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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.

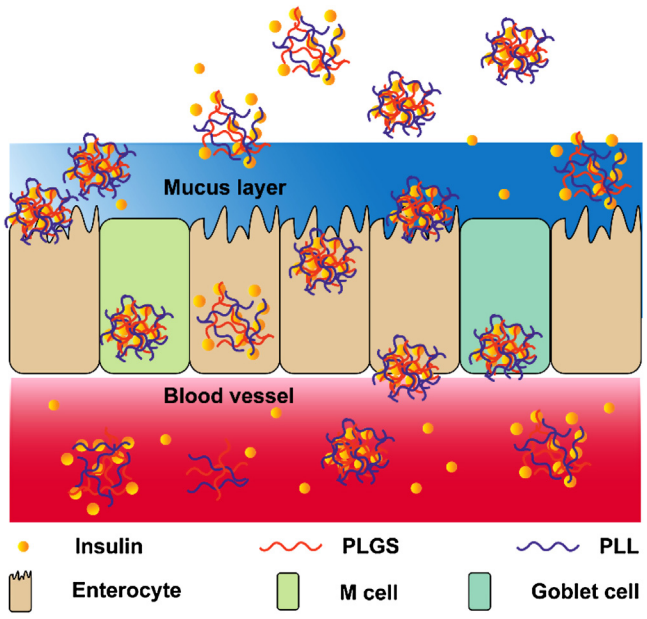

Fig. 1. Schematic illustration of the local part of the small intestine with the polyelectrolyte complexes, which can effectively deliver insulin through an oral route.

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

ethyl ether three times and dried at RT. The obtained polypeptide, poly(5-benzyl-L-glutamate) (PBG), was 3.95 g.

Deprotection of benzyl groups and trifluoroacetic anhydride (TFA) protection

PBG (1 g) was dissolved in tetrahydrofuran. Sodium hydroxide (0.47 g) was dissolved in deionized water. Two solutions were mixed and the mixture was stirred at RT for overnight. After the deprotection reaction, ammonium chloride was added into the mixture to neutralize the basic condition. The mixture was dialyzed against deionized water overnight. The dialyzed mixture was then lyophilized and poly-L-glutamate (PLG) (0.63 g) was obtained. Trifluoroacetic anhydride (TFA) (0.0175 g) was added into a PLG (0.63 g) dissolved dimethyl sulfoxide solution. The mixture was reacted at RT for 3 h with stirring, and poured into excess acetone to wash out unreacted impurities. The precipitate was washed with acetone three times and dried at RT. TFA protected PLG (0.44 g) was obtained.

Synthesis of poly(L-glutamate-co-N-3-L-glutamylsulfanilic acid) (PLGS)

TFA protected PLG (0.20 g) was dissolved into DMF, and NHS (0.54 g) was added into the PLG solution. EDC·HCl (1.20 g) was also added to activate carboxyl groups for carbodiimide coupling reactions. The activation was carried out for 3 h, and then sulfanilic acid (0.75 g) dissolved. A 1 M pH 5 pyridine buffer was then added into the mixture. After 2 days reaction at RT with magnetic stirring, the mixture was dialyzed (MWCO 3500 Da) against deionized water for 4 days and followed by lyophilization. The dried polypeptides were dissolved into methanol and the same amount of sodium carbonate was added into the solution to detach the TFA protecting group. The mixture was stirred at RT for 1 day. After the reaction, the mixture was washed with ethyl ether three times, and finally, poly(L-glutatmate-*co*-*N*-3-L-glutamylsulfanilic acid) (PLGS) (0.32 g) was obtained.

Synthesis of poly-L-lysine (PLL)

By following the previously described method, N^{ε}-trifluoroacetyl-L-lysine *N*-carboxyanhydride (NCA) was synthesized [23]. Similar with PLGS synthesis, N^{ε}-trifluoroacetyl-L-lysine NCA (5.0 g) was dissolved in anhydrous DMF with hexamethyldisilazane (65 μL) in a glove box. The molar ratio of N^{ε}-trifluoroacetyl-L-lysine NCA to HMDS was 60. The mixture was polymerized at RT for 2 days, and the mixture was then purified by a washing process that uses ethyl ether. 3.95 g of Poly-L-lysine (PLL) was obtained.

Characterization of PLGS and PLL

Using gel permeation chromatography, the molecular weights of PBG and PLL were determined. The Younglin YL9100 HPLC system (Younglin instrument, Korea) with a 10^3–10^5 g/mol DMF GPC column (Shodex KD-803) was used, and the mobile phase was a 0.01 M LiBr *N*,*N*-dimethylformamide (DMF) solution at 35 °C with a flow rate of 1 mL/min [22]. To analyze the structure of the polypeptides, ^{1}H NMR spectra were measured with Agilent 400 MHz 54 mm NMR DD2 (Aglient Technology, USA). DMSO-d_6 or D_2O was used as an NMR solvent. Element analysis was conducted using Flash 2000 series (Thermo Scientific, USA) to calculate the degree of modification in PLGS. Each weight percentage of carbon, nitrogen, sulfur, and hydrogen was measured, and these measurements were used to calculate the number of repeating units that were combined with the sulfanilic acid group. The helicity of both polypeptides was confirmed using J-815 circular dichroism spectrometry (JASCO, Japan) with a 0.01 cm pathlength quartz cell. Both polypeptides, PLGS and PLL, were dissolved in pH 1.2 or pH 6.8 solutions to achieve the final concentration of 1 mg/mL, while molecular ellipticity was measured from 190 nm to 260 nm.

Preparation and characterization of the polyelectrolyte complexes (PCs)

The polyelectrolyte complexes (PCs) were prepared by a simple mixing process. The polypeptides were dissolved in a pH 4 solution, and insulin was dissolved in a 0.01 N HCl solution. All the solutions were prepared at a concentration of 1 mg/mL. At first, the insulin solution was slowly added to the PLGS solution and pipetted for 5 min to form uniformly distributed complexes. The PLGS-insulin complexes were incubated at RT for 15 min to stabilize the complexes. After 15 min incubation, the PLL solution was added to the mixture of PLGS and insulin to form the PCs. The mixture was also pipetted for 5 min, and additionally incubated at RT for 15 min to form stable PCs. The size and polydispersity index of the PCs was measured by a dynamic light scattering method using Malvern Zetasizer Nano ZS (Malvern Instruments, UK). The zeta potential of the PCs was also measured by the same instrument. Each measurement was performed in triplicate. To optimize the PCs, the size and zeta potential of the PCs were measured by varying the amount of PLL while the ratio between PLGS and insulin was fixed at 5:1. The morphology of the optimized PCs was examined by a transmission electron microscope (Tecnai F20, FEI, USA). The polyelectrolyte solution was diluted to achieve 0.1 mg/mL, and the diluted solution was dropped onto a copper grid to prepare the TEM sample. The grid was then dried at RT to evaporate the water solvent.

2.8 In vitro *FITC insulin release test*

FITC labeled insulin was prepared by the method previously described with little modifications [24]. The FITC insulin encapsulated PCs were then prepared by a simple mixing method. The release was examined in two different pH values: one for simulating the stomach (pH 1.2) and the other one for the small intestine (pH 6.8). Both pH buffers were formulated as described in USP. The FITC insulin loaded PCs were dissolved in one of the pH buffers, and 2 mL of the PCs solution was located in a dialysis bag (MWCO 8000 Da). The dialysis bag was then put in the tube, which was filled with 5 mL of the pH 1.2 buffer or pH 6.8 buffer. The buffer solution in the tube completely soaked the dialysis bag, and the tube was placed in the shaking incubator at 37 °C and 85 rpm. At the predetermined time points (0, 0.5, 1, 2, 3, 4, 6, 8, 12 and 24 h), the entire 5 mL of the external buffer solution was replaced by a fresh buffer solution to maintain the total volume. The collected external buffer solution was used to measure the fluorescence intensity with a fluorescence spectrometry. Through the fluorescence intensity, the released amount of FITC insulin during the determined time period was calculated. The release percentage was also calculated by dividing the released amount by the total loaded amount of FITC insulin in the PCs.

2.9 In vitro *cytotoxicity study*

The biocompatibility of the PCs was evaluated by methyl thiazolyl tetrazolium (MTT) assay with Caco-2 cells. The Caco-2 cells were cultivated in a Modified Medium (DMEM) with 10% fetal bovine serum, 1% antibiotic solution, and 10% nonessential amino acid. To investigate the cytotoxicity of the PCs, Caco-2 cells were seeded onto 96-well plates at a density of 50,000 cells/well. The cells were incubated with 100 μL of culture medium at 37 °C,

5% CO_2 for 48 h. After 48 h, the culture medium was completely removed, and the confluent cells were then treated with 100 μL of three different samples: PLL-insulin mixture, PLGS-insulin complexes, and the PCs. The concentration of each sample was adjusted to 0.1–1000 μg/mL by varying the volume of the cell culture medium. After 20 h incubation with the samples, 100 μL of the MTT solution (0.5 mg/mL in PBS) was additionally added to each well. The cells were further incubated for 4 h, and the whole medium was replaced by DMSO to dissolve formazan. Using Multiskan GO (Thermo Scientific, USA), the absorbance of each well was measured and recorded. Cells that were treated only with the culture medium were used as a control and the absorbance of sample treated cells was divided by the absorbance of control to calculate the cell viability.

In vitro *cellular uptake study*

To investigate the cellular uptake enhancement of the PCs, Caco-2 cells at a density of 50000 cells/well were seeded onto the polycarbonate membrane of the SPLInsert transwell (SPL, Korea). The cells were incubated at 37 °C, 5% CO_2 for 3 weeks to fully cover the polycarbonate membrane with a cell monolayer. The confluence of the cells was demonstrated by measuring the trans-epithelial electrical resistance value using an electrical resistance meter. The Caco-2 cell monolayer was then used for the *in vitro* cellular uptake study. To analyze the cellular uptake of insulin, FITC labeled insulin was used. FITC insulin solution, FITC insulin mixed with PLL, FITC insulin combined with PLGS, and FITC insulin loaded PCs were treated on the apical side of the Caco-2 cell monolayer. All the samples were adjusted to have the same amount of FITC insulin, which was 0.07 mg/mL. The cells were incubated with four different samples at 37 °C with 5% CO_2. After 2 h, PBS was treated on the cells to remove the samples that could not uptake into the cells. For quantitative analysis of the cellular uptake of FITC insulin, the cell membrane was disrupted using a cell lysis buffer. After detaching Caco-2 cells from the polycarbonate membrane with trypsin, the RIPA buffer was added as a cell lysis buffer for rupturing the cell membrane. The fluorescence intensity of each well was then measured, and the relative absorption enhancement was calculated by dividing each intensity value by the fluorescence intensity of the FITC insulin solution treated well. To visualize the cellular internalization of the FITC insulin, the confocal laser scanning microscopy (CLSM) was adopted. The nuclei of the sample treated cells were stained with Hoechst 33342 followed by PBS washing. The membrane of the cell culture transwell was detached and examined under CLSM to get the uptake images of FITC insulin.

In vivo *hypoglycemic effect study*

STZ induced diabetic mice were prepared as previously described with little modification [25]. After 1 week, the mice fasted overnight to check the blood glucose level, and the mice, which had a fasting blood glucose level over 270 mg/dL, were considered as diabetic models [26]. The diabetic mice fasted overnight but were allowed water ad libitum before sample administration and an additional 24 h during the experiment. Free insulin solution, PLGS-insulin complexes, and the PCs at an insulin dose of 150 IU/kg were orally delivered using a gavage, while the PBS administered group was used as a blank control. Right before sample administration and at predetermined time points of 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 16, 20 and 24 h, blood samples were collected from the tail veins. The blood glucose level was measured using the Accu-Chek blood glucose meter (F. Hoffmann-La Roche Ltd., Swiss). Animal care and experimental procedures were performed under approval from the Animal Care Committee of KAIST.

Statistical analysis

Statistical analysis was performed for an *in vivo* study using one-way analysis of variance (ANOVA). A $p < 0.05$ was considered to be statistically significant.

Results and discussion

Synthesis and characterization of PLGS and PLL

Polyelectrolyte complexes with charged proteins and polymers easily form stable nanoparticles by a simple mixing process. Therefore, polyelectrolyte complexes are considered as effective candidates for the delivery of drugs or proteins. To form polyelectrolyte complexes (PCs) with insulin, negatively charged polypeptide poly(L-glutatmate-*co*-*N*-3-L-glutamylsulfanilic acid) (PLGS) and positively charged polypeptide poly-L-lysine (PLL) were synthesized by NCA polymerization and followed by carbodiimide reaction (Fig. 2). In the case of PLGS, 5-benzyl-L-glutamate NCA was polymerized with an initiator, hexamethyldisilazane (HMDS), and the chemical structure poly(5-benzyl-L-glutamate) (PBG) was confirmed by ^{1}H NMR spectrometry (Fig. 3a). Moreover, through gel permeation chromatography, the molecular weight (12700 Da) and polydispersity index (1.05) of PBG were determined (Supplementary Fig. S1a). With the obtained molecular weight, the number of repeating units of 5-benzyl-L-glutamate blocks was calculated as 58. Since the feeding ratio of 5-benzyl-L-glutamate NCA to HMDS was 60, it was possible to find out that the polymerization went precisely as designed, and a polydispersity index nearly 1 indicated that the obtained polymer was almost monodispersed. Therefore, controlled synthesis of monodispersed polypeptides was achieved using HMDS as an initiator. After the polymerization, the benzyl groups in the PBG side chain were deprotected to introduce negative charges to the main PBG backbone. The result of benzyl group deprotection was demonstrated by comparing both chemical structures of PBG and poly-L-glutamate (PLG) with ^{1}H NMR spectrometry. The disappearances of both the benzyl position peak at 4.8 ppm and phenyl peak at 7.2 ppm in the chemical structure of PLG revealed the successful removal of benzyl groups (Fig. 3a). To introduce anionic groups, sulfanilic acid was conjugated with the carboxyl groups of PLG by a simple carbodiimide reaction, and finally, PLGS was synthesized. The conjugation of sulfanilic acid was confirmed through ^{1}H NMR spectrometry by identifying the benzyl peak from 7.2 ppm to 7.5 ppm (Fig. 3a). In addition, to calculate the degree of sulfanilic acid conjugation, element analysis was used (Supplementary Fig. S2), and it was calculated as 13 blocks out of 58 total glutamate blocks. To enhance the insulin absorption in the small intestine, positively charged helical polypeptide poly-L-lysine (PLL) was also synthesized. Similar with PLGS, PLL was synthesized by NCA polymerization. With the HMDS initiator, poly(N^{ε}-trifluoroacetyl-L-lysine) (PTFL) was synthesized, and further deprotected to yield PLL. The synthesis of PLL was also established through ^{1}H NMR spectrometry (Fig. 3b) and gel permeation chromatography (Supplementary Fig. S1b).

Alpha helical polypeptides have a key role in the delivery of macromolecules into the cells. Therefore, the helicity of polypeptides was additionally confirmed through circular dichroism spectra. As shown in the graph, both spectra of PLGS (Fig. 4a) and PLL (Fig. 4b) indicated the alpha helical characteristics of polypeptides. In the case of PLGS, a slightly increased helicity content was observed in the pH 6.8 condition compared to the pH 1.2 condition, but the difference was not significant. In contrast, the PLL exhibited a higher absolute molar ellipticity at 222 nm in an acidic condition. This might have contributed to the high concentration of proton within the acidic solution, which can

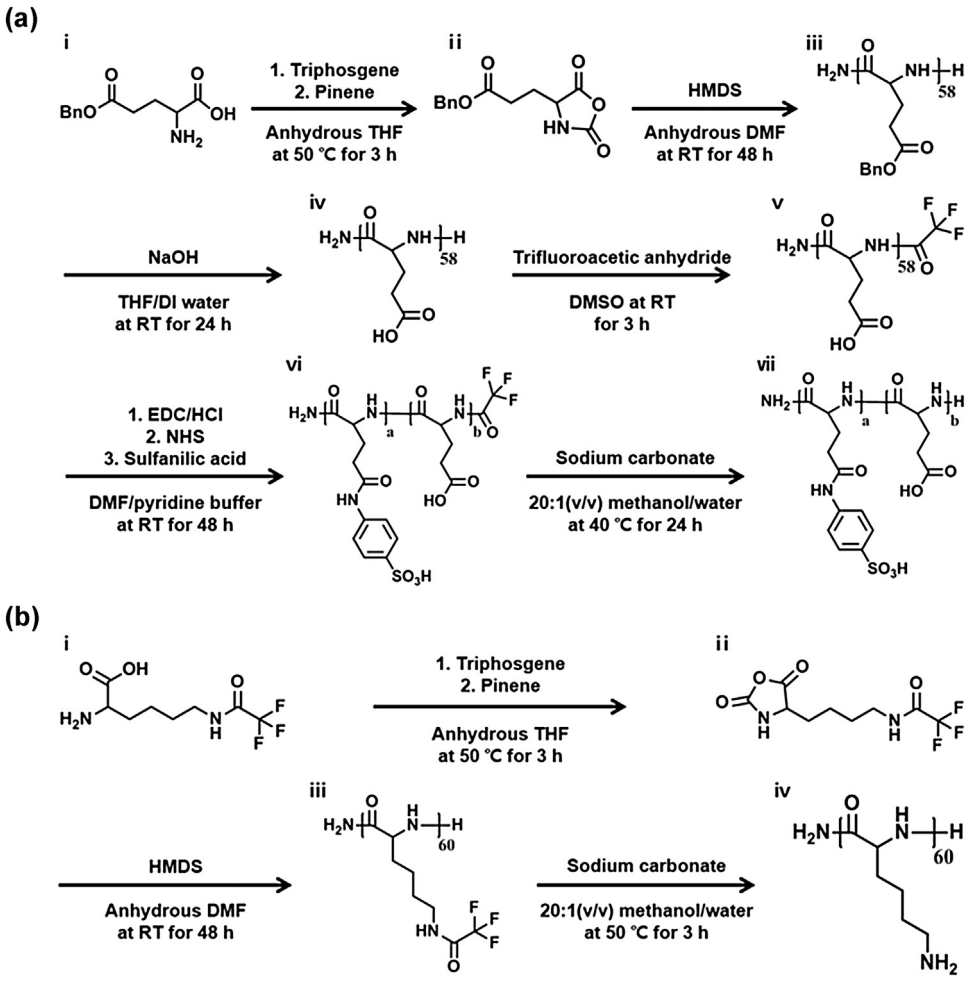

Fig. 2. Synthetic scheme of PLGS (a) and PLL (b).

stabilize the helical structure of PLL more securely. Though the helicity reduction at pH 6.8, the PLL still exhibited a helical structure, and its positive charges are expected to improve the penetration of PCs.

Optimization and characterization of the PCs

Since the isoelectric point of insulin is 5.3, it exhibits a positive charge at a low pH below the isoelectric point. Therefore, insulin

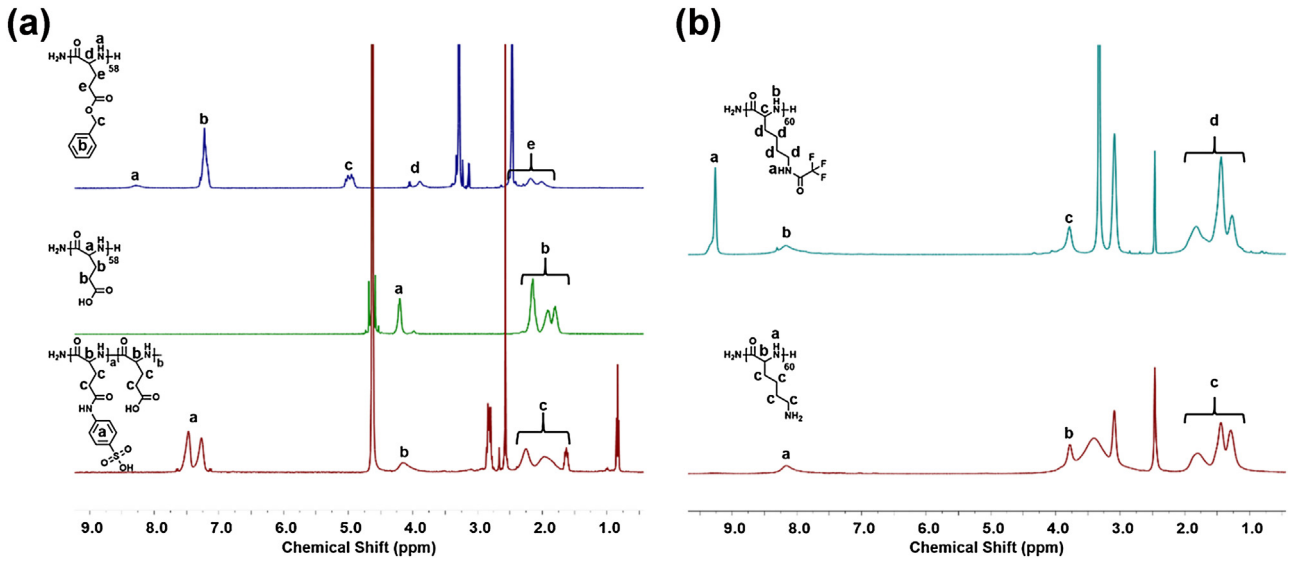

Fig. 3. (a) NMR spectra of PBLG (DMSO-d_6), PLG (D_2O) and PLGS (D_2O), (b) NMR spectra of PTFL (DMSO-d_6) and PLL (DMSO-d_6).

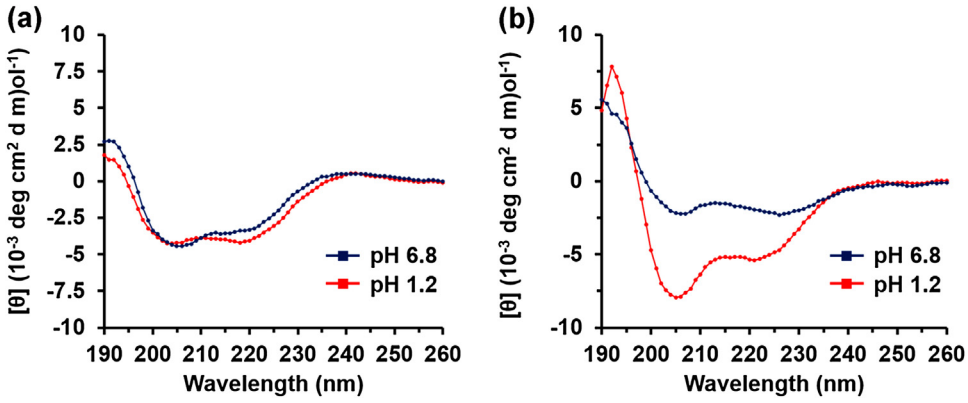

Fig. 4. CD spectra of (a) PLGS and (b) PLL at pH 1.2 and pH 6.8.

can form complexes with anionic polypeptide PLGS by simple mixing and an additional cationic PLL can also be attached to the complexes. It is important for the PCs to exhibit positive charges while maintaining the minimal size to enhance the penetration through the small intestine. Since the PCs should strongly interact with the negatively charged intestinal epithelial cells to enhance the cellular absorption, the maintenance of positive charges for the PCs is essential. In addition, the permeation into the epithelial cells is preferable for the smaller particles, so that condition which forms the smallest PCs should be found out.

To identify the optimum condition, the size and zeta potential value of PCs were measured by changing the ratio of added PLL (Fig. 5a and b). Since only PLL is involved in the formation of the outer coating, the weight ratio of PLGS to insulin was fixed to 5 while the adding ratio of PLL was changed. When the weight ratio of PLL to PLGS was adjusted to 1, the mean size of the PCs was the smallest, and it was about 215 nm (Fig. 5a). Also, moderate positive charges of the PCs proved by zeta potential value indicated the feasibility of the enhanced intestinal uptake of the complexes while minimizing the cellular toxicity (Fig. 5b). Through the characterization, the optimum weight ratio of PLGS to PLL to insulin was determined to be 5:5:1.

The trend shown in Fig. 5a and b indicated the successful addition of PLL to the complexes of PLGS and insulin. Until the optimal point, the mean size decreased as the amount of the added PLL increased. It can be speculated that the reason for this trend is that added PLL not only attached to the outer surface of the complexes, but also functioned to stabilize the complexes. Therefore, even though PLL was added, the mean particle size decreased until the most stable PCs formed. When more PLL was

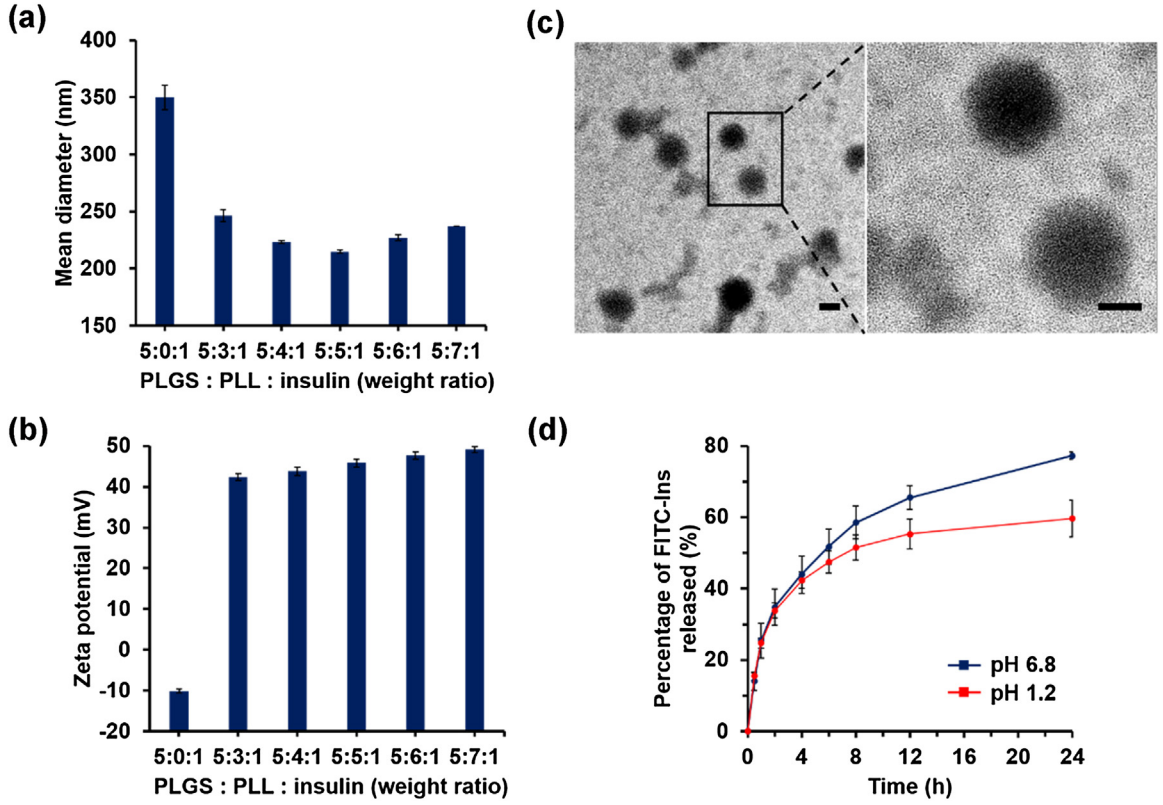

Fig. 5. Particle mean diameters (a) and zeta potential values (b) depending on the different weight ratios (PLGS:PLL:insulin). (c) TEM images of PCS (left scale bar: 200 nm, right scale bar: 100 nm). (d) Profiles of *in vitro* FITC insulin release from the PCs in two different pH conditions simulating the stomach (pH 1.2) and the small intestine (pH 6.8). The error bars indicate standard deviation ($n = 3$).

added over an optimum point, the mean size started to increase as the amount of PLL increased. This confirms that after the optimum point, no more stabilization happened because of PLL. All the others were located on the surface, so that more bulky PCs can form. Moreover, the change in zeta potential value revealed that PLL was successfully attached to PCs. Although the combination of PLGS with insulin exhibited negative charges that cannot strongly interact with the negatively charged epithelial cells, the addition of PLL changed the surface charge to positive.

In addition to the size and zeta potential, the PCs were further characterized by the TEM images (Fig. 5b). The size measured through the TEM images was similar with that measured by a dynamic light scattering method while little shrinkage was observed. This may have been caused by the removal of the water solvent during the sample preparation. Also, numerous globular particles indicated the successful formation of uniformly distributed PCs. The uniform distribution of the PCs was additionally confirmed through the polydispersity index of the optimum PCs (Fig. 5a).

In vitro *FITC insulin release study from the PCs*

To confirm the controlled insulin release property of the PCs, an *in vitro* FITC insulin release test took place. Instead of intact insulin, FITC insulin was encapsulated in the PCs in particular to visualize the released amount from each condition. As can be seen in the release profile, the release kinetics showed similar aspects in both pH conditions (Fig. 5d). A burst release was observed until the first 2 h, while a more slow release was achieved during the rest of the time periods. However, the total released amounts of FITC insulin after 24 h were considerably different depending on the external pH values (Fig. 5d). Until the first 4 h, no significant differences were observed between pH 1.2 and pH 6.8. At both pH conditions, about 40% of FITC insulin was released from the PCs. Nevertheless, after 4 h, the PCs started to release more FITC insulin at pH 6.8 compared to pH 1.2, and the release gap between pH 1.2 and pH 6.8 condition became enlarged over time. At pH 6.8, though the rate of increment decreased after 2 h, FITC insulin was continuously released, so that more than 77% of FITC insulin was released after 24 h. In contrast at pH 1.2, the release kinetics reached a plateau. Therefore, less than 60% of FITC insulin was leaked out from the PCs within 24 h at pH 1.2 (Fig. 5d).

This difference can be explained by the charge change of insulin that occurred because of differences in the pH. At pH 1.2, protonated insulin bears a positive charge, so that it can interact more strongly with negatively charged PLGS. Thus, the core PCs that were formed between PLGS and insulin can be stabilized in the acidic condition, and this stabilization can protect insulin by preventing the insulin leakage and the access of proteolytic enzymes. However, when pH increases to 6.8, which is a higher value than the isoelectric point of insulin of 5.3, the insulin changes the positive charge to the negative one, and it makes repulsion with PLGS. The repulsion releases insulin from the PCs, so that it can penetrate through the intestinal epithelial cells. By analyzing the results, the insulin release controllability of the PCs was confirmed. The loaded insulin can be protected from the harsh environment of the stomach, while can achieve sustained release in the intestinal condition.

In vitro *cellular uptake study using Caco-2 cells*

The PCs were designed to enhance the intestinal absorption of insulin. To demonstrate the insulin penetration enhancing ability of PCs, an intracellular uptake study was performed using Caco-2 cells that mimic the intestinal epithelial cells. To analyze this ability quantitatively, FITC insulin encapsulated PCs were incubated on Caco-2 cells for 2 h, and the cells were lysed to measure the fluorescent intensity of the absorbed FITC insulin. As expected, the PCs showed a more enhanced internalization of FITC insulin compared to the groups treated with the FITC insulin solution and the combination of PLGS with FITC insulin (Fig. 6b). Compared with the FITC insulin solution treated control group, the PCs enhanced the FITC insulin permeation more than 3-fold. On the other hand, the single use of PLGS could not promote the absorption of FITC insulin. The results well described the function of PLL that was used for enhancing the cellular uptake of the PCs themselves or the released insulin. Positively charged helical polypeptide PLL effectively functioned to improve the insulin permeation through the epithelial cells, and it also assisted the PCs. By virtue of PLL, the complexes showed a significantly enhanced penetration of insulin than the group incubated with the FITC insulin solution. Unlike PLL, polyanion PLGS cannot strongly attach itself to the negatively charged intestinal epithelial cells, so that the insulin combined with PLGS could not encourage the penetration of insulin. In addition to the quantitative analysis, the internalization of FITC insulin was also visualized through the CLSM images (Fig. 6a). The FITC insulin absorption trend observed in the CLSM images was in accordance with the quantitative interpretation. Although the FITC signal was rarely observed in the cells incubated with the FITC insulin solution or the combination of PLGS with FITC insulin, the PCs treated group showed a green FITC signal. A strong FITC signal around the nuclei indicated the improved insulin cellular internalization with the help of the PCs. The driving force of the enhanced internalization was that PLL with strong cationic charges strongly interacted with the plasma membranes, which was likely to trigger both endocytosis and macropinocytosis pathways

Confirmation of in vitro *cytotoxicity of the PCs*

Biocompatibility is one of the most important factors for novel materials to be used in biological purposes. Since the materials themselves should not cause any harmful health problems, the development of biocompatible and biodegradable materials is critical. To confirm the biocompatibility of the novel PCs, MTT assay was performed with Caco-2 cells. In addition to the PCs, raw materials PLL and PLGS were also investigated separately to confirm the cytotoxicity. The Caco-2 cells were treated with all the groups for 24 h, and the relative toxicity was measured by comparing with media that was solely treated as control. As can be seen in Fig. 6c, the PCs showed minimal toxicity, even at high concentrations. No toxicity was observed until the concentration of PCs reached 100 μg/mL, and finally, the viability of Caco-2 cells decreased only by about 10% at high concentration (1000 μg/mL) (Fig. 6c). In contrast, the cells treated with PLGS or PLL showed dramatically decreased cell viability as the concentration increased. PLGS showed strong toxicity at the highest concentration, and PLL exhibited more toxic characteristics, even at diluted concentrations (100 μg/mL) (Fig. 6c). At the highest concentration, no more than 50% of PLGS treated Caco-2 cells survived after 24 h incubation, and less than 10% of the cells survived in the case of PLL (Fig. 6c). Even at the lower concentration (100 μg/mL), PLL had high toxicity, and less than 30% of the cells survived (Fig. 6c). It can be speculated that the high toxicity of the raw materials might be attributed to the strong charges of the raw materials. Since PLGS and PLL were designed as polyanion and polycation, respectively, those polypeptides themselves are strongly charged, so that severe cytotoxicity can be observed (Fig. 6c). Among those polypeptides, PLL in particular showed more serious toxicity than PLGS. PLL, which bears positive charges, can actively interact with the negatively charged cell membrane, and this interaction can destabilize the membrane. The destabilization of the cell

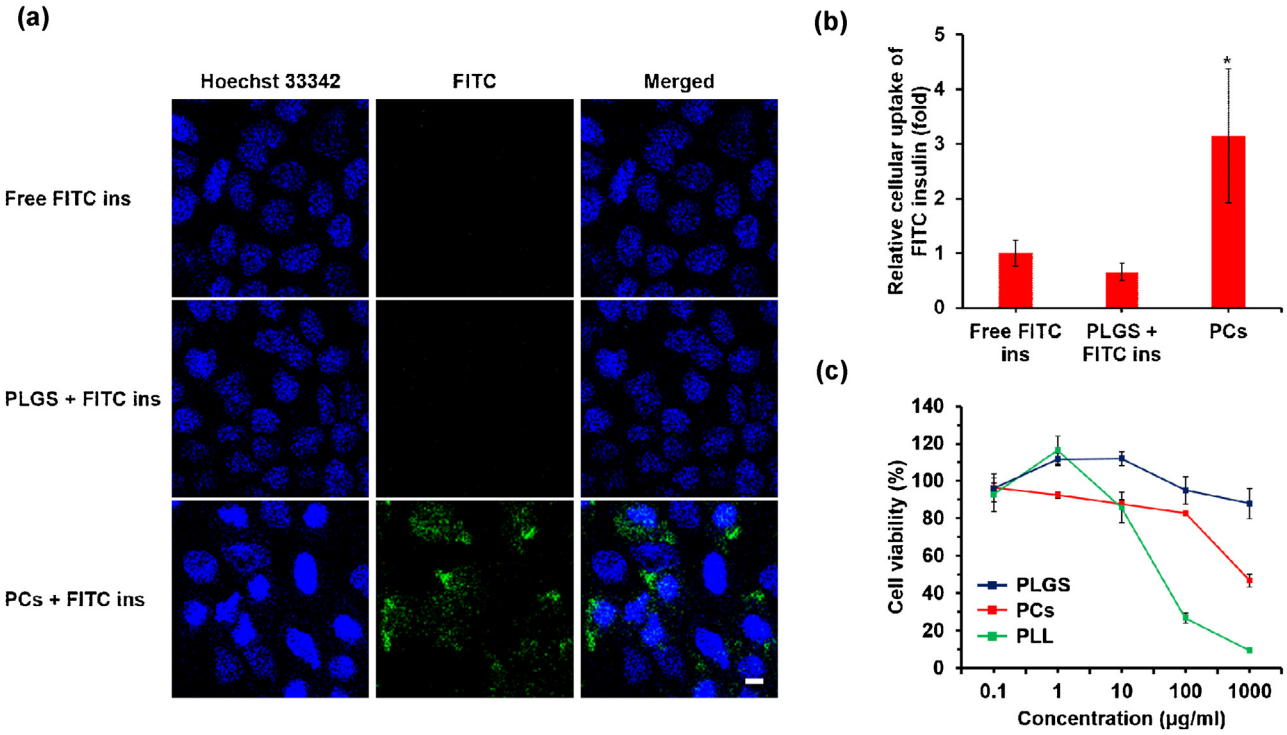

Fig. 6. (a) CLSM images of Free FITC ins, PLGS + FITC ins and PCs + FITC ins using Caco-2 cells (Scale bar: 10 μm). (b) Relative cellular uptake of FITC insulin by the treatment of free FITC ins, PLGS + FITC ins, PLL + FITC ins and PCs + FITC ins against Caco-2 cells. ($n = 3$) Significant differences are expressed as follows: *, compared with free FITC ins and PLGS + FITC. *$p < 0.05$ (c) Relative cell viability of PLGS, PLL and PCs employing MTT assay against Caco-2 cells with different concentrations ($n = 3$). All the error bars were expressed as standard deviation. (For interpretation of the references to colour in the text, the reader is referred to the web version of this article.).

membrane can lead to the destruction of the cell structure, and finally to the cell death. Unlike PLL, polyanion PLGS does not interact strongly with the cell membrane. Therefore, the severity of PLGS was markedly reduced compared to PLL, even though the highly charged PLGS showed cytotoxicity. Though the raw materials exhibited cytotoxicity, almost no toxic effect was observed in the PCs. Since the PCs were formed by combining PLGS and PLL together, the strong charges of both polypeptides can be canceled out to yield more moderately charged complexes. Thanks to the minimal toxicity of the PCs, a high dose can be achieved for clinical study without any undesirable health problems.

In vivo *hypoglycemic effect study with diabetic mice*

To investigate the oral insulin delivery efficiency of the PCs, an *in vivo* study was carried out with streptozotocin (STZ) induced diabetic mice. As expected, the PBS administered group, which was used as a blank control, did not show any significant changes in the blood glucose level for 24 h, indicating successful induction of the diabetic models (Fig. 7). A similar trend was observed in the group that orally administered the insulin solution, revealing the complete degradation of the administered insulin. Unlike PBS or the insulin solution treated group, the group that orally administered the combination of insulin and PLGS decreased the blood glucose level to more than 10% of the initial blood glucose level. Although the same insulin dose was used in both the insulin solution and the combination of PLGS with insulin, there was a recognizable difference between the blood glucose levels of both groups. The PLGS in the insulin treated group showed a convincing decrease of the blood glucose level while the insulin solution administered group did not. The difference in the blood glucose level is meaningful since it implies the proper protection of insulin with PLGS. As designed, polyanion PLGS succeeded in achieving the controlled release of insulin, so that insulin can be delivered to the small intestine without any critical damage. A more considerable decline in the blood glucose level was observed in the PCs administered group. After the oral administration of the PCs, the blood glucose level gradually decreased until 80% of its initial value, which is a notable decrease compared to the other groups. The significant decreasing trend of the blood glucose level might be attributed to the synergistic effect of the two polypeptides: PLGS and PLL. In order to prepare the PCs, intestinal penetration enhancing polypeptide PLL was additionally added to the combination of PLGS and insulin. Therefore, the insulin that was delivered to the small intestine with the help of PLGS can easily

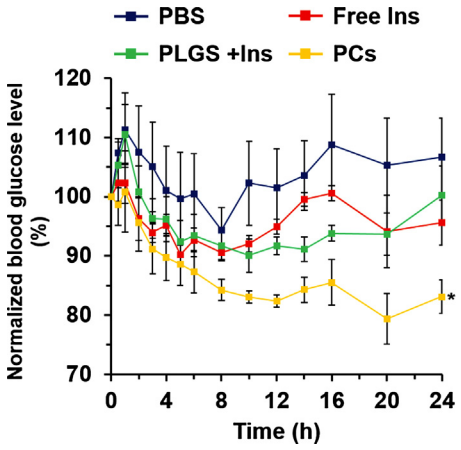

Fig. 7. Profiles of blood glucose level after oral administration of insulin solution (Free Ins), insulin combined with PLGS (PLGS + Ins) or the PCs at an insulin dose of 150 IU/kg. Oral administration of PBS was used as a blank control. The error bars indicate standard error ($n = 3$–6). Significant differences are expressed as follows: *, compared with blank control PBS administered group. *$p < 0.05$.

penetrate the intestinal epithelial cells by virtue of PLL. Furthermore, the blood glucose level maintained its lowest value for more than 14 h, indicating the long lasting hypoglycemic effect of the PCs. The long lasting property of the PCs can reduce the number of administrations, so that patients can be free from the inconvenience of frequent administration. In addition, the actual insulin dose might be notably reduced in human studies since the sensitivity of mice to the porcine insulin is relatively low. The function of PLL was used for enhancing the cellular uptake of the PCs themselves or the released insulin. A positively charged helical polypeptide PLL effectively functioned to improve the insulin permeation through the epithelial cells, and it also assisted the PCs. By virtue of PLL, the complexes showed a significantly enhanced penetration of insulin than the group incubated with the FITC insulin solution. Unlike PLL, polyanion PLGS cannot strongly attach itself to the negatively charged intestinal epithelial cells, so that the insulin combination with PLGS could not encourage the penetration of insulin. In addition to the quantitative analysis, the internalization of FITC insulin was also visualized through the CLSM images (Fig. 6a). The FITC insulin absorption trend observed in the CLSM images was in accordance with the quantitative interpretation. Although the FITC signal was rarely observed in the cells incubated with the FITC insulin solution or the combination of PLGS with FITC insulin, the PCs treated group showed a green FITC signal. A strong FITC signal around the nuclei indicated the improved insulin cellular internalization with the help of the PCs.

Conclusions

Artificial monodispersed polypeptides PLGS and PLL were successfully synthesized by NCA polymerization. The molecular weight of polypeptides was well controlled by changing the feeding ratio of NCA monomer to HMDS. Also, both polypeptides showed an alpha helical property, which is essential for enhancing the cell permeability. The PCs were then formed by a simple mixing process between two different polypeptides and insulin. Through the *in vitro* FITC insulin release study, the controlled insulin release property of the PCs was confirmed. The PCs succeeded in releasing the loaded insulin in the intestinal pH condition while hindering the leakage of the insulin in the acidic stomach condition. Furthermore, the cell penetrating ability of the PCs on Caco-2 cells was examined by the *in vitro* cellular uptake study. The PCs enhanced the permeation of the loaded FITC labeled insulin in consequence of the cell penetration enhancing ability of cationic helical peptides, PLL. The cellular uptake enhancing property of the PCs was confirmed *via* both quantitative analysis and the CLSM images. The cytotoxicity study with Caco-2 cells verified the minimal toxicity of the PCs, even at a high concentration representing the biocompatibility of the complexes. Finally, an *in vivo* hypoglycemia study was conducted with diabetic mice, and the PCs significantly reduced the blood glucose level. The decrease of the blood glucose level indicated the successful delivery and penetration of orally administered insulin. In addition, the long lasting hypoglycemic effect of the PCs is expected to reduce the number of insulin administrations, which can provide better patient compliance. The non-toxic PCs verified the feasibility of it being a novel applicant for oral insulin delivery. For future study, the *in vivo* hypoglycemic effect of the PCs can be enhanced by developing a peptide that improves the cell penetrating property more than PLL.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jiec.2016.12.022.

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