procedure was applied to the preparation of NPs at different concentrations of CMCS and mass ratio of CMCS to $CaCl_2$ was listed in Table 1.

2.3. Size and surface charge characterizations of the NPs

The size and surface charge of blank NPs and insulin loaded NPs were characterized and measured by dynamic light scattering (DLS) with a Malvern Zetasize Nano ZS 90 (Malvern Instruments Ltd., Malvern, UK) at a detector angle of 90, 670 nm, and 25 °C. The samples were prepared with deionized water at appropriate concentrations. All measurements were performed in triplicate.

2.4. Transmission electron microscopy (TEM)

The shapes of the insulin free and insulin loaded NPs were observed by transmission electron microscope (TEM). The CMCS NPs suspensions were stained by phosphotungstic acid, placed onto copper grid and dried at room temperature, then observed by TEM (100 CX II, Japan).

2.5. Encapsulation efficiencies (EE) and loading capacity (LC) of insulin

The EE refers to the amount of insulin associated with formed NPs, expressed as the percentage of the total amount of insulin added in the process, while LC is defined as the percentage by the weight of the associated insulin in the resultant NPs. To determine these parameters, NPs were separated by centrifugation (15,000 rmp/min, 30 min and 4 °C) from the aqueous preparation medium containing the free insulin. And the free insulin content was estimated by BCA method at 562 nm in a microplate reader (MCC340, Multiskan, Belgium) [26]. The EE and LC were calculated using the following formulas [27]:

$$\mathrm{EE}(\%) = ((\text{total amount of insulin added-free insulin})/(\text{total amount of insulin added})) \times 100\%$$

$$\mathrm{LE}(\%) = ((\text{total amount of insulin added-free insulin})/(\text{weight of nanoparticles})) \times 100\%$$

2.6. Cytotoxicity

The cytotoxicity of the NPs was evaluated using MTT assay conducted with colon adenocarcinoma (Caco 2) cell line and L929 cell line, which were obtained from the American Type Culture Collection (Manassas, USA, between 25 and 35 passages). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; high glucose) and supplemented with 10% (v/v) fetal bovine serum and penicillin/streptomycin at 37 °C in 5% CO_2. Briefly, cells in the logarithmic growth phase were added to 96-well culture plates of 5×10^4 cells/mL at 100 μL per well and incubated overnight. The culture medium was then replaced by the serum-free medium with various concentrations (500 μg/mL, 250 μg/mL, 125 μg/mL, 62.5 μg/mL and 31.25 μg/mL) of insulin loaded CMCS NPs, and MTT assays were performed at 24 h, 48 h and 72 h after the addition of the NPs dilutions. Cells without the NPs treatment were as control. Cell viability (%) was calculated as follows[28]:

$$\text{cell viability}\,(\%) = \left(\mathrm{OD_{test}}/\mathrm{OD_{control}}\right) \times 100\%$$

Where OD_{test} and $OD_{control}$ are the absorbance of the tested sample and the control sample.

2.7. In vitro drug release

Insulin released from NPs was analyzed by incubating NPs in PBS buffer solutions at pH 1.2 and pH 6.8 media. NPs suspension (5 mL) was placed into a cellulose membrane dialysis tube (MW cutoff 8000–14,000). The dialysis tube was placed in 45 mL of PBS at pH 1.2 and pH 6.8 at 37 °C while shaking (100 rpm), respectively. At specific time intervals (between 0 h and 4 h), 1 mL medium was removed and replaced by 1 mL fresh release medium. The amount of insulin released in the medium was determined by BCA method at 562 nm in a microplate reader. And a calibration curve was generated using blank NPs to correct for the intrinsic absorption of the NPs.

2.8. Statistical analysis

All the results were expressed as mean ± SD, n = 3. The significance level was determined by one-way ANOVA following Tukey'spost hoc test. $p < 0.05$ was considered as significant.