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Original Article

Application of self assembling peptide as drug carrier for extending the GLP-1 stability

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Abstract: The multiple physiological characterizations of glucagon-like peptide-1 (GLP-1) make it a promising drug candidate for the therapy of type 2 diabetes. However, the extremely poor stability of GLP-1, proteolysis by dipeptidyl peptidase-IV (DPP IV), limits its development in the clinical utility significantly. In order to avoid the pocket docking of GLP-1 against DPP IV, self assembling peptide was employed in this study. Self assembling peptide (pp1) is capable of forming supramolecule contain a cavity which load GLP-1 molecules inside. In this study, the formation procedure of GLP-1/pp1 complex was computationally simulated, in order to further investigate the interaction between GLP-1 and pp1. In this study, the amino acid residues in GLP-1 and pp1 those involved in forming hydrogen bonds were ascertained which benefits the further mutation and improvement on pp1. In addition, the glucose tolerance test in rodent animals was employed to ascertain the prolonged stability of GLP-1 followed the complex formation with pp1. Our results clarified the mechanism of the formation of GLP-1 and pp1 stable complex, which is significant for its development into clinical trial.

Keywords: Self-assembled peptide, molecular dynamics, simulations, GLP-1, sustained complex

Introduction

Glucagon-like peptide 1 (GLP-1) is a gut hormone released from intestinal L cells after oral glucose administration [1]. It serves as an incretin factor stimulating the secretion of insulin, and reduces the levels of blood glucose in normal subjects and patients with type 2 diabetes mellitus [2]. GLP-1 stimulates the secretion of insulin and suppresses glucagon secretion in glucose-dependent manner, which minimizes the risk of hypoglycemia [3, 4]. It was also found that GLP-1 restores the glucose sensitivity of pancreatic β-cells, which possibly depend on the increasing level of GLUT2 and glucokinase expression [5]. Furthermore, GLP-1 promotes β-cell regeneration, proposing that it is effective for the treatment of type 1 diabetes [6, 7]. Report proved that GLP-1 inhibits apoptosis (programmed cell death) in cells, consequently improves their survival [8]. Evidence has demonstrated that GLP-1 was identified to play a crucial role in the regulation of glucose metabolism, and the infusion of GLP-1 induces the decreasing level of blood glucose [9, 10]. The deficiency of GLP-1 secretion in type 2 diabetes suggested that GLP-1 acts as a promising potential therapy for type 2 diabetes [11]. The most serious challenge is that GLP-1 has an exceptionally short half-life of less than 2 mins *in vivo* [12], due to the rapid degradation of the enzyme dipeptidyl peptidase IV (DPP-IV) [13]. This caused that the therapeutic administration of GLP-1 seemed impractical, thus many efforts have focused on amending the pharmacokinetic properties of GLP-1 in a series of derivatives and analogues [14].

Crystallography data showed that GLP-1 is a continuous α-helix from Thr13 to Val33, with a kink around Gly22 [15-17]. It is established that the residues between Ala24 and Val33 interact with the Extra-Cellular Domain (ECD) of GLP-1 receptor (GLP-1R) [18, 19]. The hydrophilic face of this α-helix is comprised by residues Gln23, Lys26, Glu27, and Lys34; the hydrophobic face of it is defined by Ala24, Ala25, Phe28, Ile29, Leu32, and Val33 [16, 17]. The hydrophobic

residues of this amphiphilic α-helix all interact with the ECD, whereas Lys26 is the only one of the hydrophilic residues is possible to interact directly with the ECD. Previous Ala scanning assay has demonstrated the importance of Phe28, Ile29, and Leu32 in GLP-1 binding [20]. The mutation of the residues on the hydrophobic side of GLP-1 causes loss of ECD binding affinity, while that on the hydrophilic face does not [21, 22]. Moreover, the N-terminal truncation of GLP-1 are unable to bind and activate GLP-1R [23], this segment is an obstacle for long-acting GLP-1 analogs as the main site of DPP-IV degradation. Two GLP-1 analogous, Exenatide and Liraglutide, were approved by FDA for the treatment of type 2 diabetes in 2005 and 2010, respectively [24, 25]. Compared with Exendin-4 and two GLP-1 analogs, the utility of hGLP-1 is quite probably to avoid any possible immunotoxicity of non-mammalian product and improve the biotolerance in turn.

In our previous study, the self-assemble peptide (pp1) was employed to form a stable complex with native hGLP-1 [26]. The serum stabilization assay of GLP-1/pp1 complex illuminated that the complex formation leads to an improvement of GLP-1 stability *in vitro* and *in vivo* [26]. It was presumed that pp1 formed a complex which provided with a 'protecting surface' protects the GLP-1 molecule inside against the enzymatic degradation [26]. In order to clarify the formation of stable complex of GLP-1 and pp1 deeply, the molecular dynamics simulations were performed and the interior hydrogen bonds were analyzed via molecular dynamics simulations. Upon the simulations, hydrogen bonds and torsion angle were analyzed; the amino acid residues of either pp1 or GLP-1 involved in the formation of hydrogen bonds were ascertained as well. The simulations data helped us to investigate the mechanism of the complex formation and perform the further mutation for improved assembling activity. Combined with simulation data, the glucose tolerance test was performed in order to ascertain the prolonged stability of complex in experimental animals.

Self-assembled peptides display the advantageous properties of stability, injectability, biodegradability and biocompatibility. These peptides, by self-assembling, can be widely applied in such fields as drug delivery (small molecules and large molecules), regenerative medicine and nanobiotechnology. The various physical, chemical and biological aspects of self assembling peptide family lead to different application field. Clarification the mechanism of self assembling peptide makes these novel biomedical materials to be applied much more available.

Materials and methods

Animals

All studies were carried out with permits from the Animal Experiments Inspectorate, China. Male SD rats were obtained from SLAC Laboratory Animal, China Academy of Sciences (Shanghai, China).

Simulation procedures

In the current work, molecular dynamics (MD) simulations were carried out for two different systems containing different amount of pp1 peptides and one GLP-1 peptide. The two systems represent GLP-1 in pp1 solutions with different concentrations. AMBER force field and GBSA model, which count solvent effect implicitly, were chosen for the simulations. Simulations were performed by AMBER 99 software package. In the MD run, Langvin thermostat method was used to fix the temperature at 300 K. Large box size, which equivalents to no periodic boundary condition, was used in the runs. The initial array of system I was that the GLP-1 was immersed in ten and twenty randomly arrayed pp1 peptides. For each system, MD simulations were carried out for 1 ms. The time-step size for the integration was 1 ms, and trajectories at each of 500 time-steps were collected for analysis.

Peptide synthesis

GLP-1 (sequence as: HAEGTFTSDVSSYLEGQAAKEFIAWLVKGRG) and pp1 (sequence as: GLWWKAWWKAWWKSLWWRKRKR) were purchased from Sangon Biotech (Shanghai, China) Co., Ltd (HPLC-purified; purity >90%, identified by MS). The freeze-dried peptides were weighed and dissolved in pure water to make 10 mg/ml stock solutions for further analysis.

Glucose tolerance test upon single dose administration

A single dose of GLP-1/pp1 complex (1:10 and 1:20) (GLP-1: 300 μg/kg body weight) was

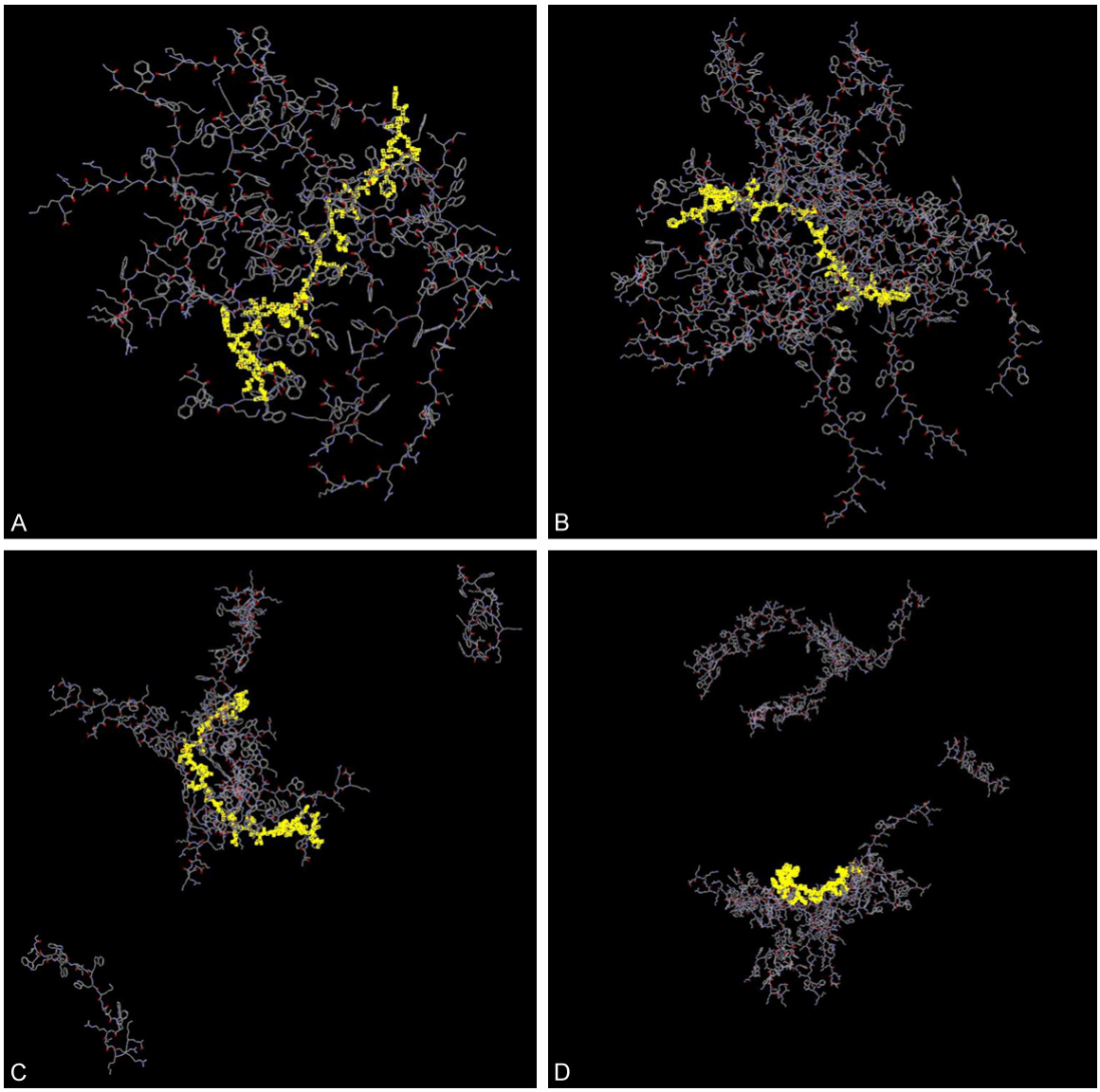

Figure 1. Molecular dynamics simulation of GLP-1 and pp1. A. Initial array of system I. GLP-1 (yellow) was immersed in 10 randomly arrayed pp1 peptides (grey). B. Initial array of system II. GLP-1 (yellow) was immersed in 20 randomly arrayed pp1 peptides (grey). C. Snapshot of the GLP-1 and pp1 complex at the end of MD simulation in system I. D. Snapshot of the GLP-1 and pp1 complex at the end of MD simulation in system II. Conditions: AMBER force field and GBSA model which counts solvent effect implicitly were chosen for the simulations. Simulations were performed by AMBER 99 software package. In the MD run, Langvin thermostat method was used to fix the temperature at 300 K. Large box size which equivalents to no periodic boundary condition was used in the runs. For each system, MD simulations were carried out for 1 ms. The time-step size for the integration was 1 ms and trajectories at each of 500 time-steps were collected for analysis. Legend: GLP-1 was wrapped by pp1 peptide in system I and system II. The formation of this complex protected the GLP-1 degradation against DPP IV proteolysis.

injected subcutaneously into fasting SD rats (n=10 per group, male) 30 min prior to glucose administration. GLP-1 was injected into the control animals. Rats were given 2 g glucose/kg body weight via intraperitoneal injection. Blood was drawn from the tail vein, and glucose levels were measured using a glucometer 30 min after glucose administration. Chronic glucose (2 g/kg body weight) was administrated 30 min prior to each blood glucose measurement time point during the 100-h experimental period.

Statistical analyses

Student's *t*-test was employed for analyses of the data. Unless otherwise stated, the results were reported as the means ± standard error. *P*

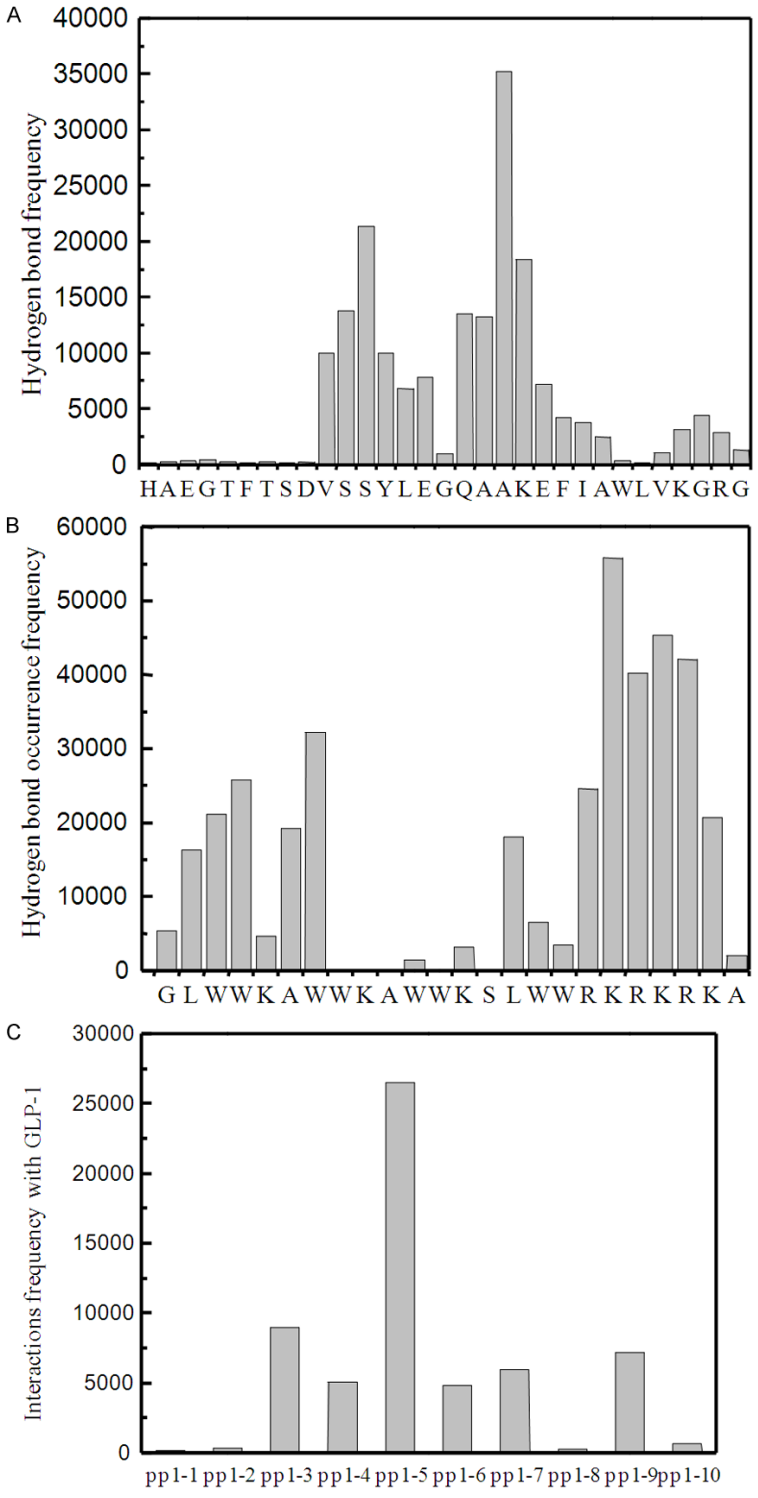

Figure 2. Hydrogen bonds analysis of pp1 and GLP-1 complex in system I. A. Hydrogen bonds formation frequency of GLP-1 residues with pp1 peptide. B. Hydrogen bonds formation frequency of pp1 residues with GLP-1. C. The numbered pp1 peptides formed complex with GLP-1 by hydrogen bonds. Conditions: The hydrogen bonds were determined by checking the distance between polarized hydrogen (H-N, H-O) and electronegative atoms O and N. Legend: The hydrogen bonds between residues of GLP-1 and residues of neighboring pp1 peptides in systems I was analyzed. It was observed that hydrogen bonds were formed mainly in the middle region and C-terminus of GLP-1 as showed in panel A. The N-terminus of GLP-1 exhibited very low probability of forming hydrogen bond with closed pp1 peptides. In addition, the residues of pp1 peptide involved in the forming hydrogen bond with GLP-1 were analyzed, as showed in panel B. It was found that both N- and C-terminus of pp1 played important role in the formation of hydrogen bonds with GLP-1. These amphipathic terminuses are key domains interacting with GLP-1. Our result revealed that the residues W, K, and R in pp1 formed most of hydrogen bonds with GLP-1. The molecular dynamic simulation data revealed that there are 7 pp1 molecules involving in the complex formation in system I (totally 10 pp1 peptide molecules).

values less than 0.05 were considered significant.

Results and discussions

Binding mode of GLP-1 and pp1 peptide

Our previous findings indicated the formation of a supramolecular complex of GLP-1 and pp1 by HPLC assays. The incubating mixture of pp1 and GLP-1 was loaded onto C18 column and the spectra feature was compared to those from GLP-1 and pp1 alone, respectively. Data demonstrated the existence of supramolecule and it was clarified as complex of GLP-1 and pp1 upon the ratio of 1:6 using MALDI-TOF. In current study, **Figure 1C** and **1D** showed the snapshots of systems I (initial assay as **Figure 1A**) and II (initial assay as **Figure 1B**) at the ends of the MD runs. We found that GLP-1 was wrapped by five pp1 peptides in system I and seven pp1 peptides in system II. From **Figure 1C** and

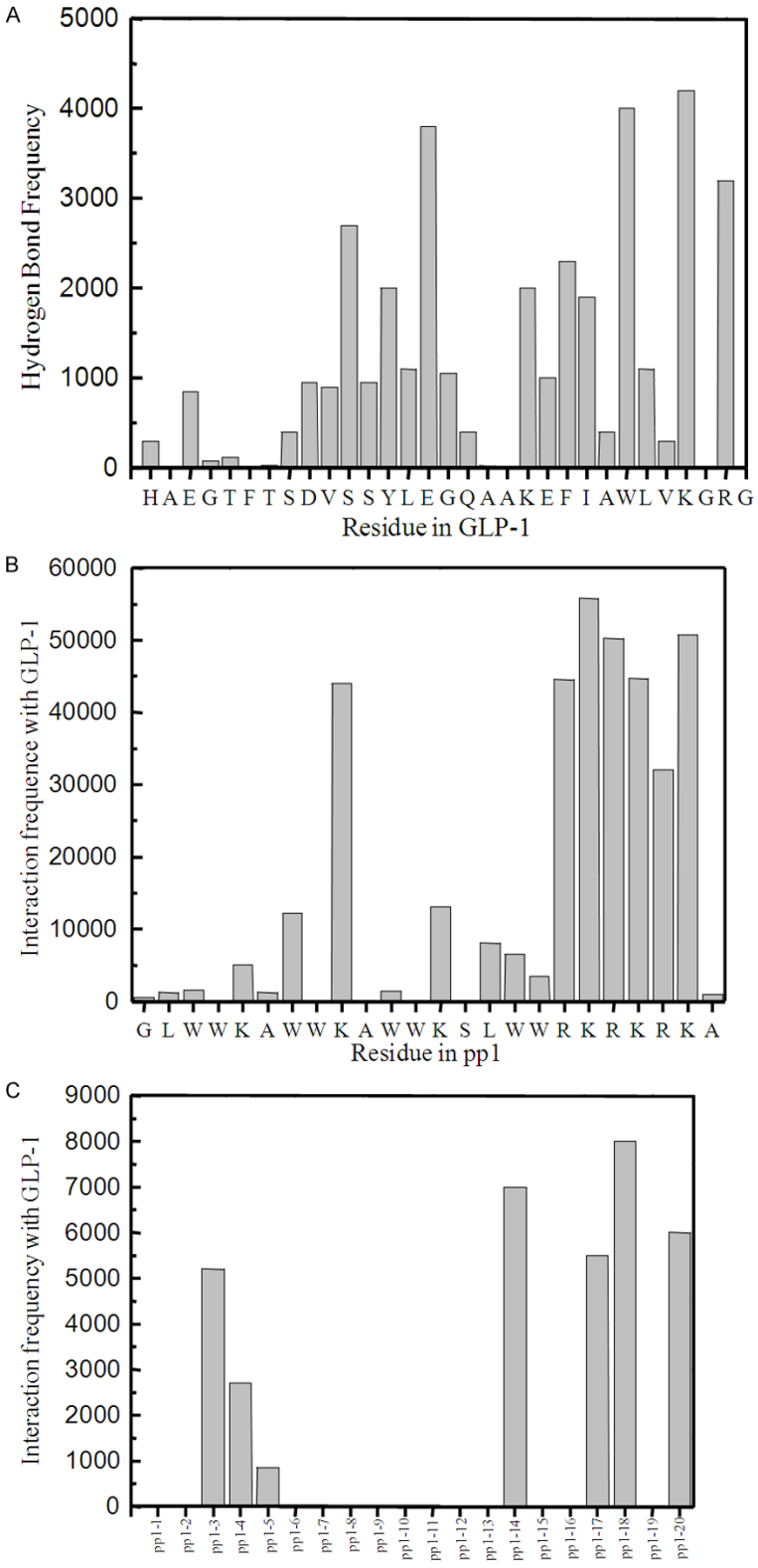

Figure 3. Hydrogen bonds analysis of pp1 and GLP-1 complex in system II. A. Hydrogen bonds formation frequency of GLP-1 residues with pp1 peptide. B. Hydrogen bonds formation frequency of pp1 residues with GLP-1. C. The numbered pp1 peptides formed complex with GLP-1 by hydrogen bonds. Conditions: The hydrogen bonds were determined by checking the distance between polarized hydrogen (H-N, H-O) and electronegative atoms O and N. Legend: The hydrogen bonds between residues of GLP-1 and residues of neighboring pp1 peptides in systems I was analyzed. It was observed that hydrogen bonds were formed mainly in the middle region and C-terminus of GLP-1 as showed in panel A. The N-terminus of GLP-1 exhibited very low probability of forming hydrogen bond with closed pp1 peptides. In addition, the residues of pp1 peptide involved in the forming hydrogen bond with GLP-1 were analyzed, as showed in panel B. It was found that both N- and C-terminus of pp1 played important role in the formation of hydrogen bonds with GLP-1. These amphipathic terminuses are key domains interacting with GLP-1. Our result revealed that the residues W, K, and R in pp1 formed most of hydrogen bonds with GLP-1. The molecular dynamic simulation data revealed that there are 8 pp1 molecules involving in the complex formation in system II.

1D, it was observed that GLP-1 could form a complex with pp1 in system I and system II. Like showed in **Figure 1**, the GLP-1 molecule was wrapped by pp1 peptides to form a stable complex which might provide the inside GLP-1 molecule against the degradation of DPP IV.

The frequency of occurrence of hydrogen bonds between GLP-1 and pp1

It was identified that the pp1 peptides bound GLP-1 via hydrogen bonds, by investigating the distance between polarized hydrogen (H-N, H-O) and electronegative atoms O and N (**Figures 2** and **3**). Bar charts showed the frequency of occurrence of the hydrogen bonds between residues of GLP-1 and residues of neighboring pp1 peptides in sys-

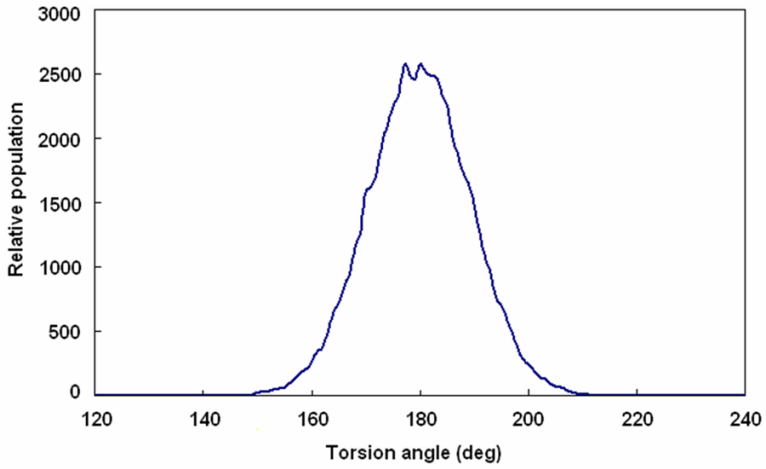

Figure 4. Analysis of torsion angles in GLP-1 and pp1 complex. Legend: It showed that these angles are all populated around 180° indicating that GLP-1 maintaining its active form even upon the wrapping of pp1 peptides. Results illuminated that the existence of pp1 unable to induce the conformational change of GLP-1.

tems I and II. It was observed that hydrogen bonds were formed mainly in the middle region and C-terminus of GLP-1 as showed in **Figures 2A** and **3A**. Interestingly, the N-terminus of GLP-1 exhibited very low probability of forming hydrogen bond with closed pp1 peptides. The simulation data provided irrefutable evidence to the previous finding: the N-terminal of GLP-1 plays an important role in their physiological functions by interacting with ECD of GLP-1 receptor. Thus it was considered that the formation of hydrogen bonds in N-terminal segments of GLP-1 would impair the docking of GLP-1 to receptor inevitably, but not in C-terminal. These presumed that pp1 provided a protection role for the GLP-1 inside and remained their activities successfully. **Figures 2C** and **3C** indicated that 7 (peptide numbered 3, 4, 5, 6, 7, 9 and 10 in system I, **Figure 2C**) or 8 (peptide numbered 3, 4, 5, 14, 17, 18, 19 and 20 in system II, **Figure 3C**) interacted with GLP-1 upon high hydrogen bonds frequency, which is coincided with our previous results that GLP-1 could form stable complexes at the molar ratio of 1:6 by reverse phase HPLC and S-200 gel filtration column [26].

In addition, results presented that both N- and C-terminus of pp1 showed high hydrogen bond occurrence frequency, suggesting that they are vital for the formation of hydrogen bonds with GLP-1 (**Figures 2B** and **3B**). Structural analysis of pp1 illuminated that pp1 possesses 'L shape' helix containing hydrophobic and hydrophilic tail end, furthermore, the amphipathic terminuses are key domains interacting with GLP-1. Our result also revealed that the residues W, K, and R of pp1 have high probability of the formation of hydrogen bonds with GLP-1.

The occurrence of hydrogen bonds in each residue of pp1 provides information about hydrogen donors and acceptors, in **Figure 4**. Result indicated that residues Glu, Leu and Asp in pp1 can only be hydrogen bond acceptors, while the rest of residues can either be acceptor or donor. Data proved that these three residues interacted with GLP-1 only, and importantly suggested the mutations at these positions should be carefully in order to avoid the self-aggregation of pp1 or loss of binding affinity with GLP-1.

Torsion angle analysis of GLP-1/pp1

Torsion angle analysis was carried out to investigate whether pp1 binding changed the conformation of GLP-1. **Figure 5** showed that the main frame torsion angles of GLP-1 and closed pp1 peptides in system I are all populated around 180°, indicating that GLP-1 maintained its active form even upon the wrapping of pp1 peptides and the existence of pp1 is unable to influence the conformational of GLP-1 and its physiological activities, such as insulin secretion, cAMP and blood glucose regulation.

Anti-diabetic activity of complex of GLP-1 and pp1 in long-acting manner

To ascertain the long-lasting effects on glucose tolerance of complex of GLP-1 and pp1, glucose tolerance tests (GTTs) were performed 120 h after single dose administration into Sprague Dawley rats (n=6 per group, male). As shown in **Figure 6**, the rats treated with wild-type GLP-1 had high blood glucose levels at 11 mmol/L approximately in the whole experimental period of 100 h since the GLP-1 had been degraded rapidly. However, as predicted, the rats injected

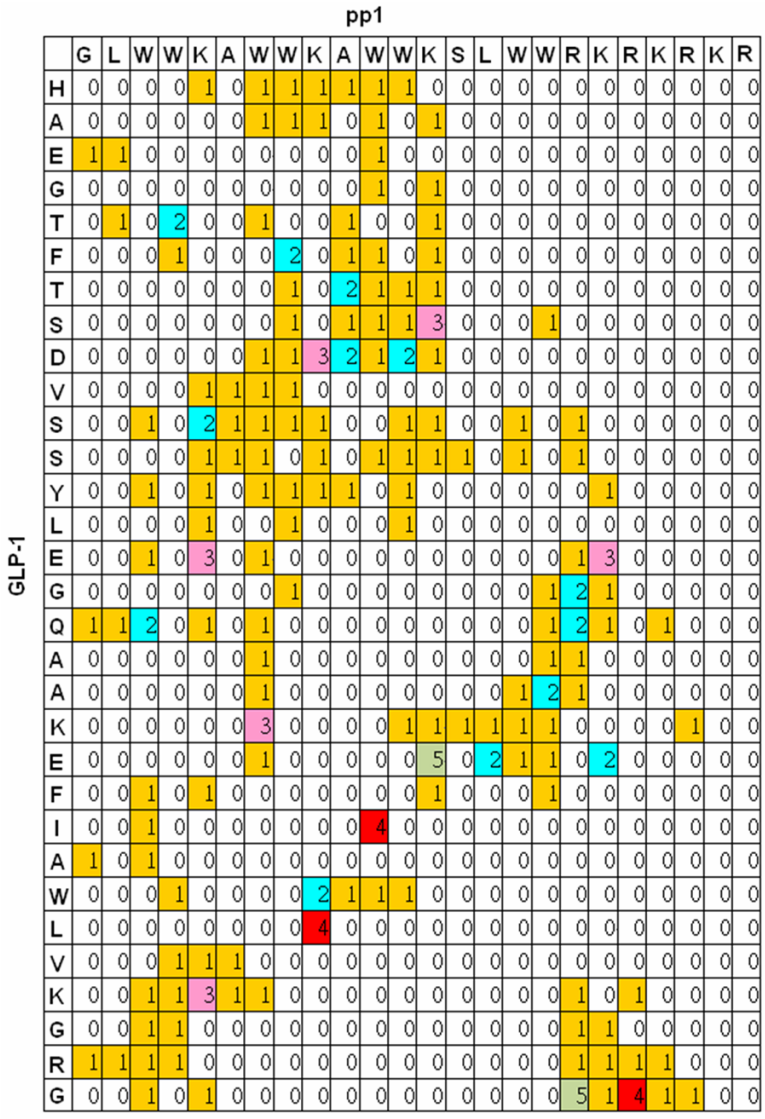

Figure 5. Occurrences of hydrogen bonds between GLP-1 and pp1 peptides (the numbers in the matrix are the counting numbers divided by 1000). Legend: The occurrences of hydrogen bonds formed between GLP-1 and pp1 molecule was obtained by MD simulations. Data illuminated the residues in either GLP-1 or pp1 with higher frequency for hydrogen bonds formation.

with GLP-1 complex showed improved glucose tolerance in this experiment. The blood glucose levels were maintained at 8 mmol/L after 60 h, $P<0.01$.

Conclusion

The discovery of self-assemble peptides provides a platform for a drug sustained releasing system. Self-assemble peptides possess a self-assembly property which depends on amphipathic domains. GLP-1 is considered to be potent tool for treatment of type 2 diabetes since distinct blood glucose regulation properties, such as insulin secretion in blood glucose dependent manner etc. However, the clinical utility of GLP-1 is significantly limited by its short half-life in vivo (2 minutes approximately). According to this, many scientists focus on its long-acting analogue or sustained releasing formulation in order to realize the clinical utility of GLP-1. In our previous study, we found self-assemble peptide (pp1) is able to form a stable complex with hGLP-1, and this complex extends the half-life of GLP-1 to 72 hours in vivo [26]. Beside the prolonged half-life of GLP-1, the insulin secretion assay and glucose tolerance test reveal the remained physiological properties of wrapped GLP-1. Depending on previous finding, the computational simulations were performed in this study in order to investigate the detailed interaction between GLP-1 and pp1. In current study, the computational simulation investigated the interaction of pp1 and GLP-1 in detail and data indicated that five pp1 molecules are sufficient for providing a fully protection of GLP-1 against the protease degradation. The interaction of pp1 and GLP-1 blocked the recognition of DPP IV to GLP-1 and then prolonged the stability of GLP-1. We also performed further analysis to clarify the residue(s) involved in this interaction in both pp1 and GLP-1 peptides. Elucidation the crucial residues for this interaction will be helpful to further mutate of pp1 peptide in order to achieve an improved the assembling property.

Acknowledgements

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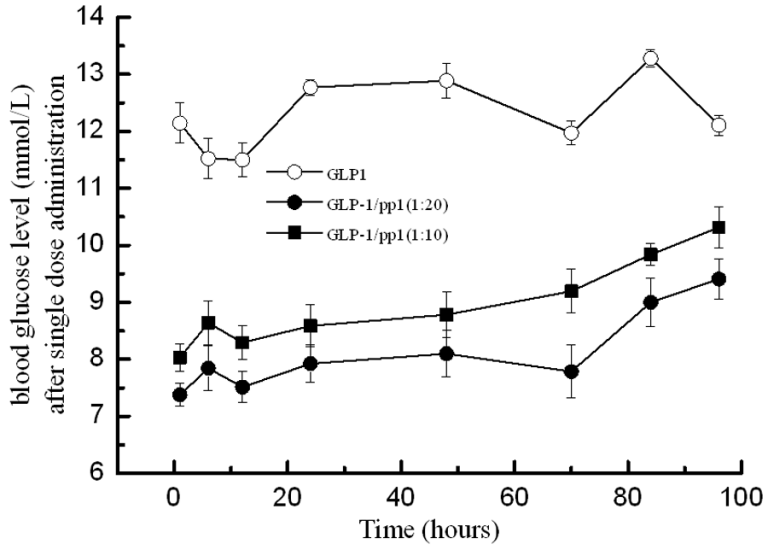

Figure 6. GLP-1/peptide complexes improve glucose regulation in ZDF rats. Conditions: The intraperitoneal injections of GLP-1/peptide 1 complex (molecular ratio at 1:10 ●; and ratio at 1:20 ■) (300 μg GLP-1/kg body weight) were administrated once in the whole experimental period of 100 hours and then glucose levels were measured by a glucometer at indicated times. GLP-1 (▲) (300 mg/kg body weight) was administrated daily and Exenatide (♦) (100 mg/kg body weight) was injected intraperitoneally twice daily. Saline (□) was injected in controls. Legend: Results indicated that the GLP-1/peptide 1 treated rats maintained relatively constant glucose levels within 70 hours.

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Disclosure of conflict of interest

None.

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