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β-Cyclodextrin grafting hyperbranched polyglycerols as carriers for nasal insulin delivery

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ABSTRACT

We prepared a β-CD functionalized hyperbranched polyglycerol (HPG) with the purpose of enhancing the nasal transport of insulin in rats. Insulin-loaded HPG-*g*-CD nanoparticles (NPs) were prepared and the size of the NPs ranged from 198 to 340 nm with a positive charge. The NPs exhibited a great capacity of associating insulin, reaching the efficiency as high as 88.21%. *In vitro* release showed that the release rate of insulin was much faster under acidic condition than physiological condition. *In vitro* cytotoxicity against Caco-2 cells showed that HPG-*g*-CDs had good biocompatibility. The *in vivo* evaluation in rats demonstrated that insulin-loaded HPG-*g*-CD NPs had the ability to significantly decrease the blood glucose concentrations. Furthermore, the capability of HPG-*g*-CD NPs to cross the nasal mucosal epithelia was proved by confocal laser scanning microscopy (CLSM). Consequently, the results suggest that the HPG-*g*-CD NPs are promising carriers for nasal insulin delivery.

1. Introduction

Peptide and protein drugs, including insulin, are usually used as parenteral formulations, but this method of administration is sometimes associated with tissue invasion and infection. Oral delivery of insulin can replace daily injections to diabetic patients. However, poses unique problem of stability and susceptibility to proteolysis, which reduce their bioavailability (Shelma, Paul, & Sharma, 2010). Recently, researches have been focused on the nasal mucosa as an alternative route to the oral and parenteral routes, since it has many advantages. These include a large absorptive surface area and the high vascularity of the nasal mucosa, where drugs absorbed from the nasal cavity pass directly into systemic circulation, thereby avoiding first-pass liver metabolism (Chein & Chang, 1987).

However, the bioavailability of intranasal administered peptide and protein drugs may be low due to their high molecular weight and hydrophilicity (Merkus, Verhoef, Romeijn, & Schipper, 1991). In addition, the normal physiology of the nasal cavity presents several barriers to peptide and protein drugs absorption, including the physical removal by mucociliary clearance mechanism, enzymatic degradation and low permeability of the nasal epithelium (Wu, Wei, Wang, Su, & Ma, 2007).

Many strategies have been explored to improve the absorption of these drugs through the nasal mucosa, including the use of chemical penetration enhancers and proteolytic enzyme inhibitors, and designing suitable dosage formulations. Among these approaches, the use of absorption enhancers has been proven to be effective (Wang et al., 2002). Unfortunately, most of the traditional absorption enhancers, such as surfactants and bile salts, have limited clinical use because of the irreversible damage to the nasal mucosa when used at effective concentrations, particularly under long-term exposure (Khafagy, Morishita, Onuki, & Takayama, 2007).

Cyclodextrin (CD) can form non-covalent inclusion complexes with a large variety of drugs/proteins. Complexation represents a unique and effective strategy for improving protein therapy by stabilizing the drugs against aggregation, thermal denaturation and degradation (Sajeesh & Sharma, 2006). Moreover, CDs are believed to enhance nasal absorption of peptides by opening tight junctions and/or solubilizing membrane components (Marttin, Verhoef, & Merkus, 1998), which perturbs membrane integrity in a rather nonspecific manner. It is inevitable that varying extents of assault would occur to the mucosal tissue in intimate contact with CDs (Uekama, Hirayama, & Irie, 1998). However, when compared with other absorption-promoting agents and preservatives used commonly in nasal formulations, CDs exert a rather mild and reversible effect on the surface morphology of nasal mucosa

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and the ciliary beating (Adriaens, Voorspoels, Mertens, & Remon, 1997; Marttin, Verhoef, Romeijn, Zwart, & Merkus, 1996; Romeijn, Verhoef, Marttin, & Merkus, 1996). An additional challenge is the aqueous solubility of β-CD (1.8 g/100 mL at 25 °C), which is often insufficient at therapeutic doses (Gupta, Agashe, Asthana, & Jain, 2006). Therefore, it is essential to further decrease their harmful effects on the mucosal tissue and increase the solubility of β-CD.

Hyperbranched polyglycerols (HPGs) are readily available, well-defined polymer with dendritic branching obtained by controlled anionic polymerization of glycidol (Sunder, Hanselmann, Frey, & Mulhaupt, 1999; Sunder, Mulhaupt, Haag, & Frey, 2000). Moreover, they are promising biomaterials owing to their high water solubility, chemical reactivity, high biocompatibility (as high as that of PEG), and thermally and oxidatively stability (more stable than PEG) (Siegers, Biesalski, & Haag, 2004). Furthermore, the multiple free hydroxyl groups of HPG can be further functionalized with various groups for specific use like many other hyperbranched polymers (Calderón, Warnecke, Gräser, Haag, & Kratz, 2008; Gottschalk, Wolf, & Frey, 2007; Jones, Gao, & Leroux, 2008). It has been reported that hydrophobically derivatized hyperbranched polyglycerols are mucoadhesive, since the surface of this dHPG contains numerous hydroxyl groups and PEG chains (Mugabe et al., 2008). Additionally, in the previous report, we had designed an effective protein delivery system using HPG, which was functionalized with polylactic acid (Gao et al., 2009).

In the present study, we developed a novel nanoparticle (NP) system based on the coupling of β-CD and HPG with the objective of exploring its application as a drug delivery vehicle for insulin. High loading capacity (LC) and encapsulation efficiency (EE) were achieved. The physicochemical properties of the insulin-loaded NPs and the *in vitro* release of insulin were evaluated. Furthermore, the ability to enhance the nasal absorption of insulin was investigated by determining the decrease in blood glucose levels following nasal administration. Finally, the interaction of HPG-*g*-CD NPs with the nasal epithelium was investigated by confocal laser scanning microscopy (CLSM).

2. Materials and methods

2.1. Materials

Glycidol was purchased from Shenyang Jinjiuqi Chemical Co., Ltd. (Shenyang, China). 4-Toluene sulfonyl chloride (TsCl) was recrystallized in petroleum ether before use. Dimethyl sulfoxide (DMSO) and 1,2-ethylenediamine (EDA) were freshly distilled with anhydrous magnesium sulfate and sodium sulfate, respectively. β-CD was obtained from the Sinopharm Chemical Reagent Co., Ltd., China. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was purchased from J&K-Acros Chemical Ltd. (China) without further purification. The dialysis membrane (3.5 kDa and 8 kDa cutoff) was purchased from Shanghai Green Bird Science and Technology Co., Ltd. (Shanghai, China). Pure crystalline porcine insulin (with a nominal activity of 28 IU/mg) was obtained from Xuzhou Wanbang Biochemical Co., Ltd. (Jiangsu, China) and used without further purification. Fluorescein isothiocyanate (FITC) was purchased from Tianjin Lianxing Biotechnology Co., Ltd. (Tianjin, China). The other chemicals were of analytical grade and used as received. The animal experiments had been proved by animal ethical committee in Experimental Animal Center of Tianjin.

2.2. Synthesis of HPG-g-CD

HPG, with a molecular weight of 70 kDa (M_w/M_n = 2.0) was synthesized by the anionic polymerization of glycidol in the presence of alkoxides according to the previous published method (Sunder et al., 1999). The HPG-*g*-CD copolymers were synthesized by the reaction of amine and mono-tosylated β-CD with some modifications (Arima, Kihara, Hirayama, & Uekama, 2001; Kihara, Arima, Tsutsumi, Hirayama, & Uekama, 2003; Kojima, Toi, Harada, & Kono, 2008) and the structure of the copolymer was analyzed by ^{1}H NMR (Varian Unity-plus 400 NMR Spectrometer) and FTIR (FTS-6000, Bio-Rad Co.) (Zhang et al., 2011). By changing the mass ratio of HPG to β-CD, samples with different β-CD grafting ratio were obtained, named HPG-*g*-CD1, HPG-*g*-CD2 and HPG-*g*-CD3, respectively. The apparent amount of β-CD was measured by the previous method (Zhang et al., 2009) and the results are shown in Table 2. However, the molecular weights of the HPG-*g*-CD copolymers failed to be determined by gel permeation chromatography (GPC), owing to the strong interaction of hydroxyl groups in the β-CD ring with GPC column (Zhou et al., 2009).

2.3. Insulin-loaded NPs preparation

Various concentrations of polymer (0.1–4 mg/mL) and insulin (0.5 and 2 mg/mL) aqueous solutions were prepared. 2 mL of insulin solution was slowly added to the same volume of polymer solution under magnetic stirring at room temperature. The solution was incubated overnight at room temperature. The NP suspension was centrifuged at 16,000 × *g* for 30 min, and the resultant NPs were freeze-dried.

2.4. Characterizations of the HPG-g-CD NPs

The hydrodynamic diameter (D_H) and size distribution of NPs were determined by dynamic light scattering (DLS) using a dynamic light scattering particle size analyzer (Brookhaven, INNDVO300/BI900AT) at 25 °C. The zeta potential of the NPs was measured using a zeta potential meter (Zetasizer 3000HS, Brookhaven) at 25 °C. The morphological characteristic of the insulin-loaded NPs was determined by transmission electron microscopy (TEM, Philips, EM400ST).

2.5. Stability of the insulin-loaded HPG-g-CD NPs

In order to evaluate the colloidal stability of the insulin-loaded HPG-*g*-CD NPs in acetate buffer (pH 4.0), the variation of hydrodynamic diameter (D_H) with time at 25 °C was assessed. Size measurements at given time intervals were recorded using a dynamic light scattering particle size analyzer (Brookhaven, INNDVO300/BI900AT).

2.6. Evaluation of the insulin-loading capacity of the NPs

The association capacity of insulin was determined after isolating the NPs from the NP suspension containing free insulin, which was achieved by three cycles of dispersion–recentrifugation (16,000 × *g*, 30 min, 4 °C). The amount of free insulin in the collected supernatant was measured by the Bradford method using a UV spectrometer (Shimadzu UV-2550) at 595 nm (Bradford, 1976). The EE and LC of insulin were calculated using the following equations:

$$\text{EE}\ (\%) = \frac{\text{Total insulin} - \text{free insulin}}{\text{Total insulin}} \times 100\% \quad (1)$$

$$\text{LC}\ (\%) = \frac{\text{Total insulin} - \text{free insulin}}{\text{NPs weight}} \times 100\% \quad (2)$$

2.7. In vitro dug release

Insulin release from the HPG-*g*-CD NPs was analyzed by incubating insulin-loaded NPs (5 mg) at 37 °C in 2 mL of phosphate buffer solution (PBS, 0.1 M, pH 7.4) or acetate buffer (0.1 M, pH 4.0) while

Table 1
Diameter and size distribution of the insulin-loaded HPG-*g*-CD3 NPs with various polymer to insulin mass ratios using insulin concentration of 0.5 and 2 mg/mL ($n = 3$; mean ± SD).

Polymer/insulin (w/w)	Insulin concentration 0.5 mg/mL		Insulin concentration 2 mg/mL	
	Average diameter (nm)	PDI	Average diameter (nm)	PDI
0.2:1	474.3 (±6.9)	0.270 (±0.025)	874.4 (±9.7)	0.366 (±0.033)
0.4:1	449.4 (±5.4)	0.275 (±0.036)	518.1 (±7.8)	0.252 (±0.027)
1:1	340.6 (±5.8)	0.123 (±0.029)	372.5 (±7.4)	0.156 (±0.045)
1.5:1	354.7 (±6.1)	0.148 (±0.041)	>1 μ	–
2:1	>1 μ	–	>1 μ	–

shaking (100 rpm). At predetermined time-points, samples were centrifuged at 16,000 × *g* for 5 min and 100 μL of the supernatant was removed and replenished by fresh buffer solution. The amount of free insulin was determined by the Bradford method and a calibration curve was generated using the non-loaded NPs to correct for the intrinsic absorption of the polymer. All the samples were analyzed in triplicate and error bars in the plot represent the standard deviation.

2.8. Cell toxicity assays

Cell viability was evaluated according to the MTT method by using Caco-2 cells under ranges of concentrations (25, 50, 100, and 200 μg/mL) of blank NPs as previously described (Jin et al., 2009). The incubation time was 48 h and 96 h, and the untreated cells were used as control groups and their viability rate was set to 100%. The cell viability in each wall was calculated as follows:

$$\text{Cell viability}\ (\%) = \frac{\text{Average optical density values in experimental groups}}{\text{Average optical density values in the control groups}} \times 100\%$$

Each experiment was performed in triplicate and the results were reported as mean standard deviation.

2.9. Intranasal administration

Animal experiments were conducted using streptozotocin (STZ)-induced hyperglycemic rats (230–260 g), one week after rats received 45 mg/kg of STZ dissolved in sodium citrate buffer (50 mM, pH 4.5) intravenously. The freeze dried insulin-loaded HPG-*g*-CD NPs were suspended in 0.5 mL acetate buffer (pH 4.0). Insulin dissolved in acetate buffer (pH 4.0) was used as a control. The blank and insulin-loaded NP suspension and insulin solution were administered to the nasal cavity in a volume of 100 μL (50 μL in each nostril) via 3 cm of polyethylene tubing (0.5 mm diameter), which was inserted into the nasal cavity by about 5 mm. Blood samples (0.2 mL) were collected from the eyes 30 min before the nasal administration to establish the baseline glucose level and at predetermined time points after dosing for up to 6 h. Blood glucose levels were determined by the glucose–oxidase method. Results are presented as mean average values (% of basal level ± SD) for $n = 5$.

2.10. Interaction of HPG-g-CD NPs with the nasal epithelial cells by confocal laser-scanning microscopy (CLSM)

HPG-*g*-CDs were labeled with FITC based on the reaction between the isothiocyanate group of FITC and the primary amino groups of HPG-*g*-CDs following the method described in the literature (Lin, Mi, Chen, Chang, & Liang, 2007). The FITC-labeled HPG-*g*-CD NPs were dispersed in PBS (0.5 mg/mL), and then the NPs suspension was intranasally administered to rats (50 μL per nostril). One hour after the administration, the rats were killed by cervical dislocation. After removing the skin around the nasal region, the whole nasal mucosa was removed and washed immediately with PBS (0.1 M, pH 7.4) three times and directly observed by CLSM using an argon laser at 488 nm excitation wavelength.

2.11. Statistical analysis of in vivo data

Statistical differences were determined by using one-way ANOVA followed by the Student–Newman–Keuls method for multiple comparisons. Differences were assigned to be significant for values of $P < 0.05$.

3. Results and discussion

3.1. The particle size and morphology of NPs

The size of the insulin-loaded HPG-*g*-CD3 polymeric NPs at different polymer to insulin mass ratio was studied by DLS. As can be seen from Table 1, at higher polymer ratios, the size of NPs exceeded 1 μm. It was possible that the NPs were prone to aggregate when the concentration of copolymer was high. Aggregation also occurred at a higher insulin concentration (2 mg/mL).

At polymer to insulin mass ratio of 1:1, the insulin-loaded HPG-*g*-CD3 NPs were formed with a relatively low polydispersity index (PDI) at both of the two insulin concentrations. Therefore, we chose the ratio of 1:1 to conduct the following investigations.

The size of blank and insulin-loaded HPG-*g*-CD polymeric NPs is depicted in Table 2. It is evident that the size of the blank NPs increased from 177.5 to 253.9 nm, depending on the polymer composition (grafting ratio of β-CD). This could be probably explained by the increasing amount of β-CD grafted onto the HPG.

The size of insulin-loaded NPs was somewhat above the range observed with the blank NPs, varying from 198.5 to 340.6 nm, which was the result of the entrapment of insulin molecules. From the morphology of insulin-loaded HPG-*g*-CD2 NPs in Fig. 1, we could

Table 2
The physicochemical properties of HPG-*g*-CDs NPs ($n = 3$; mean ± SD).

Sample	HPG-TS/mono-6-OTs-β-CD (mg/mg)	β-CD (μmol/g)	Average diameter (PDI) (nm)		Zeta potential (mV)	
			Blank	Insulin-loaded	Blank	Insulin-loaded
HPG-*g*-CD1	100/100	18.07	177.5 ± 5.2 (0.303)	198.2 ± 3.5 (0.313)	+25.30 ± 2.9	+9.95 ± 1.9
HPG-*g*-CD2	100/250	19.97	229.8 ± 3.8 (0.220)	295.9 ± 4.1 (0.245)	+39.40 ± 3.1	+10.46 ± 1.4
HPG-*g*-CD3	100/500	30	253.9 ± 4.8 (0.208)	340.6 ± 5.7 (0.123)	+43.20 ± 2.5	+12.69 ± 0.9

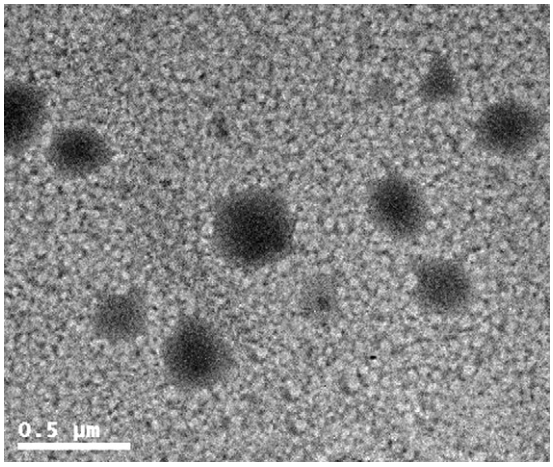

Fig. 1. Transmission electron microscopy (TEM) image of insulin-loaded HPG-*g*-CD2 NPs.

see the well-dispersed micelles with regular spherical shape. From the TEM image, the size of the micelles was around 200–300 nm, which was in accordance with the result of DLS.

3.2. The zeta potential of NPs

As shown in Table 2, the zeta potential of blank NPs ranged from +25.3 to +43.2 mV. The positive surface charge is considered to be an advantage from the biopharmaceutical viewpoint, as it favors the muco- and bioadhesion of the NPs to negatively charged sites on cell surfaces and tight junctions (Kotze et al., 1997, 1998; Smith, Wood, & Dornish, 2004). Addition of insulin resulted in the reduction of zeta potential, suggesting that some insulin molecules coated the surface of the particle, which was able to shield the positive charge of HPG-*g*-CD NPs (Elfinger et al., 2009).

3.3. Stability of the insulin-loaded HPG-g-CD NPs

We selected acetate buffer (pH 4.0) to administer insulin-loaded NPs intranasally to diabetic rats, since this pH was adequate to solubilize insulin (p*I* 5.3) and lower than that of nasal secretions (pH 5.5–6.5) (Goycoolea, Lollo, Remunan-Lopez, Quaglia, & Alonso, 2009). Therefore, it was necessary to investigate the stability of our formulations in this buffered medium before *in vivo* studies. The results were demonstrated in Supporting information S2. The NPs were seen to be stable after 100 min in a buffered medium (acetate buffer, pH 4.0), indicating that they could be maintained in a stable state for up to 100 min.

3.4. LC and EE of NPs

Insulin-loaded HPG-*g*-CD NPs was prepared by mixing the polymer and insulin solution together. The process had a major advantage in that the insulin loading could be achieved without the aid of organic solvents or other harmful treatment. Since HPG-*g*-CD was positively charged at pH 7.4, its electrostatic interaction with negatively charged insulin (p*I* 5.3) was favored. We incubated HPG-*g*-CD with insulin at a range of pHs, in order to determine the effect of electrostatic interaction on the association of insulin with HPG-*g*-CD. As shown in Table 3, when pH value decreased from 7.4 to 4.0, the LC of insulin decreased from 18.21% to 11.33% for HPG-*g*-CD1, 19.4% to 13.05% for HPG-*g*-CD2, and 20.37% to 14.96% for HPG-*g*-CD3 NPs ($P < 0.05$). The same trend was observed for EE. The results suggest that higher LC and EE were obtained when the insulin was dissolved at a pH above its isoeletric point (pH 7.4), so that the insulin was predominantly negatively charged (Calvo, Remunan-Lopez, Vila-Jato, & Alonso, 1997). This indicates that the association of insulin to HPG-*g*-CD NPs was partially mediated by an electrostatic interaction between insulin and positively charged HPG-*g*-CD. This mechanism was in accordance with the partial neutralization of the surface charge of NPs due to the loading of insulin. The EE of HPG-*g*-CD increased with the increasing content of β-CD from 18.07 to 30 μmol/g ($P < 0.05$). The probable reason was considered to be formation of more inclusion complexes between cyclodextrin and insulin. However, there was no remarkable difference among the LC of the three types of NPs. This might be attributed to the different yield of NPs when centrifuged. In fact, cyclodextrins and their hydrophilic derivatives are known to enhance the loading capacity of liposomes and polymeric nano/microparticles (Duchene, Ponchel, & Wouessidjewe, 1999).

3.5. In vitro release of insulin

Fig. 2 shows the profiles of the cumulative release of insulin from insulin-loaded NPs with different β-CD content at pH 7.4 and 4.0, respectively. The release rate of insulin was initially rapid and then decreased several hours later. The initial burst release of insulin was attributed to the adsorption of insulin on the periphery of the NPs. In addition, the drug density in the NPs also exerted great influence on the insulin release rate. The higher the drug density in NPs was, the faster the insulin was released from NPs. This was due to the fact that the diffusion process of insulin from polymeric NPs to release medium was easier for the higher drug density sample, resulting in a faster drug release rate (Chen et al., 2008). Because the release also reduced the drug density inside the NPs, the later stage of insulin release became slow. Moreover, as it can be seen from Fig. 2B, the release of insulin was affected by β-CD content. Both the cumulative release percentage and release rate of insulin were enhanced with increasing β-CD content. This indicates that a significant role was played by β-CD on the controlled release of insulin.

Comparing the release profiles of insulin from the same copolymer NPs at different pH values, we could see that the release of insulin was much slower at pH 7.4 than at pH 4.0. In addition, only 40% of loaded insulin could be release at pH 7.4, which was much lower than the amount of insulin released from the NPs at pH 4.0. It was attributed to the strong electrostatic attraction existed between the negatively charged insulin (p*I* 5.3) and positively charged HPG-*g*-CD at pH 7.4, which would restrain the release of insulin, and the increased electrostatic repulsion between positively charged HPG-*g*-CD and insulin at pH 4.0, which would accelerate the insulin release. These results suggest that the electrostatic interaction between insulin and HPG-*g*-CD was an important factor in determining the release rate of insulin.

Table 3
Effect of pH on insulin loading capacity and encapsulation efficiency of HPG-*g*-CDs ($n = 3$; mean ± SD).

pH	HPG-*g*-CD1		HPG-*g*-CD2		HPG-*g*-CD3	
	LC (%)	EE (%)	LC (%)	EE (%)	LC (%)	EE (%)
7.4	18.21 ± 1.15	76.54 ± 3.22	19.41 ± 2.14	80.35 ± 3.88	20.37 ± 3.05	88.21 ± 4.21
4.0	11.33 ± 1.32	54.36 ± 2.99	13.05 ± 1.69	60.26 ± 2.94	14.96 ± 2.49	66.43 ± 3.69

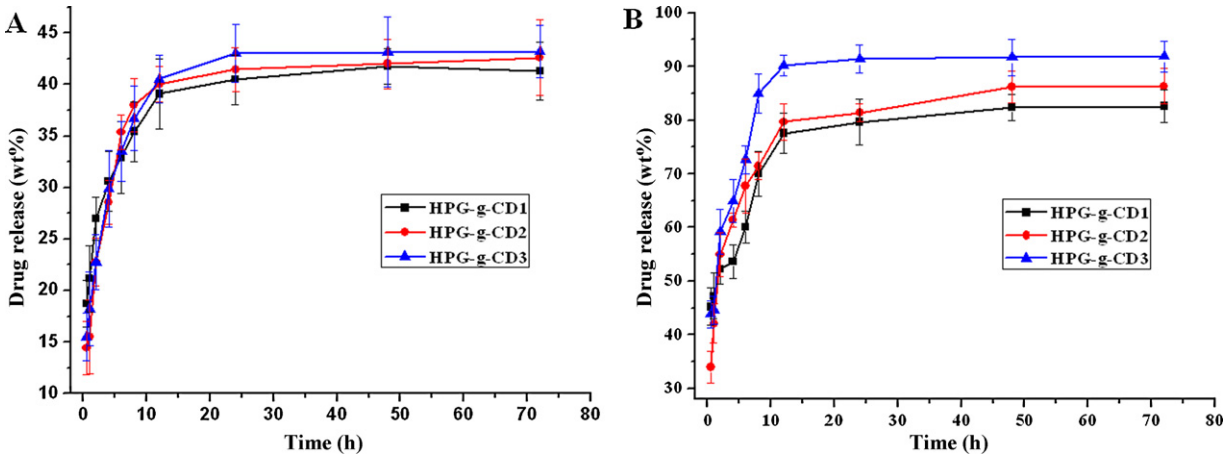

Fig. 2. *In vitro* cumulative percent release of insulin from the HPG-*g*-CD NPs: the effect of polymeric composition on the insulin release in PBS of pH 7.4 (A) and acetate buffer of pH 4.0 (B). Triplicates for each sample were analyzed and each datum point represents the mean value ± SD ($n = 3$).

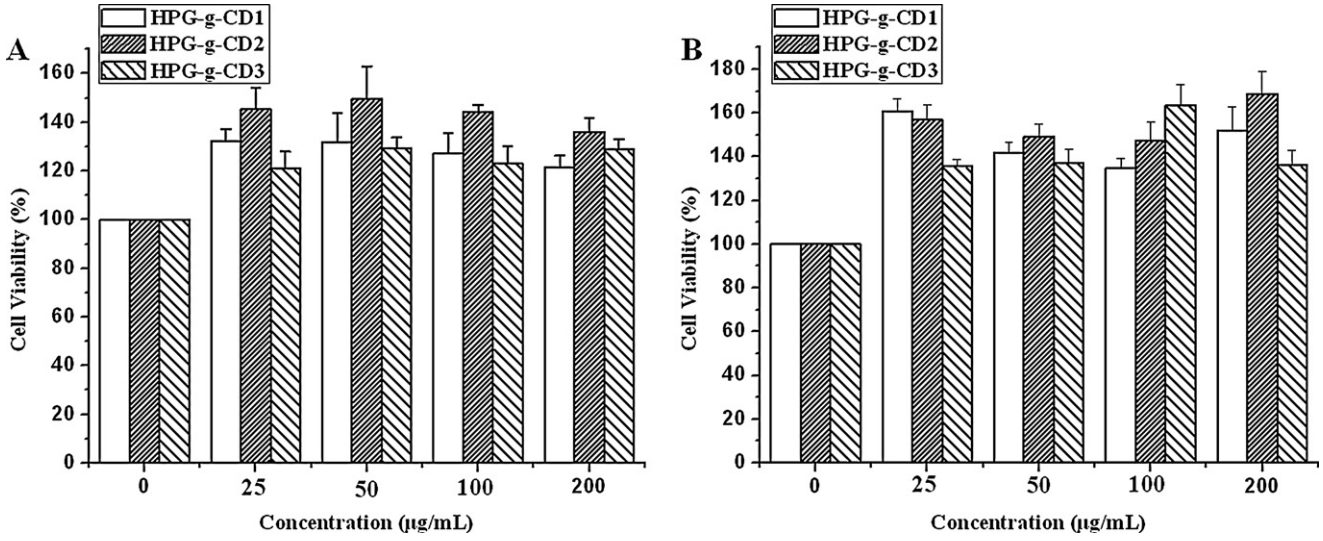

Fig. 3. Cell viability assay in Caco-2 cell line. Cell viability was determined by the MTT assay: various materials (HPG-*g*-CD1, HPG-*g*-CD2 and HPG-*g*-CD3) at different concentrations (25, 50, 100 and 200 μg/mL) for 48 h (A) and 96 h (B). (The cell viability of control groups was set to 100%.)

3.6. Cell viability

HPG showed very good biocompatibility according to the previous report (Kainthan, Janzen, Levin, Devine, & Brooks, 2006) and the toxicity of CDs was dependent on the route of administration, the cavity size (α-, β- or γ-CD), and chemical modification (hydroxypropyl-CD, sulfobutylether-CD, CD sulfate and methylated CD) (Zhang et al., 2009). Therefore, it was important in the present study to verify the innocuous nature of the polymerized β-CD on the surface of HPG in order to prevent the deactivation of the encapsulated insulin.

Fig. 3 shows the results of cytotoxicity measurements of the samples analyzed by the MTT method in Caco-2 cell line. As shown in Fig. 3A, there was no toxic effect of all HPG-*g*-CD NPs up to a concentration of 200 μg/mL (cell viability above 100%) after incubating for 48 h. This might be attributed to the reduction of the density of amino groups by the introduction of β-CD to HPG-*g*-CDs. Davis et al. reported that the introduction of cyclodextrin lowered the toxicity of polyether-imide (PEI) by decreasing the density of amino groups. In addition, the large cyclodextrin molecules can sterically reduce nonspecific binding affinities (Pun et al., 2008). From Fig. 3B, we could see that the cell viability of Caco-2 cells was maintained over 100% for 96 h and no toxicity was shown with an increase

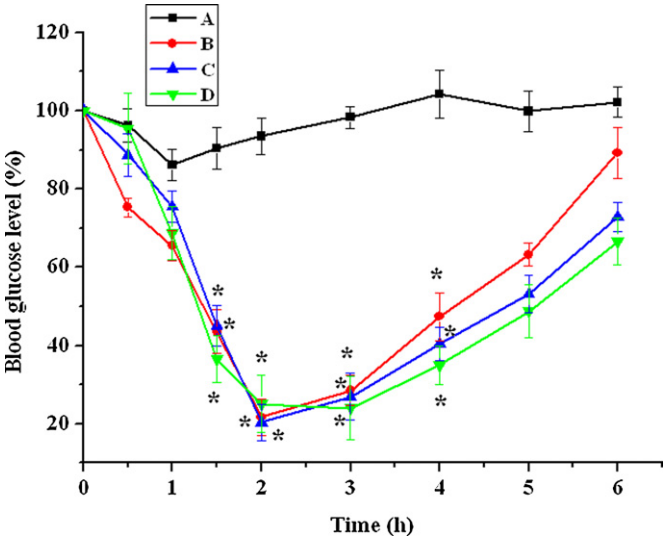

Fig. 4. Blood glucose levels in rats following nasal administration of insulin solution (A), insulin-loaded HPG-*g*-CD1 (B), insulin-loaded HPG-*g*-CD2 (C), and insulin-loaded HPG-*g*-CD3 NPs (D) in acetate buffer (pH 4.0). Mean ± SD, $n = 5$. *Statistically significant differences from control insulin solution ($P < 0.05$).

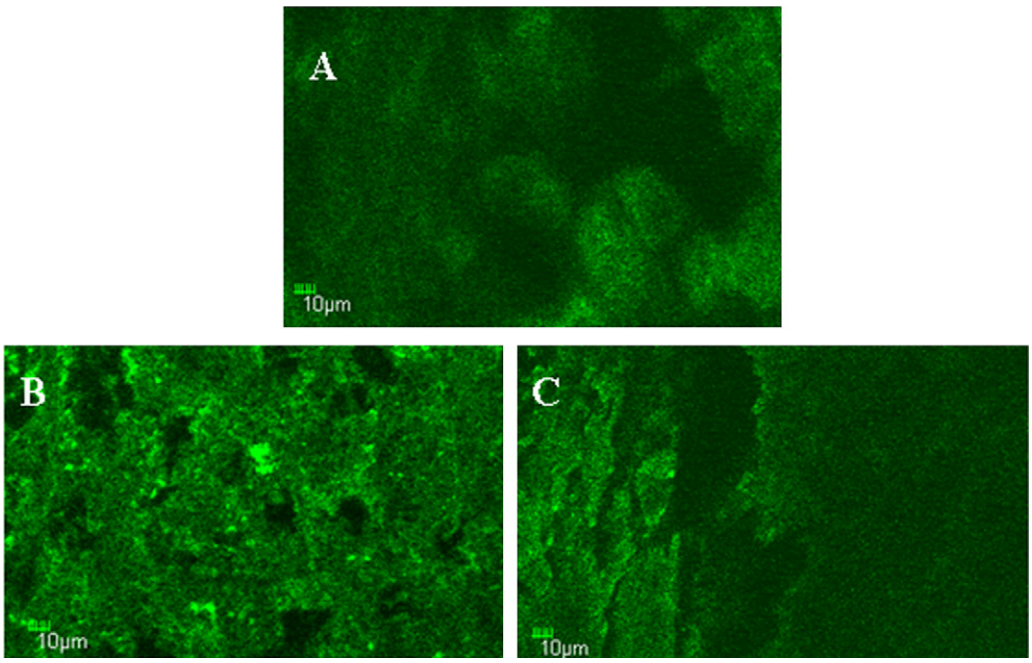

Fig. 5. Confocal laser scanning microscopy of the rat nasal tissue excised following administration of HPG-*g*-CD1 NPs (A), HPG-*g*-CD2 NPs (B) and HPG-*g*-CD3 NPs (C).

in the concentration. Therefore, the results show that the HPG-*g*-CD NPs were biocompatible and had high potential for *in vivo* use.

3.7. In vivo efficiency of insulin-loaded HPG-g-CD NPs

Blank and insulin-loaded HPG-*g*-CD NPs suspension and insulin solution were administered intranasally to diabetic rats (dose of insulin: 5 IU/kg). From the experimental results, we found hardly effect was exerted on the blood glucose level by blank NPs (data not shown). As shown in Fig. 4, the blood glucose levels of diabetic rats treated with free insulin were not reduced significantly (maximum glucose decrease of 14%) at 1 h post-administration and increased again immediately. In contrast, the insulin-loaded HPG-*g*-CD2 NPs remarkably enhanced the hypoglycemic effect (maximum glucose decrease of 80% at 2 h after dosing) and a low blood glucose level was maintained for about 6 h. A similar hypoglycaemic effect was observed following administration of HPG-*g*-CD1 and HPG-*g*-CD3 NPs. Interestingly, the hypoglycaemic effect was prolonged as the amount of β-CD increased. These results confirm that HPG-*g*-CD NPs could improve the systemic absorption of insulin in its active form after administration and the method of preparing NPs did not damage the drug.

The possible mechanisms for the enhancement of the nasal absorption by HPG-*g*-CD NPs might be due to the following reasons. Firstly, CDs are believed to enhance nasal absorption of peptides and proteins by inhibiting their enzymatic degradation, disrupting the epithelial membrane by extraction of phospholipids and proteins, and/or opening tight junctions (Marttin, Verhoef, Romeijn, & Merkus, 1995; Marttin et al., 1998; Merkus et al., 1999). Secondly, the positive charge of the NPs might also play an important role, since the interaction of positively charged material with the negatively charged epithelium membrane would be helpful for opening the tight junction and facilitating the absorption of drugs across the paracellular pathway (Schipper et al., 1997). In the previous publications, it has been reported that nasal insulin absorption is remarkably promoted by the hydrophilic derivatives of cyclodextrin (Merkus et al., 1991; Schipper, Romeijn, Verhoef, & Merkus, 1993; Teijeiro-Osorio, Remunan-Lopez, & Alonso, 2009).

3.8. Interaction of NPs with the nasal epithelial cells

FITC-labeled HPG-*g*-CDs NPs were administered into the nasal cavity of rats. The CLSM images of vertical cross-sections of nasal epithelium (Fig. 5) showed strong fluorescence intensity for the three materials, illustrating that the NPs were able to enter the nasal epithelium. It has been reported that cyclodextrins could cause the opening of tight junctions, thereby accounting for the increased paracellular transport (Hinchcliffe & Illum, 1999).

4. Conclusion

The hyperbranched copolymer HPG-*g*-CD was synthesized and showed good biocompatibility. The process of preparing the insulin-loaded HPG-*g*-CD NPs was carried out under a mild condition, achieving a high loading capacity. The *in vitro* insulin release was affected by the pH of the release medium and the content of β-CD in HPG-*g*-CD. Moreover, the insulin-loaded HPG-*g*-CD NPs were able to transport insulin across the nasal barrier and enhance the nasal absorption of insulin, resulting in a significant decrease in blood glucose concentrations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2011.01.057.

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