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Noninvasive imaging oral absorption of insulin delivered by nanoparticles and its stimulated glucose utilization in controlling postprandial hyperglycemia during OGTT in diabetic rats☆

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ABSTRACT

This work examined the feasibility of preparing a pH-responsive nanoparticle (NP) system composed of chitosan and poly(γ-glutamic acid) conjugated with ethylene glycol tetraacetic acid (γPGA–EGTA) for oral insulin delivery in diabetic rats during an oral glucose tolerance test (OGTT). OGTT has been used largely as a model to mimic the period that comprises and follows a meal, which is often associated with postprandial hyperglycemia. Based on Förster resonance energy transfer (FRET), this work also demonstrated the ability of γPGA–EGTA to protect insulin from an intestinal proteolytic attack in living rats, owing to its ability to deprive the environmental calcium. Additionally, EGTA-conjugated NPs were effective in disrupting the epithelial tight junctions, consequently facilitating the paracellular permeation of insulin throughout the entire small intestine. Moreover, results of positron emission tomography and computer tomography demonstrated the effective absorption of the permeated insulin into the systemic circulation as well as promotion of the glucose utilization in the myocardium, and skeletal muscles of the chest wall, forelimbs and hindlimbs, resulting in a significant glucose-lowering effect. Above results indicate that as-prepared EGTA-conjugated NPs are a promising oral insulin delivery system to control postprandial hyperglycemia and thus may potentially prevent the related diabetic complications.

1. Introduction

Blood glucose levels in diabetic patients often increase promptly and significantly after a meal, possibly related to the development of diabetic complications [1]. As the most effective glucose-lowering agent, insulin can stimulate glucose uptake in skeletal muscles, myocardium, and other tissues in order to control glucose homeostasis [2]. Although subcutaneous (SC) administration of insulin remains the most prevalent means of treating diabetic patients, its patient compliance is poor, owing to their phobia of needles and local pain [3–5].

Alternatively, oral delivery of insulin is a promising means of improving compliance among diabetic patients. However, the bioavailability of insulin *via* oral ingestion is limited, owing to its presystematic degradation and the absorption barriers posed by the intestinal epithelium [3]. To address these limitations, our recent work developed a pH-responsive nanoparticle (NP) system comprising chitosan (CS) and poly(γ-glutamic acid) conjugated with ethylene glycol tetraacetic acid (γPGA–EGTA) for oral insulin delivery [6]. According to those results, the CS/γPGA–EGTA NPs (EGTA-conjugated NPs) could inhibit the proteolytic degradation of insulin and improve its paracellular permeability *in vitro*, as well as enhance the oral bioavailability of insulin in fasting diabetic rats.

As is well known, postprandial hyperglycemia is a more sensitive indicator of diabetic control than fasting glucose levels [7]. The oral glucose tolerance test (OGTT) has been used as a model to mimic the postprandial state of hyperglycemia as well as provides information on the glucose-lowering effect as a consequence of insulin action on the glucose utilization [8].

We hypothesize that insulin absorbed into the systemic circulation, as delivered by the EGTA-conjugated NPs orally, can effectively

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promote the glucose utilization and then control the postprandial hyperglycemia during OGTT in diabetic rats. The ability of γPGA–EGTA to inhibit insulin from enzymatic degradation was also evaluated *via* an *in vivo* imaging system (IVIS), based on the concept of Förster resonance energy transfer (FRET). Effects of the EGTA-conjugated NPs on disrupting the ultra-structures of epithelial tight junctions (TJs) and enhancing the intestinal absorption of insulin were visualized directly *in vivo* by transmission electron microscopy (TEM) and confocal laser scanning microscopy (CLSM), respectively. Biodistribution of the absorbed insulin and its subsequent stimulation of glucose uptake were imaged noninvasively by using positron emission tomography (PET)/computer tomography (CT). Their pharmacokinetic (PK) and pharmacodynamic (PD) profiles during OGTT were also analyzed.

2. Materials and methods

2.1. Preparation and characterization of EGTA-conjugated NPs

The 85% deacetylated CS (MW 60 kDa) was purchased from Koyo Chemical Co. Ltd. (Japan), whereas γPGA (MW 100 kDa) was acquired from Vedan Co. (Taichung, Taiwan). Insulin and EGTA were obtained from Sigma-Aldrich (St. Louis, MO, USA). The γPGA–EGTA conjugate was synthesized based on the procedure described in our previous study [6]. Insulin-loaded EGTA-conjugated NPs were prepared using an ionic-gelation method. Briefly, bovine insulin (2 mg/mL, 0.5 mL) was premixed with an aqueous CS solution (1.2 mg/mL, 4 mL, pH 6.0) in the presence of sodium tripolyphosphate (TPP, 1 mg/mL, 0.5 mL) by magnetic stirring at 4 °C for 15 min. Subsequently, the mixed solution was added, *via* flush mixing with a pipette tip, into an aqueous γPGA–EGTA solution (4 mg/mL, 0.5 mL, pH 7.4) and then blended thoroughly at room temperature to form the test NPs. Finally, the obtained NPs were collected *via* centrifugation at 8000 rpm for 50 min, resuspended in deionized (DI) water, and freeze-dried with trehalose (*i.e.*, a cryoprotective agent) for further use [9].

The particle size and zeta potential of the as-prepared NPs were measured by dynamic light scattering (Zetasizer 3000HS, Malvern Instruments Ltd., Worcestershire, UK). The loading efficiency and loading content of insulin in NPs were determined by assaying the amounts of free insulin in supernatants by using high-performance liquid chromatography [6]. The drug loading efficiency (LE) and loading content (LC) were calculated as shown below [5].

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

$$\mathrm{LC}(\%) = \frac{\text{Total amount of insulin added} - \text{free insulin}}{\text{weight of NPs}} \times 100\%$$

2.2. Detection of insulin degradation by FRET analyses

FRET involves the non-radiative transfer of energy from the excited state of a donor to the empty levels of an acceptor placed in close proximity (<10 nm), and, subsequently, the conformational changes of proteins can be probed using its efficiency [10]. To detect the degradation of insulin (or its conformational changes) based on FRET, an insulin derivative conjugating the fluorescent cyanine 3 (Cy3) and cyanine 5 (Cy5) moieties (Cy3–insulin–Cy5) was prepared. Briefly, equimolar N-hydroxy-succinimide-functionalized Cy3 and Cy5 (2.6 mg/mL, 0.1 mL, GE Healthcare, Little Chalfont, UK) were reacted with insulin (10 mg/mL, 1 mL, 27.4 IU/mg) at pH 9.5 in an ice bath for 24 h [11]. The unconjugated Cy3 and Cy5 molecules were removed *via* dialysis in distilled water at 4 °C for 3 days. Following dialysis, the obtained Cy3–insulin–Cy5 was lyophilized for the subsequent *in vitro* and *in vivo* degradation studies.

In the *in vitro* study, the synthesized Cy3–insulin–Cy5 (1 mg) was incubated with trypsin (4 mg) in a Krebs-Ringer buffer (KRB, 1 mL, pH 7.4) containing $CaCl_2$ (0.06 mg) with or without γPGA–EGTA (40 mg) at 37 °C for 2 h [12]. Insulin is highly sensitive to trypsin, which is a Ca^{2+}-dependent enzyme present in the intestinal fluid and mucus layer [13]. Next, the degradation of Cy3–insulin–Cy5 was monitored by FRET analyses, in which the donor (Cy3) was excited at 535 nm and the emission spectra of the donor–acceptor were recorded at all wavelengths simultaneously by using a fluorescence spectrometer (Horiba Jobin Yvon, Edison, NJ, USA).

The *in vivo* animal study was conducted in compliance with the "Guide for the Care and Use of Laboratory Animals", which was prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press in 1996, and approved by the Institutional Animal Care and Use Committee of National Tsing Hua University (protocol number 10046). The enzymatic degradation of insulin was investigated *in vivo* using an intestinal closed-loop method [14]. Briefly, overnight-fasted rats (Wistar, ~250 g, n = 5) were anesthetized *via* the intramuscular injection of Zoletil® (50 mg/kg, Virbac Laboratories, Carros, France). The abdominal cavity of animals was opened by a midline incision, and the small intestine was exposed. A 5-cm segment of the duodenum was then selected for producing a closed loop. The proximal end of the intestinal loop was first tied up, and the Cy3–insulin–Cy5 (2 mg/mL, 0.5 mL) in the absence or presence of γPGA–EGTA (40 mg) was then introduced *via* a syringe. The distal end was subsequently ligated to form a closed loop.

Fluorescent signals of Cy3–insulin–Cy5 in the intestinal loop were acquired by using an IVIS (Xenogen, Alameda, CA, USA). The test animal was irradiated at a wavelength of 535 nm (the excitation wavelength of Cy3) and imaged with an appropriate emission filter (680 nm) to obtain Cy5 images. The fluorescent intensities from regions of interest were quantified using Living Image 3.1 software.

2.3. Ultra-structural examination of the permeability of epithelial TJs

The epithelial TJ permeability was observed directly by examining the ultra-structural changes induced by the EGTA-conjugated NPs at different segments of the small intestine by using TEM. In the work, the loops created in the duodenum, jejunum and ileum were individually treated with the test NPs (2 mg/mL, 0.5 mL). Three hours later, the rats were sacrificed; the intestinal loops were subsequently dissected and washed three times with isotonic saline. Next, the dissected loops were fixed in 4% paraformaldehyde and washed with s-Collidine buffer. To illustrate the paracellular permeability, the fixed loops were further incubated with 2% lanthanum, an electron-dense tracer, for 2 h [15]. After rinsing in s-Collidine and phosphate buffered saline (PBS), tissue samples were processed for TEM examination, as detailed previously [16].

2.4. Structural reorganization of TJs and intestinal absorption of insulin

The fluorescein isothiocyanate-labeled insulin (FITC-insulin) was synthesized [17] and then loaded in the EGTA-conjugated NPs. Next, as-prepared fluorescent NPs were introduced into the intestinal loops created in rats (n = 6), as described above. At 2 h following treatment, three of the studied rats were sacrificed, and their loop segments were dissected to examine the corresponding structural reorganization of TJs and intestinal absorption of insulin. Additionally, to study the recovery of TJ structures after removal of test NPs, the ligations of the intestinal loops created in the other three rats were removed and then eluted with deionized (DI) water thoroughly. Twenty-four hours later, rats were sacrificed, and their intestinal loops were dissected.

The dissected intestinal loops were washed with PBS and then fixed in paraformaldehyde. Following fixation, tissue samples were

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