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Polypeptide-based polyelectrolyte complexes overcoming the biological barriers of oral insulin delivery

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ABSTRACT

In this study, a novel oral insulin delivery system was prepared by combining two different artificial polypeptides with insulin. Negatively charged poly(L-glutamate-*co*-*N*-3-L-glutamylsulfanilic acid) (PLGS), cationic alpha helical peptide poly-L-lysine (PLL), and insulin formed polyelectrolyte complexes (PCs) were characterized. The property of the PCs was examined by an *in vitro* study. A significantly higher amount of the loaded FITC insulin was released in the intestinal condition, suggesting the controlled release of the PCs to protect insulin in the acidic stomach condition while releasing it in the small intestine. The *in vitro* cellular uptake study with Caco-2 cells also revealed the improved penetration of the loaded FITC labeled insulin. By virtue of the cell penetration enhancing ability of PLL, the permeation of insulin in the small intestine was notably augmented. Furthermore, the feasibility of the PCs was confirmed through an *in vivo* hypoglycemic effect study. The PCs showed an improved hypoglycemic effect suggesting the success of the delivery and penetration of the loaded insulin. The blood glucose level was lowered to 80% of its initial value after the oral administration of the PCs, and the hypoglycemia lasted for more than 14 h. The long lasting hypoglycemic effect of the PCs can reduce the number of administrations, and it will contribute to improving the quality of patients' lives. The PCs provided reasonable results as a competitive candidate for oral insulin delivery. The introduction of the PCs will promote the oral delivery of charged proteins or drugs.

Introduction

Diabetes mellitus, one of the most common and dangerous diseases, is a metabolic disease caused by insufficient insulin secretion or reduced insulin sensitivity [1]. The number of diabetic patients is dramatically increasing, and these patients suffer from some serious complications [2–5]. To treat diabetes, insulin should be introduced into the body, and usually it is done by subcutaneous injection. However, subcutaneous injection can cause some serious problems such as patient compliance problems, a shorter circulation of injected insulin, and strong pain [6]. Therefore, various routes for insulin administration have been suggested such as nasal [7], transdermal [8], and oral routes [9].

Among the various routes, the oral route is considered the most reasonable delivery route because of its convenience and having the same delivery mechanism as that of endogenously secreted insulin [10]. Nonetheless, there exist two major problems with achieving the oral insulin delivery. One is the insulin degradation problem within the harsh environment of the gastrointestinal (GI) tract. The large pH gradient and numerous enzymes in the GI tract make insulin lose its function, so proper protection should always be followed [11]. The other problem is the unfavorable penetration of the orally delivered insulin in the small intestine. Since the intestinal epithelium and the mucus layer inhibit the transport of macromolecules, absorption enhancement should also be considered [11]. Therefore, it is important to develop an insulin delivery system which not only protects insulin from the harsh environment, but also improves the penetration of insulin in the small intestine.

Recently, various types of oral insulin delivery systems have been introduced to overcome the problems mentioned above. Sonaje et al. synthesized a pH-responsive nanoparticle system

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composed of chitosan and poly(γ-glutamic acid) for the oral insulin delivery [12]. The orally administered insulin nanoparticles showed a prolonged hypoglycemic response compared to subcutaneous injection. Jin et al. reported goblet cell-targeting nanoparticles for the oral insulin delivery [13]. The nanoparticles were modified with goblet cell targeting peptides, and the researchers observed an improved hypoglycemic effect. Although those studies proved the feasibility of oral insulin delivery systems, the approaches still have limitations in replacing the current method, so further research is required.

Synthetic polypeptides have recently emerged as novel drug carriers due to their biocompatibility, low toxicity, and biodegradability while maintaining the proper strength to protect the drug in a harsh environment. Also, it is easy to give functionality to polypeptides by simple reactions with various types of functional groups. Therefore, polypeptides can be applied for diverse purposes by changing the functional groups. Because of these reasons, specially designed polypeptides have been developed such as charged polypeptides [14], stimuli-cleavable polypeptides [15], and structure changing polypeptides [16]. The currently reported systems composed of polypeptides have been used for various purposes, and their low toxicity has made the systems competitive alternatives.

Alpha helical polypeptides are emerging as one of the most powerful candidates for enhancing cell penetration. Although the mechanism of cell penetration is not fully understood yet, the endocytic process and direct translocation are regarded as possible mechanisms. Generally, cell-penetrating peptides (CPP), which are naturally occurring alpha helical polypeptides, have been used for delivering macromolecules. However, their high cost and low productivity limit their wide usage as delivery carriers. Instead, artificial alpha helical polypeptides have been suggested recently [17,18]. Since artificial alpha helical polypeptides can be synthesized by chemical reactions, it is more convenient to synthesize and adopt them as delivery carriers than CPPs. Polyelectrolyte complexes, which are mainly formed by electrostatic interactions, are the combination of oppositely charged materials such as polymer–polymer or polymer–protein [19]. The formation of polyelectrolyte complexes is achieved by simple mixing in an aqueous solution. Therefore, cross linking agents are not needed in the formation of polyelectrolyte complexes. In addition, the use of organic solvents or sonication is not needed to form nano-sized polyelectrolyte complexes [20]. For these reasons, nano-sized polyelectrolyte complexes can easily be formed without any toxic components that can cause unexpected health problems or reduced effects of the target molecules which are drugs or proteins.

Some of the functional groups bear positive or negative charges depending on their pK_a values and external conditions. In particular, sulfanilic acid, which has a pK_a value around 3, will always be negatively charged in physiological conditions. On the other hand, the isoelectric point of insulin is 5.3 [21], so that insulin can exhibit a positive charge at a pH lower than 5.3 while it can bear a negative charge at a higher pH. Therefore, self-assembled polyelectrolyte complexes can be formed when positively charged insulin is mixed with sulfanilic acid attached polypeptides, and their structure can be changed by the external pH condition.

Herein, we describe a novel oral insulin delivery system that can protect insulin concurrently with improving the insulin permeation. It uses artificially synthesized polypeptides: one with anionic groups and sulfanilic acid, and the other one for exhibiting cationic alpha helicity (Fig. 1). The sulfanilic acid modified polypeptides were always able to be negatively charged at a physiological pH. Insulin, whose isoelectric point is 5.3, interacted with the sulfanilic acid attached polypeptides in a different manner according to the external pH because of charge differences. Thus, the insulin loaded polypeptide complexes achieved a controlled release to protect and release insulin appropriately. Also, artificially synthesized alpha helical polypeptides functioned to enhance the intestinal absorption of insulin in the small intestine. In this study, a novel delivery system showed reasonable results comparable to current insulin delivery methods.

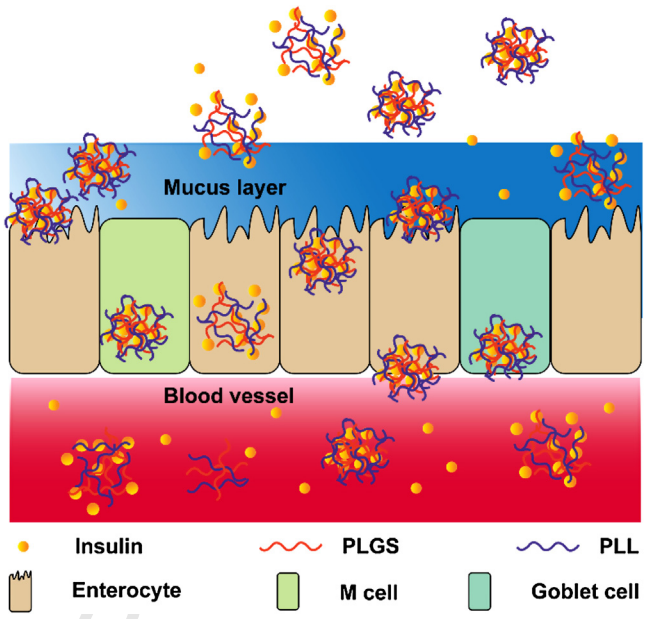

Fig. 1. Schematic illustration of the local part of the small intestine with the polyelectrolyte complexes, which can effectively deliver insulin through an oral route.

Materials and methods

Materials

Solvents, hexamethyldisilazine (HMDS), trifluoroacetic anhydride (TFA), sulfanilic acid, fluorescein isothiocyanate (FITC), bovine pancreas insulin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dulbecco's Modified Eagle's Medium (DMEM), phosphate buffered saline (PBS), fetal bovine serum (FBS), antibiotic solution (penicillin and streptomycin), and streptozotocin (STZ) were purchased from Sigma Aldrich. 5-Benzyl-L-glutamate and triphosgene were obtained from Alfa-Aesar. while N_ε-trifluoroacetyl-L-lysine, *N*-hydroxysuccinimide (NHS), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)·HCl were purchased from Tokyo Chemical Industry Co., Ltd. HPLC solvents were purchased from Daejung Co., Ltd. Molecular weight cut off (MWCO) 8000 Da and 3500 Da dialysis bags were purchased from Spectrum Laboratories, Inc. MEM non-essential amino acids solution was purchased from Gibco® by Life Technologies™. Caco-2 cells were kindly donated by Prof. Park (Dept. of Bio and Brain Eng., KAIST). Eight-week-old male C57BL/6 mice weighing 20 ± 2 g were purchased from Orient Bio.

Synthesis of poly(5-benzyl-L-glutamate) (PBG)

By following the previously described method, 5-benzyl-L-glutamate *N*-carboxyanhydride (NCA) was synthesized [22]. With hexamethyldisilazane (47 μL), 5-benzyl-L-glutamate NCA (4.7 g) was dissolved in anhydrous *N*,*N*-dimethylformamide (DMF) in a glove box. The molar ratio of 5-benzyl-L-glutamate NCA to HMDS was 80. The mixture was stirred at RT for 2 days. After the polymerization, the mixture was poured into an excess ethyl ether to wash out unreacted impurities. The precipitate was washed with

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