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Preparation and characterization of monomethoxypoly(ethylene glycol)-insulin conjugates

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Insulin was modified with monomethoxypoly(ethylene glycol) 5000 (MPEG5000) by reaction of insulin amino groups with activated MPEG5000, and mono-, di- and tri-PEGylated insulin were obtained. Circular dichroism demonstrated that the attachment of MPEG5000 to insulin did not alter the tertiary structure of insulin. PEGylation of insulin could inhibit the self-association tendency of insulin, which decreased with the increase of substitution degree. The physical stability and proteolytic stability of insulin increased after MPEG conjugation, and the stabilities of the conjugates depended on the PEGylation degree. *In vivo* biological activity of mono-PEGylated insulin following parenteral administration both in mice and rats was preserved while di- and tri-PEGylated insulin showed a great loss in biological activity. In addition, mono-PEGylated insulin administered subcutaneously in mice as well as intravenously in rats displayed extended action profiles. These results indicated that mono-substituted MPEG5000-insulin conjugate was a good candidate for insulin replacement therapy.

1. Introduction

It is well known that covalent attachment of poly(ethylene glycol) (PEG) or monomethoxypoly(ethylene glycol) (MPEG) to therapeutic proteins and peptides, a process known as PEGylation, could increase their serum half-lives and improve their stabilities (Bailon and Berthold 1998). The PEGylation technology has been applied to insulin with various degrees of success (Hinds et al. 2000; Gordon-Still 2002; Calceti et al. 2004; Hinds et al. 2005). Low-molecular-weight MPEG was used in most of previous studies, and a two-stepped chemical procedure was employed on insulin starting with a reversible protection of some residues that could be involved in PEGylation. This procedure produced MPEG (750 and 2000)-insulin conjugates that displayed improved physical stability, prolonged half-life, and there is no loss in biological activity after conjugation of MPEG750 to insulin (Hinds et al. 2002; Calceti et al. 2004). However, the conjugates are more susceptible to enzymatic degradation by α-chymotrypsin (Hinds 2001), and this method is not commonly suitable for proteins, because the protein's structure can be affected during the protection and deprotection reactions (Veronese and Pasut 2006).

Previous studies implied that by increasing the polymer mass on the protein surface either by increasing the PEG molecular mass or the number of PEG chains bound, further prolongation of action and enhanced stability could be achieved (Veronese et al. 1996; Uchio et al. 1999). In this study, insulin was selected as a model drug, and MPEG5000-insulin conjugates were synthesized by reaction of insulin with active ester of MPEG5000 under controlled conditions in order to increase its stabilities and prolong its action profile. Separation of the conjugates was achieved by ion-exchange chromatography and reversed-phase HPLC. The self-association behavior, physical stability, proteolytic stability and biological activity of each conjugate were examined as compared to native insulin.

2. Investigations, results and discussion

2.1. Preparation of MPEG5000-insulin conjugates

Succinimidyl ester of carboxymethyl monomethoxypoly(ethylene glycol) 5000 (MPEG5000-SCM) was used for conjugation of PEG to amino groups of insulin under alkaline condition. The reaction mixture contained mono-, di-, and tri-PEGylated insulin because there are three free amino groups on insulin. The reaction mixture was separated to five fractions by strong cation-exchange chromatography (Fig. 1). The result of SDS-PAGE analysis (Fig. 2) indicated that fraction 1 which eluted as flow-through contained tri-PEGylated insulin, while fraction 2, 4, and 5 corresponded to di-PEGylated insulin, mono-PEGylated insulin, and unmodified insulin, respectively. Fraction 3 was a mixture of di- and mono-PEGylated insulin, which might be positional isomers of fraction 2 and 4, respectively. The fraction 1 was further purified by strong anion-exchange chromatography to remove inactive MPEG that was detected by the colorimetric method (Sims and Snape 1980). As shown in Fig. 3, the inactive MPEG was separated from tri-PEGylated insulin and eluted as flow-through. The main peak in Fig. 3 evidently corresponded to tri-PEGylated insulin.

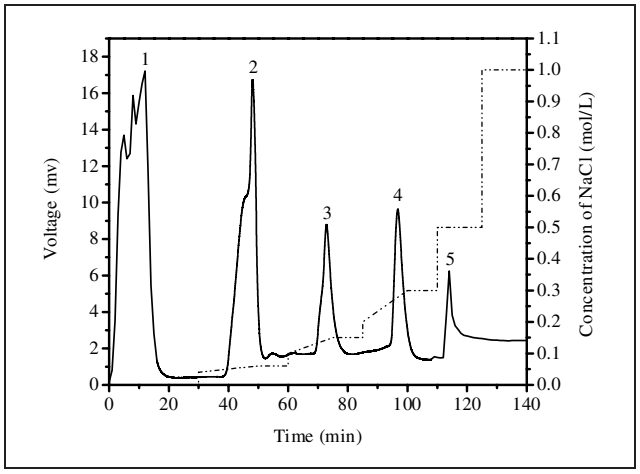

Fig. 1: Fractionation of MPEG5000-insulin conjugates by cation-exchange chromatography using SP Sepharose FF media

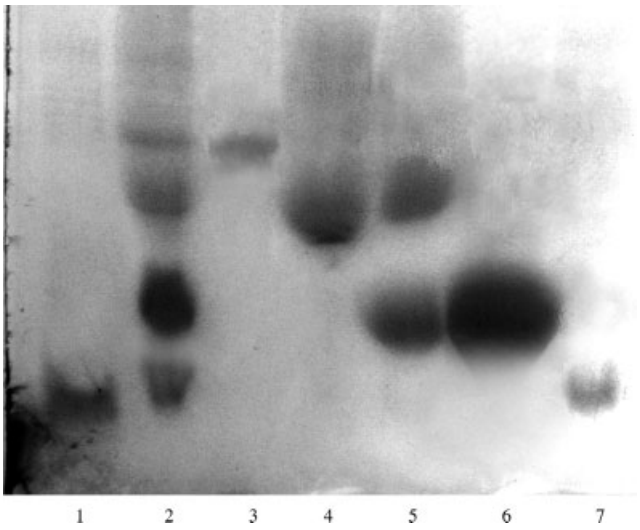

Fig. 2: SDS-PAGE analysis of fractions of MPEG5000-insulin conjugates separated by cation-exchange chromatography. Lane 1: native insulin; lane 2: MPEG5000-insulin mixture; lane 3: fraction 1 of Fig. 1; lane 4: fraction 2 of Fig. 1; lane 5: fraction 3 of Fig. 1; lane 6: fraction 4 of Fig. 1; lane 7: fraction 5 of Fig. 1

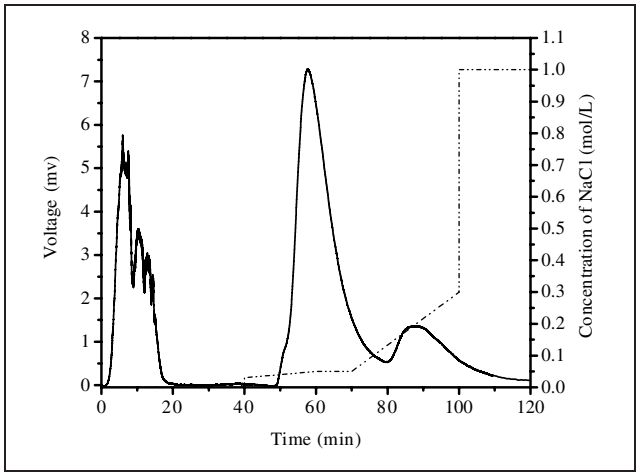

Fig. 3: Purification of tri-substituted MPEG5000-insulin conjugate by anion-exchange chromatography using Q Sepharose FF media

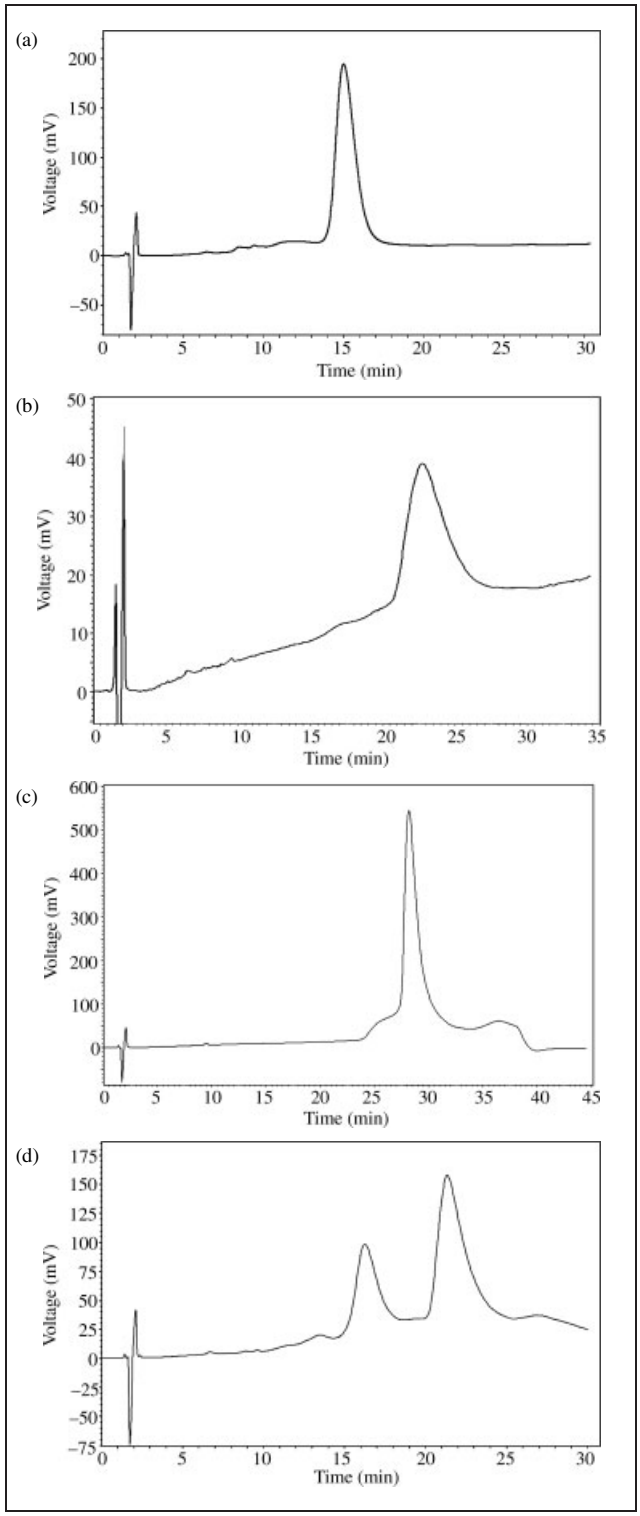

Fig. 4: Reversed-phase HPLC chromatograms of: (a) mono-PEGylated insulin, (b) di-PEGylated insulin, (c) tri-PEGylated insulin, (d) fraction 3 of Fig. 1. Native insulin had a retention time of 5.13 min

The identification of mono-PEGylated insulin and di-PEGylated insulin was confirmed by MALDI-TOF-MS that the molecular weights of them were 10.8 KDa and 15.9 KDa, respectively. MALDI-TOF-MS failed to detect tri-PEGylated insulin because much more PEG chains on the protein surface probably decreased the desorption/ionization/detection efficiency of the conjugate. The similar interference of high PEGylation degree with MALDI-TOF-MS characterization of PEG-protein conjugates was also found in other proteins (Roberts 1997).

Each conjugate's purity was assessed by RP-HPLC. There was nearly a single peak in the chromatograms of mono- and di-PEGylated insulin (Fig. 4a, 4b), which showed that the purities of these conjugates were high enough (> 95%). The chromatogram of tri-PEGylated insulin (Fig. 4c) showed a small shoulder indicating the presence of a small amount of di-PEGylated insulin in the final product. Fraction 3 of cation-exchange chromatography showed two peaks (Fig. 4d), indicating the presence of different species of mono- and di-PEGylated insulin, which was in accordance with the result of SDS-PAGE analysis. Meanwhile, it was reported that the relative reactivity of insulin amino groups decreased in the order LysB29 > GlyA1 ≫ PheB1 at pH 9.5 and higher (Uchio et al. 1999). In this study, the apparent pH value of reaction solvent was over 9.5 so that the reactivity of LysB29 was dominant. Consequently, it was believed that the purified mono-PEGylated insulin was mainly constituted by mono-substituted MPEG-LysB29-insulin, and the purified di-PEGylated insulin was mainly constituted by di-substituted MPEG-LysB29, GlyA1-insulin.

2.2. Circular dichroism

Circular dichroism spectroscopy was performed to analyze the tertiary structure and self-association behaviors of insulin and its PEGylated derivatives in aqueous environments. The overall far-UV CD spectra of insulin and three species of conjugates (Fig. 5) suggested that the conjugates substantially maintained the native tertiary structure of insulin. Far-UV CD-band at 208 nm, 223 nm and near-UV CD-band at 273 nm are usually used to study the self-association states of insulin. According to the literature (Hinds 2000), the increase of ratio $[\theta]_{208}/[\theta]_{223}$ could be an indicator of decreased self-association. It was shown that ratio $[\theta]_{208}/[\theta]_{223}$ increased with an increasing number of attached PEG chains (Table 1), which demonstrated that PEGylation of insulin could inhibit the self-association tendency of insulin, and the self-association of the conjugates decreased with the increase of substitution degree.

The CD-band at 273 nm is primarily derived from restriction of rotation of the aromatic side-chains of amino acids residues between two insulin monomers in the dimer and hexamer (Hinds 2000). Therefore, it was reasonable that conditions, which favored oligomer dissociation such as dilution, would reduce the intensity of 273 nm CD-band as it was shown in Table 1. The $[\theta]_{273}$ values of tri-PEGylated insulin at two different concentrations (0.1 and 0.01 mmol/L) were similar to each other (Table 1), which was probably because tri-PEGylated insulin primarily existed as monomer in aqueous solution at the concentrations studied here. In addition, the $[\theta]_{273}$ values of the three conjugate species at a concentration of 0.01 mmol/L were similar to each other too (Table 1), which implied that the conjugates might be mainly in a monomeric state at this concentration. The $[\theta]_{273}$ of the conjugates at a protein concentration of 0.1 mmol/L decreased with an increasing number of attached PEG chains (Table 1), which paralleled such finding that PEG conjugation to insulin could weaken the self-association tendency of insulin in aqueous environments, and this behavior depended on the PEGylation degree. The decrease of self-association of the conjugates was due to the steric hindrance effect induced by the flexible PEG chains that prevented the intermolecular interaction between monomeric or dimeric conjugates.

Table 1: Summary of results from circular dichroism experiments

Sample	$[\theta]_{273}$[a]	$[\theta]_{273}$[b]	$[\theta]_{208}/[\theta]_{223}$[a]
Insulin	−273.5	−203.7	1.04
1MPEG5000-insulin	−153.2	−104.9	1.39
2MPEG5000-insulin	−143.7	−101.1	1.42
3MPEG5000-insulin	−103.3	−103.9	1.50

Solutions prepared in PBS (10 mmol/L sodium phosphate, 125 mmol/L NaCl, pH 7.4) to a protein concentration of: [a]0.1 mmol/L, [b]0.01 mmol/L

2.3. Physical stability

Insulin will undergo fibrillation process in aqueous solutions during storage, which impairs the physical stability of insulin formulation. An accelerated shaking test was carried out to examine the physical stability of each conjugate compared to that of insulin, and results are shown in Fig. 6. Most of insulin fibrillated and precipitated within 12 h and the remaining amount could not be detected by RP-HPLC. There was over 50% of mono-PEGylated insulin remaining in 8 d and over 10% remaining in 16 d. As

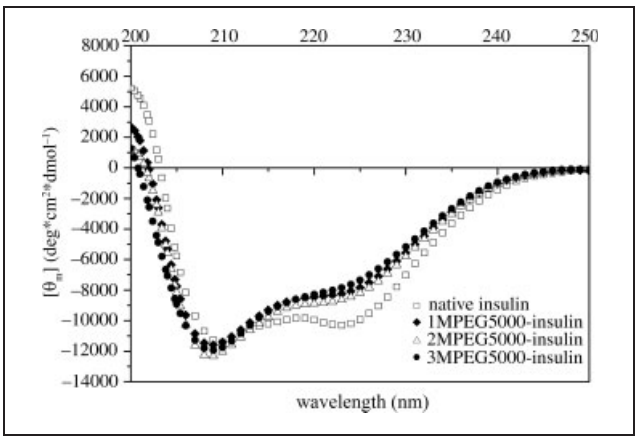

Fig. 5: Far UV-CD spectra of insulin and its PEGylated derivatives in PBS (10 mmol/L phosphate, 125 mmol/L NaCl, pH 7.4) at a concentration of 0.1 mmol/L

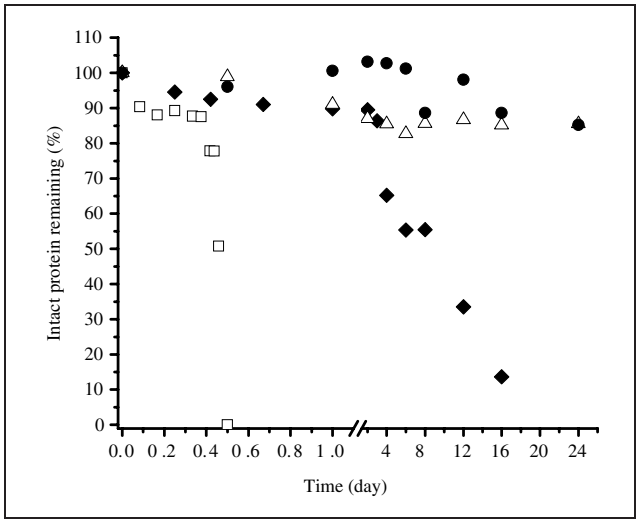

Fig. 6: Protein remaining profiles of insulin (□), mono-PEGylated insulin (◆), di-PEGylated insulin (△) and tri-PEGylated insulin (●) in PBS (10 mmol/L phosphate, 125 mmol/L NaCl, pH 7.4) at a concentration of 0.5 mg/mL. Samples were shaken at 200 rpm and 37 °C

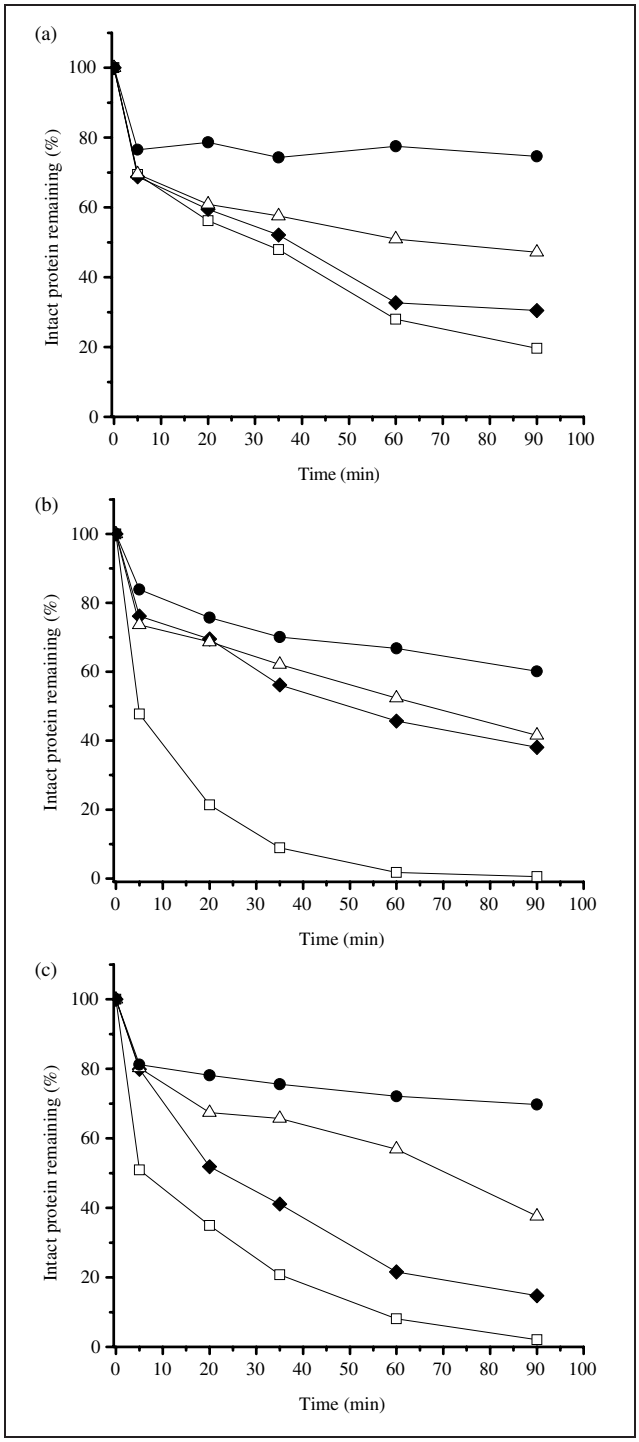

Fig. 7: The proteolytic profiles of insulin (□), mono-PEGylated insulin (◆), di-PEGylated insulin (△) and tri-PEGylated insulin (●) after incubation with pepsin (a), trypsin (b) and chymotrypsin (c)

for di- and tri-PEGylated insulin, the contents of intact conjugates were both over 80% in 24 d. It could be concluded that the conjugates possessed enhanced physical stability, which increased as the number of MPEG chains bound on insulin increased. Explanations for the increased physical stability might be the non-specific steric hindrance effect and the specific steric blockage to some nonpolar residues in the N-terminus of insulin that participated in the process of fibrillation (Brange et al. 1997; Hinds et al. 2000). The increased physical stability of mono-PEGylated insulin was mainly caused by the nonspecific steric hindrance because MPEG was mainly conjugated to LysB29. While for di- and tri-PEGylated insulin, conjugation of MPEG to GlyA1 or PheB1 resulted in the hydrophilization of N-terminus, which effectively prevented this surface from participating in the hydrophobic interactions that drove insulin fibrillation, therefore the conjugates with higher substitution degree possessed much higher physical stability.

2.4. Proteolytic stability

The proteolytic profiles of native insulin and three species of MPEG5000-insulin conjugates after incubation with pepsin, trypsin and chymotrypsin were reported in Fig. 7. Insulin was rapidly degraded in all cases, while PEGylated insulin exhibited enhanced resistance to proteolysis due to the polymer shield on the surface of insulin that partially prevented the approach of enzymes (Calceti et al. 2004). The more the number of MPEG5000 chains attached to insulin was, the higher resistance to enzymatic degradation that the conjugate had. This result agreed well with the studies reported in the literature (Veronese et al. 1996; Calceti et al. 2004), which revealed the correlation between polymer mass on the protein surface and proteolytic stability. Besides, mono-PEGylated insulin displayed a relative higher resistance to trypsin than pepsin and chymotrypsin, which could be ascribed to the fewer enzymatic cleavage sites on insulin for trypsin as compared to pepsin and chymotrypsin. In addition, conjugation of MPEG to amino acid residues that are specific enzymatic cleavage sites could further impede recognition between the proteolytic enzymes and substrates. Consequently, modification of insulin with one MPEG5000 could provide enough protection from degradation by trypsin, especially when LysB29, one of the cleavage sites of trypsin was modified with MPEG.

2.5. Biological activity

The hypoglycemic profiles obtained by s.c. administration of equivalent dose of native insulin and three species of conjugates to normal mice are depicted in Fig. 8. A relationship between time to onset of glucose-lowering activity and the number of PEG chains attached to insulin was observed. Insulin with more MPEG chains attached re-

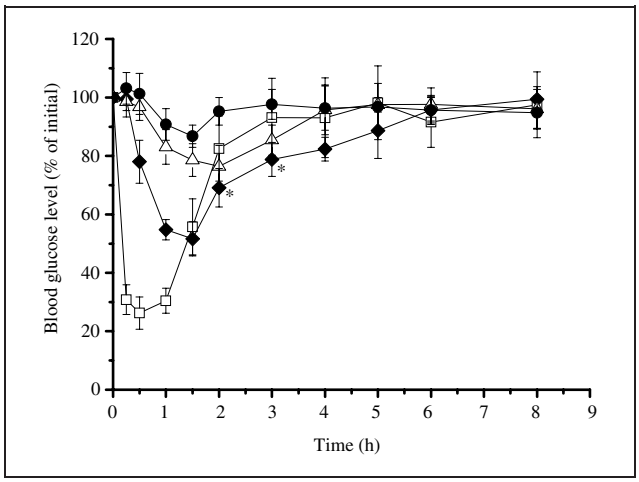

Fig. 8: Blood glucose levels in normal mice following s.c. administration (0.5 U/kg) of insulin (□), mono-PEGylated insulin (◆), di-PEGylated insulin (△) and tri-PEGylated insulin (●). Data are mean ± S.D., n = 6. (*denotes $p < 0.05$ compared to insulin group using two-tailed student's t-test)

Table 2: ***In vivo*** **mouse bioactivity study results for insulin and PEGylated insulin**

Sample	$AAC_{0-8\,h}$ (min × %)	% Bioactivity[a]
Insulin	8754	
1MPEG5000-insulin	8145	93
2MPEG5000-insulin	4046	46
3MPEG5000-insulin	2079	24

[a] Calculated relative to insulin

quired longer time to decrease blood glucose level. The lag of glucose-lowering activity was probably due to the relative slower s.c. absorption rates of conjugates. The absorption rate of insulin (conjugates) was subjected to many factors such as the association state, diffusion of insulin (conjugates) through capillary cell membranes from the injection site, local blood flow, and local temperature (Kang et al. 1991; Hinds et al. 2002). Although MPEG5000-insulin conjugates had improved solubility and decreased self-association states that should promote their s.c. absorption, the increased molecular sizes of the conjugates and the specific interactions of the PEG moiety with cell membranes (Harris 1992) restricted the absorption rate to a greater extent that conjugates with more MPEG chains attached displayed much more delayed action. After the initial lag time to full action, the maximal glucose lowering activity of three forms of conjugates in contrast to that of insulin decreased with increasing substitution degree. Furthermore, the area above glucose-lowering curve during 0–8 h was calculated by the trapezoidal method and results were summarized in Table 2. It was shown that mono-PEGylated insulin preserved most biological activity of insulin after s.c. administration in mice because the extended action of this conjugate compensated for its biological activity loss. However, bioactivities of di- and tri-PEGylated insulin were about 54% and 76% lower than that of insulin, respectively. It was in agreement with the result of previous studies (Caliceti and Veronese 1999), which demonstrated that the loss of bioactivity increased as the number of PEG chains coupled to insulin increased. The increasing steric hindrance of insulin-receptor interactions was one of contributing factors to the conjugates' decreased biological activity, and this effect became even more important for di- and tri-PEGylated insulin. In addition, the possibility that conjugation of MPEG to GlyA1 in di-PEGylated insulin was larger than that in mono-PEGylated insulin, and GlyA1 was evidently conjugated with MPEG in tri-PEGylated insulin. As GlyA1 was directly involved in the receptor recognition while PheB1 and LysB29 were not involved in the receptor binding (Murray-Rust et al. 1992), it was reasonable that biological activities of the conjugates decreased with the increasing substitution degree.

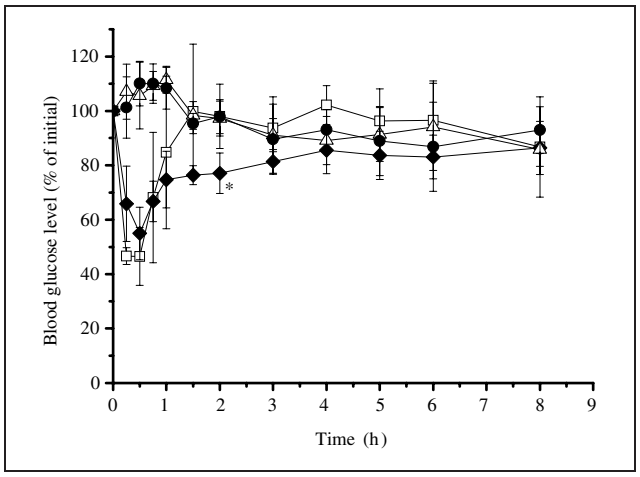

Fig. 9: Blood glucose levels in normal rats following i.v. administration (0.5 U/kg) of insulin (□), mono-PEGylated insulin (◆), di-PEGylated insulin (△) and tri-PEGylated insulin (●). Data are mean ± S.D., n = 4. (*denotes $p < 0.05$ compared to insulin group using two-tailed student's t-test)

The hypoglycemic effects after i.v. administration of equivalent dose of native insulin and conjugates to normal rats were reported in Fig. 9. The blood glucose depression of mono-PEGylated insulin was comparable to that of insulin. In addition, mono-PEGylated insulin displayed a significantly protracted blood glucose-lowering profile partly due to its increased molecular size and decreased receptor-binding ability. The increased molecular size of the conjugate might retard its renal filtration, thus increasing its circulation half-life. Furthermore, it was reported that insulin was primarily cleared through receptor-mediated mechanism (Neubauer et al. 1983). Though the decreased receptor-binding ability gave rise to a decrease of biological activity, it might contribute to the slower clearance of the conjugate at the same time. Another contributing factor was the PEG's ability to interact with blood cell membranes (Caliceti et al. 1989), which might allow the conjugate to remain in the circulation longer than native insulin. The di- and tri-substituted MPEG-insulin conjugates displayed no blood glucose-lowering activity following i.v. administration due to the effects described above for bioactivity loss and the *in vivo* counter-regulation evoked by i.v. administration.

In conclusion, conjugation of MPEG5000 to insulin was achieved and mono-, di-, and tri-PEGylated insulin were purified by ion-exchange chromatography. PEGylation of insulin did not alter the tertiary structure of native insulin but self-association states of the conjugates decreased as the number of MPEG chains attached to insulin increased. Besides, conjugation of MPEG5000 to insulin improved the physical stability and the proteolytic stability of insulin, which increased with the increasing number of MPEG chains attached to insulin. The mono-substituted MPEG5000-insulin retained most biological activity of native insulin with an extended action profile following both s.c. and i.v. administration, while a great loss of biological activity occurred in di- and tri-substituted MPEG5000-insulin. It is believed that the mono-PEGylated insulin is a good candidate for insulin replacement therapy.

3. Experimental

Crystalline porcine insulin (28 IU/mg, batch No. 000305) was purchased from Xuzhou Biochemical Co. (Xuzhou, China). MPEG5000 was purchased from Sigma Chemical Co. (St. Louis, MO, USA). MPEG5000-SCM was synthesized according to the previously reported method (Andreas et al., 1981). SP Sepharose FF cation-exchange media and Q Sepharose FF anion-exchange media were purchased from GE Healthcare Amersham Biosciences (Sweden). Acetonitrile of HPLC-grade was obtained from Merck (Germany). Pepsin from porcine gastric mucosa (3000 U/mg) was purchased from Amresco (USA). Trypsin from bovine pancreas (1000 U/mg) and α-chymotrypsin from bovine pancreas (4000 ATEE U/mg) were purchased from Shanghai Bio Life Science & Technology Co. (Shanghai, China). All other reagents were of the highest grade available and were obtained commercially.

3.1. Preparation of MPEG5000-insulin conjugates

MPEG5000-insulin conjugates were synthesized as described previously (Zhang et al. 2007). Sample solution at a concentration about 100–150 mg/mL was prepared by dissolving the crude products containing

MPEG5000-insulin conjugates in acetate buffer (0.05 mol/L, pH 4.0). Ten milliliters of the solution was loaded on a FPLC system (600E, Waters, USA) equipped with a SP Sepharose cation-exchange column (2.5×10 cm, CV = 50 ml) that was pre-equilibrated with acetate buffer. The column was eluted with a NaCl gradient in acetate buffer at a flow rate of 4.0 mL/min as follows, acetate buffer without NaCl for 30 min, 0.04 to 0.06 mol/L NaCl over 20 min, 0.06 mol/L NaCl for 10 min, 0.1 to 0.15 mol/L NaCl over 15 min, 0.15 mol/L NaCl for 10 min, 0.2 to 0.3 mol/L NaCl over 15 min, 0.3 mol/L NaCl for 10 min, 0.5 mol/L NaCl for 15 min, 1.0 mol/L NaCl for 15 min, and after equilibration for 30 min, the column was ready for the next run. The elution profile was monitored at 280 nm and the eluted fractions were pooled manually. Each fraction was dialyzed against 0.01% (m/v) NH_4HCO_3 solution and lyophilized. The flow-through fraction of cation-exchange chromatography was dissolved in Tris-HCl buffer (0.05 mol/L, pH 8.5) and was further purified using a Q Sepharose anion-exchange column (2.5×10 cm, CV = 50 ml) pre-equilibrated with Tris-HCl buffer. The column was eluted with a NaCl gradient in Tris-HCl buffer at a flow rate of 4.0 mL/min as follows, Tris-HCl buffer without NaCl for 40 min, 0.03 to 0.05 mol/L NaCl over 20 min, 0.05 mol/L NaCl for 10 min, 0.05 to 0.3 mol/L NaCl over 30 min, 1.0 mol/L NaCl for 20 min, and after equilibration for 30 min, the column was ready for the next run. The elution profile was monitored at 280 nm and the target fraction was pooled manually, dialyzed against 0.01% (m/v) NH_4HCO_3 solution and lyophilized. The fractions were identified using SDS-PAGE. The purities of fractions that corresponded to different PEGylated insulin species, namely mono-, di-, and tri-PEGylated insulin were assessed by RP-HPLC using a Shimadzu SCL-10A controller fitted with LC-10AT pump and SPD-10AD UV detector (Shimadzu, Japan). Analysis was conducted with a Diamonsil C18 (4.6×150 mm, 5 μm, Dikma, USA) column at ambient temperature. After injection of the sample (20 μL), gradient elution was carried out at a flow rate of 1.0 mL/min with 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B), and the elution profile was monitored at 220 nm. The gradient profile was applied as follows, 32% B to 38% B over 10 min, 38% B to 50% B over 20 min, 90% B for 3 min, and after additional equilibration with 32% B for 10 min, the column was ready for the next run.

3.2. SDS-PAGE analysis

SDS-PAGE was performed on a Bio-Rad Mini-Protean® III cell (Bio-Rad Laboratories Inc., USA) as described by Laemmli (1970) using 7.5–15% gel under non-reducing condition, and the gel was stained by commassie blue staining solution.

3.3. MALDI-TOF-MS

The molecular weight of PEGylated insulin was determined on a MALDI-TOF mass spectrometer (Model 4700, Applied Biosystems, USA) operated in the positive-ion linear mode detection. The samples were prepared by dissolving PEGylated insulin in 5% acetonitrile in deionized water. A saturated solution of α-cyano-4-hydroxycinnamic acid in 0.1% formic acid was used as matrix solution. Equal volumes of sample solution and matrix solution were mixed, and 2 μL of the solution was then deposited on the sample plate, which was allowed to dry by solvent evaporation under ambient conditions, and the 337 nm line of a nitrogen laser was used to shoot the sample for at least 100 times to acquire the final spectrum.

3.4. Concentration determination of insulin and PEGylated insulin

Protein concentrations of samples were determined using UV spectrophotometer (U-3000, Hitachi, Japan) at 275 nm. The concentration of PEGylated insulin was expressed in terms of the concentration of insulin for the following studies.

3.5. Circular dichroism (CD)

Four samples including native insulin, mono-, di-, and tri-PEGylated insulin were prepared in phosphate buffered saline (PBS, 10 mmol/L phosphate, 125 mmol/L NaCl, pH 7.4) at two different concentrations (0.1 and 0.01 mmol/L) and then filtered through a 0.22 μm membrane filter. A Jasco J-715 spectropolarimeter (Tokyo, Japan) was used to analyze the CD of samples and five scans were performed and averaged for each sample. The final spectrum of each sample was acquired by subtracting the PBS spectrum from the preliminary spectrum with noise reduction. The data (ellipticity in mdeg) was then transformed to mean residue ellipticity (θ_m) using the following eq. (Goldenman and Carpenter 1974): $\theta_m = (\theta M)/(Cl)$, where θ is the observed ellipticity (mdeg), M is the mean residue molecular weight of insulin (g/mol), C is the protein concentration (g/mL), and l is the optical path length (cm).

3.6. Physical stability

Native insulin and three species of PEGylated insulin were dissolved in PBS containing 0.01% sodium azide to obtain a final concentration of 0.5 mg/mL. After being filtered through 0.22 μm membrane filter, 1.5 mL of the solution was filled into 10-mL glass vial, which was sealed with rubber capper and horizontally shaken at 200 rpm and 37 °C. At prescribed time intervals, three vials were withdrawn and samples were filtered through 0.22 μm membrane filter. The remaining amounts of intact insulin and PEGylated insulin were determined by RP-HPLC as described above.

3.7. Proteolytic stability

Pepsin solution at a concentration of 120 μg/mL was prepared in 0.1 mol/L HCl. Native insulin and equivalent amounts of MPEG5000-insulin conjugates were dissolved in 0.1 mol/L HCl to a concentration of 100 μg/mL. After equilibration at 37 °C for 15 min, 50 μL of pepsin solution was added to 10 mL of insulin (conjugates) solution. The mixed solution was maintained at 37 °C. At scheduled time intervals (0, 5, 20, 35, 60, 90 min), 100 μL of samples were taken and mixed with 150 μL of Tris-HCl buffer (0.1 mol/L, pH 8.0) to stop the enzyme activity. The resulting solutions were analyzed by RP-HPLC for determination of undegraded protein content (%).

Trypsin and chymotrypsin were dissolved in Tris-HCl buffer (0.1 mol/L, pH 8.0) to a final concentration of 1.2 mg/mL and 1.6 mg/mL, respectively. Native insulin and equivalent amounts of MPEG5000-insulin conjugates were dissolved in Tris-HCl buffer containing 1 mmol/L $CaCl_2$ to a concentration of 100 μg/mL. The following processes were the same as described above except that 0.1% (v/v) TFA solution was used to stop the enzymatic reaction.

3.8. Biological activity

Normal male Spague-Dawley (SD) rats were fasted for at least 12 h before experiment with free access to water. Solutions of insulin, mono-, di-, and tri-PEGylated insulin in PBS (pH 7.4) were injected at a dose of 0.5 U/kg into rat tail veins (n = 4). Blood samples (200 μL) were obtained at 0, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, and 480 min to determine the serum glucose level using a glucose kit. The four solutions were also subcutaneously injected into normal mice (n = 6) at a dose of 0.5 U/kg. Blood samples (200 μL) were taken at 0, 15, 30, 60, 90, 120, 180, 240, 300, 360, and 480 min, and the blood glucose levels were determined as described above. The area above curve of blood glucose level versus time after s.c. administration was calculated by the trapezoidal method. Statistical data analysis was performed using the double-tailed Student's t-test with $p < 0.05$ indicating significant difference.

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