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Microwave-assisted solid phase synthesis, PEGylation, and biological activity studies of glucagon-like peptide-1(7–36) amide

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ABSTRACT

The insulinotropic hormone glucagon-like peptide-1 (GLP-1) is rapidly inactivated in the body. In order to improve its stability, we replaced the enzymatic hydrolyzation position $Ala_8$with Gly and replaced Ala_{30} with Cys firstly. Then the modified peptide was further PEGylated at thiol group of Cys_{30}. Biological activity studies showed that the resulting mPEG-MAL-Gly_8-Cys_{30}-GLP-1(7–36)-NH_2 exhibited long-lasting effect while maintaining moderate glucose-lowering activity.

1. Introduction

Glucagon-like peptide-1 (GLP-1) is an incretin released from L-cells in small intestine.[1] GLP-1(7–36)-NH_2 corresponding to proglucagon (78–107) is the predominant biological-active form of GLP-1 in humans.[2,3] The glucose-dependent insulinotropic activity of GLP-1 has been confirmed by numerous studies.[4] GLP-1 may reverse the progression of the type 2 diabetes not only by controlling blood glucose but also via several other effects. The multifaceted physiological actions of GLP-1 include (a) the glucose-dependent stimulation of insulin secretion,[5] (b) the glucose-dependent suppression of glucagon secretion,[6] (c) the reduction in appetite and deceleration of gastric emptying,[7] (d) the protection and proliferation of beta cells,[8] and (e) the improvement of insulin sensitivity.[9] As it plays an essential role in regulation of postprandial insulin release in the glucose-dependent manner, GLP-1 is considered as a potential therapeutic agent for the treatment of type 2 diabetes.[10] However, the rapid inactivation of GLP-1(7–36)-NH_2 in the body limits its usage in clinic. GLP-1(7–36)-NH_2 is rapidly degraded to GLP-1(9–36)-NH_2 by dipeptidyl peptidase IV (DPP IV), which removes the N-terminal dipeptide His_7-Ala_8.[11–13] GLP-1(9–36)-NH_2 has a greatly decreased affinity for the GLP-1(7–36)-NH_2 receptor, and may even be an antagonist.[14] Furthermore, other enzymes such as neutral endopeptidase (NEP) 24.11 have also shown to hydrolyze the GLP-1(7–36)-NH_2 at amino acid sites 15, 16, 18, 19, 20, 27, 28, 31, 32 with relatively lower speed.[15]

Various efforts have been made to retard the degradation of GLP-1(7–36)-NH_2 by DPP IV.[16–18] GLP-1(7–36)-NH_2 analogues were designed by substituting the Ala_8 of GLP-1(7–36)-NH_2 with other amino acids or chemical groups. Some GLP-1 analogues such as Gly_8-GLP-1(7–36)-NH_2 improved the stability to DPP IV greatly and showed better glucose-lowering activity compared with GLP-1(7–36)-NH_2. However, these analogues were still not stable enough because of the susceptivity to other enzymes and rapid clearance in kidney. Thus other approaches such as PEGylation were employed to prolong the biological half-life of GLP-1. It was well known that conjugation of polyethylene glycol (PEG) to peptides endowed the ability to avoid quick recognition by enzymes and kidney clearance.[19] PEGylated peptides possessed many ideal properties: very low toxicity, excellent pharmacokinetic behavior, and extremely low immunogenicity, especially the improvement of the stability to enzymes and the reduction of clearance in kidney due to the increase of steric hindrance of peptide.[20] Sang-Heon Lee reported that the PEGylation of unmodified GLP-1 by monomethoxy polyethylene glycol succinimidyl propionate (mPEG-SPA) exhibited similar stability of N-terminal modified GLP-1 analogues.[21] As the conjugation of mPEG-SPA to unmodified GLP-1 was not site-specific, it generated a mixture of heterogeneous isomers because mPEG-SPA could conjugate with Lys_{26}, Lys_{34}, and His_7 of GLP-1(7–36)-NH_2. These isomers were difficult to separate due to their very similar hydrophobicity. It was also demonstrated that site-specific PEGylation of GLP-1(7–36)-NH_2 by conjugating the monomethoxy polyethylene glycol propionaldehyde (mPEG-PALD) to the $His_7$of GLP-1(7–36)-NH_2 resulted in a marked reduction in activity, because His_7 was crucial to GLP-1 for effective signal transduction and biological activity.[22,23]

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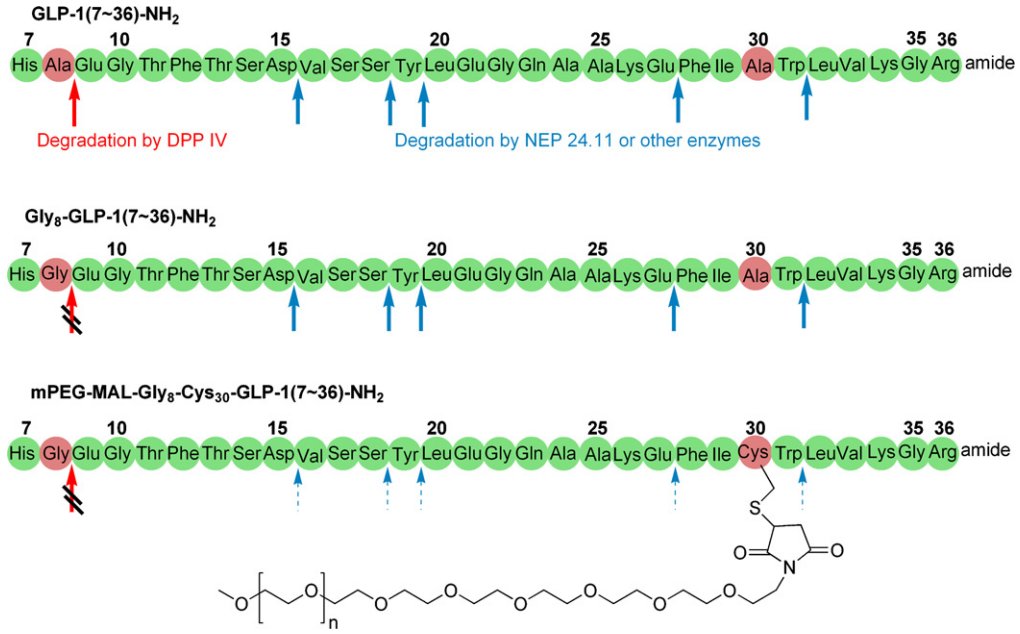


Figure 1. Amino acid sequence of GLP-1 and its derivatives, and their stability to the degradation by DPP IV or other enzymes in vivo.

Therefore, in this study both the N-terminal modification and site-specific PEGylation were used to design a novel GLP-1 derivative (see Fig. 1), which would improve the stability not only to DPP IV but also to other enzymes. It may also reduce the clearance in kidney. It was confirmed that substitution of Ala_8 by Gly_8 could significantly improve stability to DPP IV while maintaining insulinotropic potency.[24,25] Accordingly, we replaced the enzymatic hydrolyzation position $Ala_8$with Gly firstly. On the other hand, due to high chemoselectivity of maleimide to thiol group,[26] peptide containing cysteine allows for straightforward site-specific attachment by monomethoxy polyethylene glycol maleimide (mPEG-MAL). However, the site for PEGylation was very important to maintain the activity of peptide. It was confirmed that the PEGylation on N-terminal residues of GLP-1(7–36)-NH_2 resulted in an obvious reduction in activity. Thus, the modifications of N-terminal residues of GLP-1 were not considered. The secondary structure of GLP-1 has great influence on its activity, and the midst of GLP-1 contains two helical regions and a linker region. In order to avoid unfavorable effect on the secondary structure of GLP-1, we selected C-terminal residues of GLP-1 as the site for the PEGylation. There is only a marginal difference between Ala and Cys. And the Ala_{30} is not essential to glucose-lowering activity of GLP-1. Therefore, we replaced Ala_{30} with Cys for the site-specific PEGylation. Finally, the resulting peptide Gly_8-Cys_{30}-GLP-1(7–36)-NH_2 was further site-specific PEGylated by mPEG-MAL at thiol group of Cys_{30}. We reported here the synthesis of GLP-1(7–36)-NH_2 and its derivatives. The microwave-assisted peptide synthesis method was applied in the synthesis of the peptides. The stabilities of the GLP-1(7–36)-NH_2 derivatives to DPP IV and their glucose-lowering activities in vivo were evaluated in the present study.

2. Results and discussion

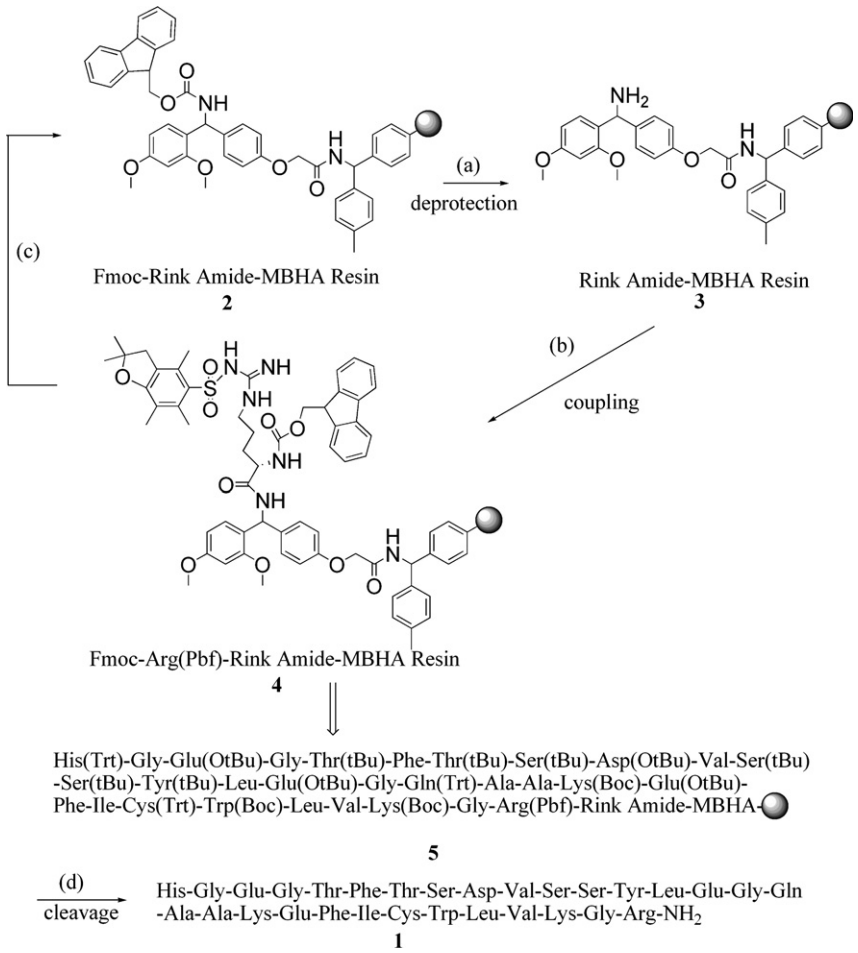
2.1. Chemistry

Since the method of solid phase peptide synthesis (SPPS) was developed by Merrifield, it has led to dramatic progress in peptide chemistry.[27] However, the use of conventional SPPS method to synthesize medium- or large-length peptides was often inefficient.[28] Since microwave technology was applied to SPPS, peptide synthesis became more efficient.[29] According to standard SPPS protocols, the coupling time per residue at room temperature usually varied between 2 and 4 h and the time was shortened to approximate 80 min by raising the temperature to 50 °C.[30] With the use of microwave irradiation, the coupling time was dramatically decreased to 5–10 min, and no racemization was detected.[31] Whereas there were only a few reports on the use of microwave irradiation in the solid phase peptide synthesis. These reports mainly focused on the high-throughput synthesis of oligopeptides in combinatorial chemistry.[32] We described here a rapid and highly efficient microwave-assisted Fmoc/tBu solid phase method to prepare GLP-1 analogues. We used Rink Amide-MBHA resin as solid phase support. The substitutions of the first amino acid attached to resin were 0.27 mmol/g (Fmoc-Arg(Pbf)-Rink Amide-MBHA resin). The method to calculate the substitution value was reported in detail in reference.[33] Microwave irradiation was performed in coupling steps and deprotection steps. The crude peptides were cleaved from resin by Reagent K. All coupling steps were monitored by qualitative ninhydrin test.

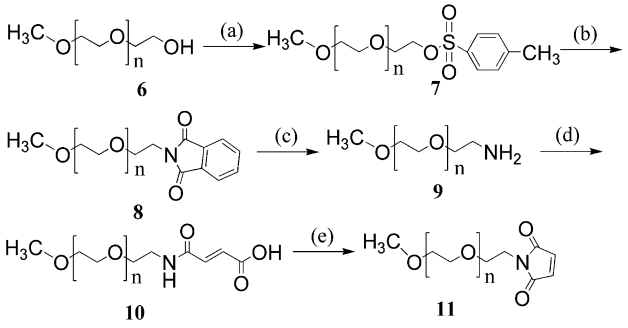
The synthetic route of Gly_8-Cys_{30}-GLP-1(7–36)-NH_2 is described in Scheme 1, and that of GLP-1(7–36)-NH_2 and Gly_8-GLP-1(7–36)-NH_2 are similar. The crude peptides were analyzed using reversed-phase high-pressure liquid chromatography (RP-HPLC), and purified by preparative RP-HPLC.

mPEG-OH (**6**) of molecular weight about 2000 Da was chosen as a convenient starting material for synthesis of mPEG-MAL (see Scheme 2). Because of only one derivatizable end group in its structure, the use of mPEG minimized crosslinking possibility in reaction. mPEG-OH reacted with *p*-toluenesulfonyl chloride in the presence of sodium hydride as acid-binding agent to produce mPEG-tosylate (**7**), which was converted to mPEG-NH_2 (**9**) through Gabriel reaction. The crucial intermediate compound **11** was obtained via reacting compound **9** with maleic acid anhydride, followed by dehydration with acetic anhydride.

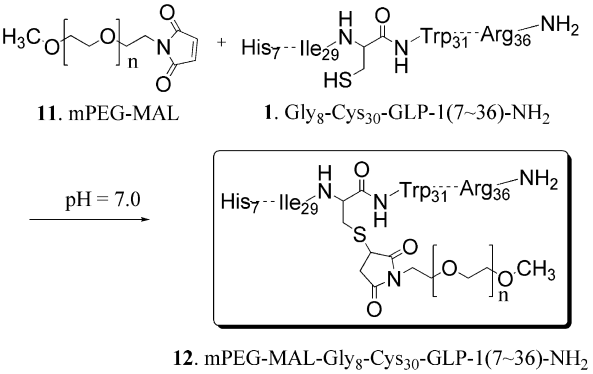
Maleimide was a highly chemoselective Michael-acceptor to thiol group at neutral pH. Gly_8-Cys_{30}-GLP-1(7–36)-NH_2 reacted with mPEG-MAL in aqueous buffer solution (pH 7.0) at room temperature to give mPEG-MAL-Gly_8-Cys_{30}-GLP-1(7–36)-NH_2 (see Scheme 3). The reaction was monitored by RP-HPLC. As shown in HPLC profiles (see Fig. 2), the reaction could be finished after



Scheme 1. Synthetic route of Gly_8-Cys_{30}-GLP-1 (7–36)-NH_2. Reagents and conditions: (a) 25% Piperidine/DMF, microwave irradiation; (b) Fmoc-Arg(Pbf)-OH (3.0 equiv), HBTU (3.0 equiv), HOBT (3.0 equiv), DIEA (6.0 equiv)/DMF, microwave irradiation; (c) repeat the cycles of deprotection and coupling with relevant Fmoc-protected amino acids. (d) Reagent K: TFA/thioanisole/water/phenol/EDT (82.5:5:5:5:2.5).



Scheme 2. Synthetic route of mPEG-MAL. Reagents and conditions: (a) 4-Toluenesulfonyl chloride, CH_2Cl_2; (b) potassium phthalimide, DMF; (c) hydrazine hydrate, C_2H_5OH; (d) maleic acid anhydride, DMAP, dioxane; (e) Ac_2O, NaOAc.



Scheme 3. Synthesis of mPEG-MAL-Gly_8-Cys_{30}-GLP-1(7–36)-NH_2.

1.5 h. The crude PEGylated peptide was purified by preparative RP-HPLC.

2.2. Characterization of GLP-1(7–36)-NH2 and its derivatives

Following the purification of GLP-1(7–36)-NH_2 and its derivatives using preparative RP-HPLC, the electrospray quadrupole mass spectrometer (ESI-Q-MS) was used for the analysis of the molecular weight values of the synthetic peptides. The mass-to-charge ratio (m/z) was found nearly identical to the theoretical m/z in each case (see Table 1).

Electrospray ion-trap time-of-flight mass spectrometer (ESI-IT/TOF-MS) was performed for the characterization of purified mPEG-MAL-Gly_8-Cys_{30}-GLP-1(7–36)-NH_2. Because the mPEG is a polymer, a series of multiple charged ion peaks are shown in Figure 3. The theoretical molecular mass of mPEG-MAL-Gly_8-Cys_{30}-GLP-1(7–36)-NH_2 was $3470.7 \pm 44n$ (n represents the number of backbone 'CH_2CH_2O' units in a PEGylated monomer), and it was in accordance with the found values (see Table 2). The average molecular mass of mPEG-MAL-Gly_8-Cys_{30}-GLP-1(7–36)-NH_2 is about 5451 Da (n = 45).

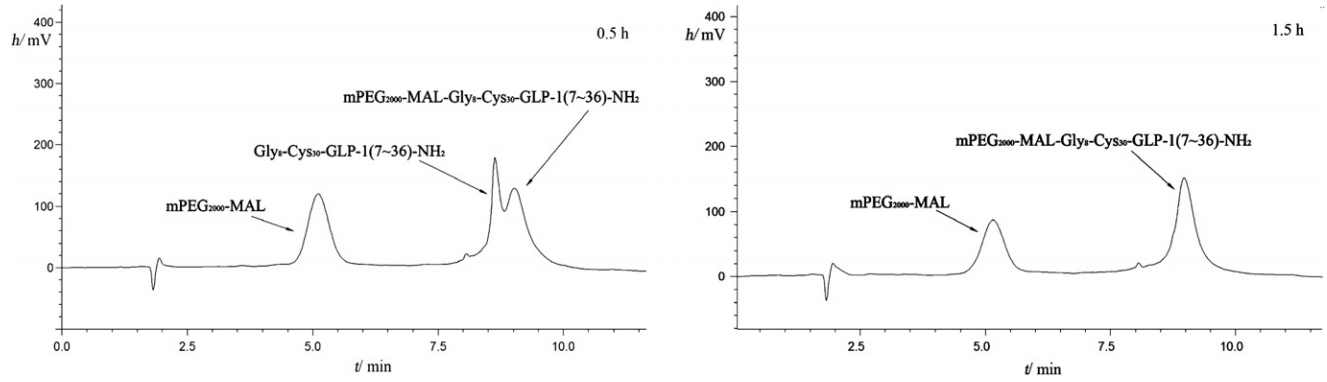


Figure 2. HPLC analysis of the reaction mixture at 0.5 and 1.5 h.

Table 1
The MS data of peptides

Peptide	Mr	(*m*/*z*) calcd	(*m*/*z*) found
GLP-1(7–36)-NH_2	3297.7	$[M+3H]^{3+}$ 1100.2, $[M+4H]^{4+}$ 825.4, $[M+5H]^{5+}$ 660.5	$[M+3H]^{3+}$ 1100.3, $[M+4H]^{4+}$ 825.3, $[M+5H]^{5+}$ 660.7
Gly_8-GLP-1(7–36)-NH_2	3283.6	$[M+3H]^{3+}$ 1095.5, $[M+4H]^{4+}$ 821.9, $[M+5H]^{5+}$ 657.7	$[M+3H]^{3+}$ 1095.5, $[M+4H]^{4+}$ 821.9, $[M+5H]^{5+}$ 657.8
Gly_8-Cys30-GLP-1(7–36)-NH_2	3315.7	$[M+3H]^{3+}$ 1106.2, $[M+4H]^{4+}$ 829.9	$[M+3H]^{3+}$ 1106.1, $[M+4H]^{4+}$ 829.8
GLP-1(9–36)-NH_2	3089.5	$[M+3H]^{3+}$ 1030.8, $[M+4H]^{4+}$ 773.3, $[M+5H]^{5+}$ 618.9	$[M+3H]^{3+}$ 1030.8, $[M+4H]^{4+}$ 773.2, $[M+5H]^{5+}$ 618.7

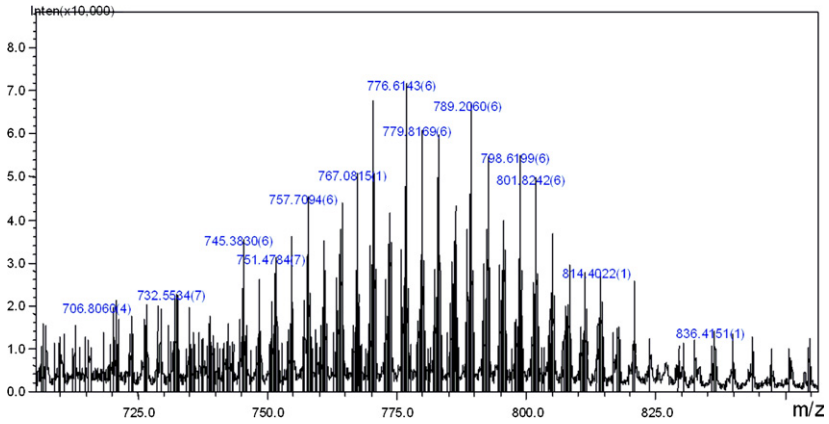


Figure 3. ESI-IT/TOF-MS spectrum of mPEG-MAL-Gly_8-Cys_{30}-GLP-1(7–36)-NH_2.

Table 2
Molecular mass data analysis of mPEG-MAL-Gly_8-Cys_{30}-GLP-1(7–36)-NH_2

n[a]	(*m*/*z*) calcd	(*m*/*z*) found
41	$[M+7H]^{7+}$ 754.5, $[M+6H+Na]^{7+}$ 757.7	$[M+7H]^{7+}$ 754.6, $[M+6H+Na]^{7+}$ 757.7
42	$[M+7H]^{7+}$ 760.8, $[M+6H+Na]^{7+}$ 764.0	$[M+7H]^{7+}$ 760.9, $[M+6H+Na]^{7+}$ 764.0
43	$[M+7H]^{7+}$ 767.1, $[M+6H+Na]^{7+}$ 770.2	$[M+7H]^{7+}$ 767.1, $[M+6H+Na]^{7+}$ 770.3
44	$[M+7H]^{7+}$ 773.4, $[M+6H+Na]^{7+}$ 776.5	$[M+7H]^{7+}$ 773.5, $[M+6H+Na]^{7+}$ 776.6
45	$[M+7H]^{7+}$ 779.7, $[M+6H+Na]^{7+}$ 782.8	$[M+7H]^{7+}$ 779.8, $[M+6H+Na]^{7+}$ 782.9
46	$[M+7H]^{7+}$ 786.0, $[M+6H+Na]^{7+}$ 789.1	$[M+7H]^{7+}$ 786.1, $[M+6H+Na]^{7+}$ 789.2
47	$[M+7H]^{7+}$ 792.2, $[M+6H+Na]^{7+}$ 795.4	$[M+7H]^{7+}$ 792.3, $[M+6H+Na]^{7+}$ 795.5
48	$[M+7H]^{7+}$ 798.5, $[M+6H+Na]^{7+}$ 801.7	$[M+7H]^{7+}$ 798.6, $[M+6H+Na]^{7+}$ 801.8

[a] n represents the number of backbone 'CH_2CH_2O' units in a PEGylated monomer.

2.3. Degradation of GLP-1(7–36)-NH_2 and its derivatives by DPP IV

DPP IV is the main enzyme responsible for GLP-1 degradation. The therapeutic potential of truncated GLP-1 metabolite was greatly diminished. N-terminal modified GLP-1 analogues such as Gly_8-GLP-1(7–36)-NH_2 were proved to be resistant to the degradation by DPP IV in previous studies. In the present study, mPEG-MAL-Gly_8-Cys_{30}-GLP-1 (7–36)-NH_2 was tested for DPP IV resistance compared with GLP-1(7–36)-NH_2 and Gly_8-GLP-1(7–36)-NH_2. The stabilities of GLP-1(7–36)-NH_2 and its derivatives were evaluated by incubation of the peptides with DPP IV at 37 °C for 4 h and followed by analysis of reaction mixtures using HPLC. As shown in Figure 4, only 11% GLP-1(7–36)-NH_2 remained intact after 4 h, and the new appearance of adjacent peak was the degradation fragment GLP-1(9–36)-NH_2. In contrast, two GLP-1 derivatives remained completely intact, and new peak was not detected. This suggested that substitution of enzymatic hydrolyzation position of GLP-1 drastically retarded the degradation by DPP IV. And the further PEGylated Gly_8-GLP-1(7–36)-NH_2 was stable to DPP IV too, and no new peak was detected.

2.4. Glucose-lowering effects of GLP-1 and its derivatives in vivo

To investigate the glucose-lowering effects of GLP-1 derivatives, the glucose in combination with GLP-1 or GLP-1 derivatives was administered by intraperitoneal injection simultaneously. The saline group was as control. As shown in Table 3, saline had no

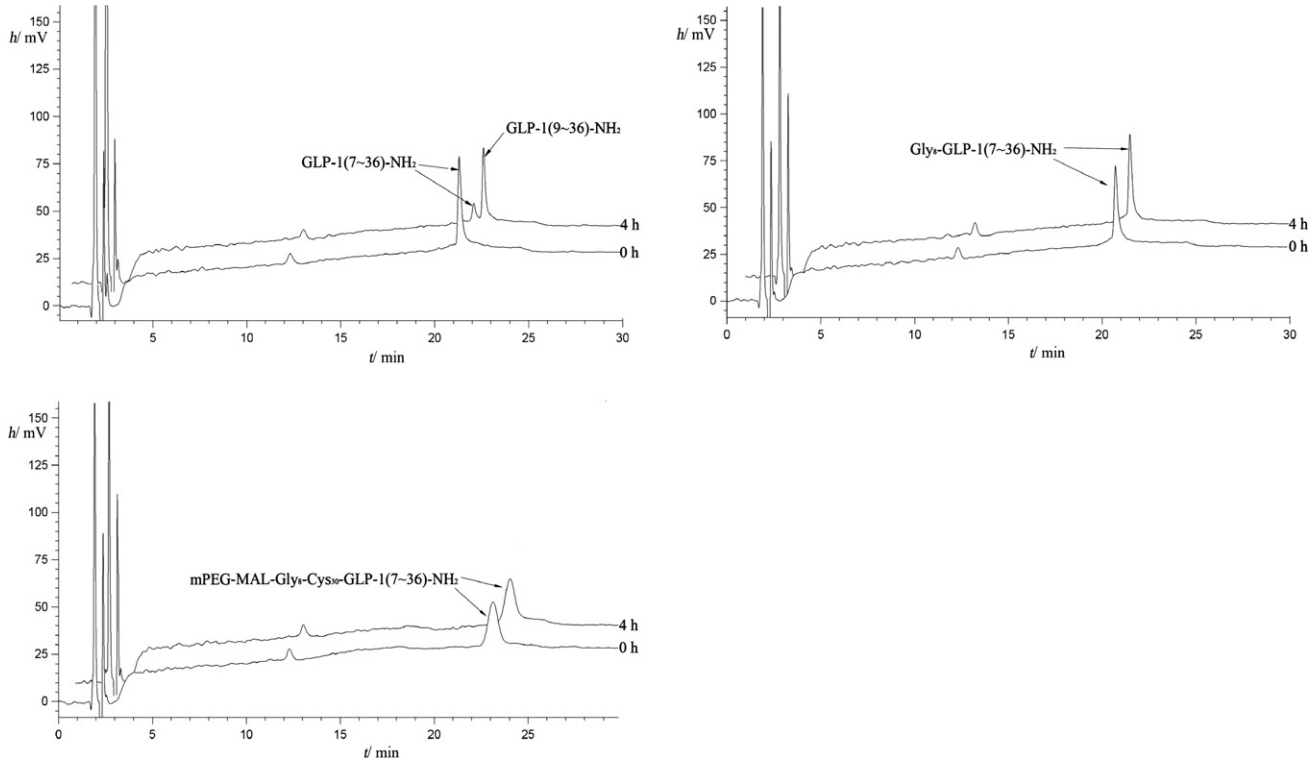


Figure 4. HPLC degradation profiles of GLP-1(7–36)-NH_2 and its derivatives after incubation with DPP IV for 0 and 4 h.

Table 3
Blood-glucose concentrations following ip administration of glucose in combination with saline, GLP-1(7–36)-NH_2, or GLP-1 derivatives simultaneously ($\bar{x} \pm s$, $n = 6$)

	Blood-glucose concentration (mmol/L)				
	0 min	15 min	30 min	45 min	60 min
Saline	6.18 ± 1.52	21.57 ± 1.08	18.98 ± 2.69	13.77 ± 1.33	11.88 ± 1.17
GLP-1(7–36)-NH_2	6.28 ± 1.36	17.12 ± 3.19**	12.10 ± 3.60**	8.08 ± 2.42***	7.68 ± 1.99**
Gly_8-GLP-1(7–36)-NH_2	5.70 ± 1.78	12.98 ± 1.31***	8.27 ± 1.47***	7.70 ± 1.78***	7.18 ± 2.23**
mPEG-MAL-Gly_8-Cys_{30}-GLP-1(7–36)-NH_2	6.45 ± 1.54	15.42 ± 3.43**	11.98 ± 2.51***	9.28 ± 2.08**	7.55 ± 2.48**

** $P < 0.01$ versus saline.
*** $P < 0.001$ versus saline.

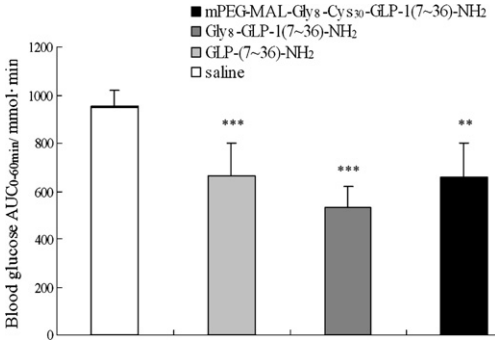


Figure 5. Blood-glucose AUC values for up to 60 min following ip administration of glucose in combination with saline, GLP-1(7–36)-NH_2, or GLP-1 derivatives simultaneously, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus saline ($\bar{x} \pm s$, $n = 6$).

effect on blood-glucose level. Compared with the control group, the blood-glucose concentrations were significantly lower following administration of GLP-1, and the *P* values were less than 0.01 at 15, 30, 60 min and less than 0.001 at 45 min. The Gly_8-GLP-1 was found to be more effective than native GLP-1, and the *P* values were less than 0.001 at 15, 30, 45 min and less than 0.01 at 60 min. Administration of mPEG-MAL-Gly_8-Cys_{30}-GLP-1 (7–36)-NH_2 resulted in a moderate blood-glucose-lowering activity, and the *P* values were less than 0.01 at 15, 45, 60 min and less than 0.001 at 30 min compared with control. As shown in Figure 5, the blood-glucose AUC was significantly lower following the administration of GLP-1(7–36)-NH_2 or its derivatives.

To investigate the duration of glucose-lowering effect of GLP-1 derivatives, saline, GLP-1(7–36)-NH_2, or GLP-1 derivatives were administered by intraperitoneal injection firstly, and glucose was ip injected alone after 90 min. Table 4 shows the blood-glucose responses at 0, 15, 30, 45, 60 min after administration of glucose. Saline and GLP-1(7–36)-NH_2 had no effect on blood-glucose level. Compared with saline group, the glucose-lowering potency of Gly_8-GLP-1(7–36)-NH_2 was statistically significant at 15, 30, and 45 min. The mPEG-MAL-Gly_8-Cys_{30}-GLP-1(7–36)-NH_2 was found to be more effective than Gly_8-GLP-1(7–36)-NH_2, and the *P* values were less than 0.01 at 15, 30 min and less than 0.05 at 45, 60 min compared with control. Compared with the control group, blood-glucose AUC of native GLP-1 had no significant difference. The *P* value of blood-glucose AUC was less than 0.05 following the administration of Gly_8-GLP-1(7–36)-NH_2. Following the administration of

Table 4
Blood-glucose concentrations following ip administration of glucose alone after ip injection of saline, GLP-1(7–36)-NH_2, or GLP-1 derivatives 90 min ($\bar{x} \pm s$, $n = 6$)

	Blood-glucose concentration (mmol/L)				
	0 min	15 min	30 min	45 min	60 min
Saline	5.78 ± 1.07	21.20 ± 2.31	17.98 ± 2.05	14.10 ± 3.15	10.97 ± 2.01
GLP-1(7–36)-NH_2	6.57 ± 1.08	23.27 ± 3.53	18.02 ± 2.12	13.48 ± 1.09	9.83 ± 1.71
Gly_8-GLP-1(7–36)-NH_2	5.18 ± 0.76	17.92 ± 1.87*	14.67 ± 2.42*	10.23 ± 2.48*	9.18 ± 1.93
mPEG-MAL-Gly_8-Cys_{30}-GLP-1(7–36)-NH_2	5.83 ± 1.36	15.90 ± 3.06**	12.43 ± 2.47**	9.07 ± 2.18**	7.70 ± 1.67*

* $P < 0.05$ versus saline.
** $P < 0.01$ versus saline.

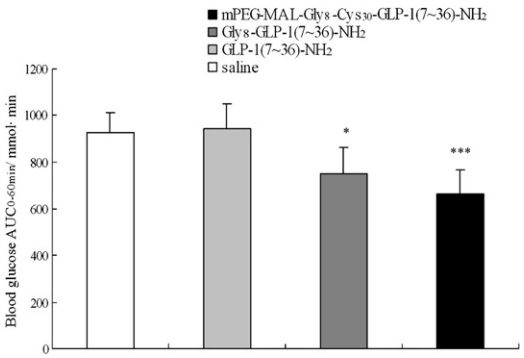


Figure 6. Blood-glucose AUC values for up to 60 min following ip administration of glucose alone after ip injection of saline, GLP-1(7–36)-NH_2, or GLP-1 derivatives 90 min $^{*}P < 0.05$, $^{***}P < 0.001$ versus saline ($\bar{x} \pm s$, $n = 6$).

mPEG-MAL-Gly_8-Cys_{30}-GLP-1(7–36)-NH_2, blood-glucose AUC was reduced more significantly than Gly_8-GLP-1(7–36)-NH_2, and the P value was less than 0.001 compared with control (see Fig. 6). This indicated that the site-specific PEGylation endowed by the N-terminal modified GLP-1(7–36)-NH_2 markedly enhanced stability in vivo.

In the case where glucose was given right after the administration of GLP-1(7–36)-NH_2 or its derivatives, Gly_8-GLP-1(7–36)-NH_2 displayed the strongest glucose-lowering effect, whereas mPEG-MAL-Gly_8-Cys_{30}-GLP-1(7–36)-NH_2 was similar to native GLP-1. The results demonstrated that substituting the Ala_8 with Gly did not impair biological activity of GLP-1. Due to the improvement of stability to DPP IV, the substitution of enzymatic hydrolyzation position Ala_8 could improve the blood-glucose-lowering effect of GLP-1 remarkably instead. PEGylation of N-terminal modified GLP-1 could still maintain moderate glucose-lowering effect although it exhibited some negative effect on N-terminal modified GLP-1 probably because the PEG chain probably bound around the peptide's active sites to a certain extent. It indicated that substituting the Ala_{30} with Cys for PEGylation had no prominent unfavorable effect on the biological activity of GLP-1. On the other hand, when glucose was given 90 min after the administration of native GLP-1 or its derivatives, mPEG-MAL-Gly_8-Cys_{30}-GLP-1(7–36)-NH_2 still showed significant glucose-lowering activity, whereas Gly_8-GLP-1(7–36)-NH_2 showed only weak activity and native GLP-1 lost the activity completely. This manifested that mPEG-MAL-Gly_8-Cys_{30}-GLP-1(7–36)-NH_2 was more stable than the merely N-terminal modified Gly_8-GLP-1(7–36)-NH_2 in vivo. Maybe it was because of the fact that further PEGylation prevented quick recognition by enzymes and clearance in kidney due to the increase of molecular size and steric hindrance of peptide. Furthermore, the purposeful site-specific PEGylation avoided shielding the active position and maintained the biological activity as far as possible.

3. Experimental

3.1. Materials

Fmoc Rink Amide-MBHA resin and Fmoc-protected amino acids were obtained from GL biochem (Shanghai, China). HPLC grade acetonitrile was purchased from Merck (Darmstadt, Germany). DPP IV was purchased from Sigma–Aldrich (St. Louis, USA). mPEG-OH was purchased from Fluka (Steinheim, Germany). Kunming mice (male, 10 weeks old) were purchased from the comparative medical center of Yangzhou University (Jiangsu, China). Other commercially available solvents and reagents were used without further purification. Microwave irradiation procedures were performed in a Discover® focused single mode microwave synthesis system (CEM, NC, USA), which produced continuous irradiation at 2450 MHz. The HPLC analysis was performed on a Shimadzu 2010C HPLC system. For purification, Shimadzu LC-10 preparative RP-HPLC system was used. The ESI-Q-MS spectra of the peptides were obtained with an Agilent Technologies Series 1100 LC/MSD SL system (Palo Alto, USA). Characterization of PEGylated peptide was performed on an ESI-IT/TOF-MS (Shimadzu, Japan). NMR spectra were recorded on a Bruker MSL 300 MHz spectrometer using deuteriochloroform as the solvent. Chemical shifts were reported in ppm relative to TMS as an internal standard.

3.2. General procedure for the prolongation of peptide on resin under microwave irradiation

Fmoc Rink Amide-MBHA resin (**2**) (0.015 mmol) was placed in a peptide synthesis vessel, swollen in DMF, and deprotected with 25% piperidine in 5 mL DMF for 4 min under microwave irradiation (microwave power: 10 W). After washing three times with DMF, a mixed solution of 0.045 mmol Fmoc-Arg(Pbf)-OH, 0.045 mmol HBTU, 0.045 mmol HOBT, and 0.090 mmol DIPEA dissolved in 4 mL DMF was added to the vessel. Then the mixture was bubbled with N_2 for 10 min under microwave irradiation (microwave power: 10 W) and was washed three times with DMF. The temperature of reaction mixture under microwave irradiation was kept below 60 °C. Qualitative ninhydrin test was applied to examine whether there were free amino groups existence. The procedures of deprotection and coupling were repeated with relevant Fmoc-protected amino acids to give peptide-resin (**5**). Then resin was washed successively with DMF three times.

3.3. General procedure for peptides cleavage from the resin

Final peptide (**1**) was cleaved from the resin by 7 mL Reagent K (TFA/thioanisole/water/phenol/EDT, 82.5:5:5:5:2.5) for 1.5 h at room temperature. Peptide was precipitated by addition of 50 mL cold ethyl ether; after centrifugation, the ethyl ether was removed, the peptide pellet was resuspended in cold ethyl ether, and this process was repeated three times. Crude peptide yield: 92% as a white powder.

3.4. General procedure for HPLC purification of crude peptides

The crude peptides were dissolved in water and purified on Shimadzu preparative RP-HPLC. The condition for purification: Shimadzu C18 reversed-phase column (5 μm, 340 mm ×28 mm), a linear gradient of mobile phase 20–75% B (mobile phase A: water with 0.1% TFA, mobile phase B: acetonitrile with 0.1% TFA) in 30 min at a flow rate of 6.0 mL/min, and ultraviolet (UV) detection at 214 nm.

3.5. Synthesis of compound 11

3.5.1. mPEG-tosylate (7)

mPEG-OH (**6**) (MW ≈ 2000, 10 g, 5 mmol) and TEA (2.02 g, 20 mmol) were dissolved in DCM (60 mL), and then stirred for 20 min at room temperature. 4-Toluenesulfonyl chloride (3.81 g 20 mmol) dissolved in 15 mL DCM was added dropwise over 30 min, and then the reaction mixture was stirred at 20 °C for 15 h. The reaction mixture was filtered, and the filtrate was washed with saturated saline (3× 50 mL). The organic layer was dried over anhydrous MgSO4, filtered, and evaporated under vacuum. Recrystallization from a mixture of Et_2O (20 mL) and DCM (4 mL) gave 7.6 g of mPEG-tosylate **7** (yield, 71.0%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 2.43 (s, 3H, $-CCH_3$), 3.36 (s, 3H, $-OCH_3$), 3.63 (s, multi H, backbone $-OCH_2CH_2-$), 4.15 (t, 2H, $-CH_2OTs$), 7.33 (d, 2H, –ArH, J = 8.1 Hz), 7.78 (d, 2H, –ArH, J = 8.1 Hz).

3.5.2. mPEG-phthalimide (8)

Compound **7** (6.5 g, 3 mmol) was dissolved in 15 mL DMF, and then potassium phthalimide (2.8 g, 15 mmol) was added. The reaction mixture was stirred at 100 °C under N_2 for 4 h. DMF was removed under reduced pressure, and the resulting yellow oil was dissolved in 15 mL of DCM. The solution was filtered, and the filtrate was dried over anhydrous $MgSO_4$, filtered, and evaporated under vacuum to a pale yellow solid. Recrystallization from a mixture of Et_2O and a small quantity of DCM gave 5.8 g (yield, 90.3%) of compound **8** as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 3.38 (s, 3H, OCH_3), 3.64 (s, multi H, backbone $-OCH_2CH_2-$), 7.68–7.91 (m, 4H, –ArH),

3.5.3. mPEG-NH_2 (9)

Compound **8** (5.8 g, 2.7 mmol) and hydrazine hydrate (8.1 mmol) were dissolved in ethanol (20 mL), and the solution was refluxed for 2 h. The reaction mixture was evaporated under vacuum, and the thick oil was crystallized from the mixture of Et_2O and DCM to give compound **9** (5.0 g, 92.6%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 2.85 (t, 2H, $-CH_2NH_2$), 3.38 (s, 3H, OCH_3), 3.64 (s, multi H, backbone $-OCH_2CH_2-$).

3.5.4. mPEG-maleic acid (10)

Compound **9** (5.0 g, 2.5 mmol) in dioxane (15 mL) was treated with maleic acid anhydride (0.98 g, 10 mmol) and DMAP (0.01 g) at 70 °C for 1 h. The reaction solution was cooled to room temperature and precipitated with cold ethyl ether. The solid was collected by filtration and washed with cold Et_2O to give 4.6 g of compound **10**, yield 88%. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 3.38 (s, 3H, OCH_3), 3.64 (s, multi H, backbone $-OCH_2CH_2-$), 6.32 (d, 1H, OCCH=CHCOOH), 6.48 (d, 1H, OCH=CHCOOH).

3.5.5 mPEG-MAL (11)

Compound **10** (4.6 g, 2.2 mmol) was dissolved in Ac_2O (10 mL), and then sodium acetate (0.36 g, 4.4 mmol) was added. The reaction mixture was stirred at 80 °C for 1.5 h and then evaporated under vacuum. The resulting thick oil was precipitated with cold ethyl ether and recrystallized twice from the mixed solvent of Et_2O and DCM to give compound **11** (2.5 g, 54%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 3.38 (s, 3H, OCH_3), 3.64 (s, multi H, backbone $-OCH_2CH_2-$), 6.71 (s, 2H, –CH=CH–).

3.6. Preparation of mPEG-MAL-Gly_8-Cys_{30}-GLP-1(7–36)-NH_2 (12)

Peptide **1** (16 mg, 5 μmol) was conjugated with compound **11** (25 mg, 12 μmol) in 5 mL of 0.05 mol/L sodium phosphate buffer (pH 7.0). The reaction mixture was stirred at 20 °C under N_2 for 1.5 h till HPLC showed completion. The analytical condition was as follows: C18 column (5 μm, 150 mm × 4.6 mm); a linear gradient of mobile phase 35–65% B in 10 min (mobile phase A: water with 0.1% TFA and mobile phase B: acetonitrile with 0.1% TFA) at a flow rate of 1 mL/min, and ultraviolet (UV) detection at 214 nm was utilized. The crude conjugate was purified on Shimadzu preparative RP-HPLC and gave 8.2 mg of purified product. The condition for purification: Shimadzu C18 reversed-phase column (5 μm, 340 mm × 28 mm), a linear gradient of mobile phase 30–70% B (mobile phase A: water with 0.1% TFA, mobile phase B: acetonitrile with 0.1% TFA) in 30 min at a flow rate of 6.0 mL/min, and ultraviolet (UV) detection at 214 nm.

3.7. Characterization by mass spectrometry

Purified compound **12** was performed on a hybrid ion-trap time-of-flight mass spectrometer equipped with an ESI source in positive ion mode (Shimadzu ESI-IT/TOF-MS). The analytical condition was as follows: scan range, m/z 600–1000; detector voltage, 1.60 KV; nebulizing gas (N_2) flow, 1.5 L/min; dry gas (N_2) flow, 5 L/min; pressure of TOF region, 1.5×10^{-4} Pa; ion-trap pressure, 1.7×10^{-2} Pa; ion accumulated time, 30 ms; precursor ion selected width, 3.0 amu. Accurate mass was corrected by calibration using the standard material sodium trifluoroacetate cluster as internal reference.

An Agilent Technologies Series 1100 LC/MSD SL system was utilized for analysis of synthetic peptides and degradation fragments of GLP-1. The MS condition was as follows: positive ion mode; scan range, m/z 500–1200; dry gas (N_2) flow, 10 L/min; nebulizer pressure 45 psi; capillary voltage 3.00 KV.

3.8. Incubation of GLP-1(7–36)-NH2 and its derivatives with DPP IV

GLP-1(7–36)-NH_2 and its derivatives (5 nmol) were incubated at 37 °C with purified DPP IV corresponding to an enzymatic activity of 5 mU for 4 h in 50 μL of 0.05 mol/L triethylamine–HCl buffer, pH 7.8. The enzymatic reactions were stopped by the addition of 3 μL of 10% trifluoroacetic acid. The mixtures were analyzed by using reverse-phase gradient elution with a Shimadzu C18 reversed-phase column (5 μm, 150 mm × 4.6 mm). A linear gradient of mobile phase 20–60% B in 20 min (mobile phase A: water with 0.1% TFA and mobile phase B: acetonitrile with 0.1% TFA) at a flow rate of 1 mL/min, and ultraviolet (UV) detection at 214 nm was utilized. The GLP-1(9–36)-NH_2 was collected after the analysis of the incubation mixture for ESI-MS analysis.

3.9. Blood-glucose-lowering activities of the GLP-1(7–36)-NH2 and its derivatives

Studies on blood-glucose-lowering activities of the GLP-1(7–36)-NH_2 and its derivatives were undertaken using 10-week-old male Kunming mice. Forty eight mice were randomly divided into eight groups. The animals were housed individually in an air-conditioned room at 22 ± 2 °C with a 12-h light/12-h dark cycle. The mice were fasted for 8 h but free access to drinking water before ip.

Administration ($n = 6$) of glucose (18 mmol/kg body weight) in combination with saline (0.9% NaCl) as control, GLP-1(7–36)-NH_2 or its derivatives (25 nmol/kg body weight) at 0 min. On the other four groups, male Kunming mice received ip administration ($n = 6$) of saline (0.9% NaCl), GLP-1(7–36)-NH_2, or its derivatives (25 nmol/kg body weight) firstly, and glucose was ip injected alone after 90 min. Blood was collected from the cut tip of the tail vein at 0, 15, 30, 45, and 60 min after ip administration of glucose, and the blood-glucose levels were measured by using a blood-glucose monitor (Ascensia® Breeze 2, Bayer, Germany). All data were expressed as means ± SEM and analyzed statistically by using Student's *t*-test. Value of $P < 0.05$ was taken to imply statistical significance.

4. Conclusion

In this study, mPEG-MAL-Gly_8-Cys_{30}-GLP-1(7–36)-NH_2 was synthesized and evaluated for the stability to DPP IV in vitro and glucose-lowering activity in vivo. The results demonstrated that site-specific PEGylation of N-terminal modified GLP-1 extended the acting time of GLP-1 analogue while maintaining moderate glucose-lowering effect, which revealed that this novel GLP-1 derivative may serve as a candidate for diabetes therapy.

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