Drug Delivery

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A Protease-Based Strategy for the Controlled Release of Therapeutic Peptides**

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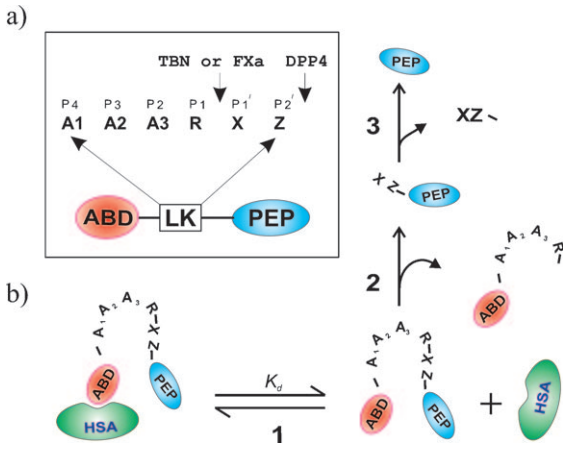
Peptides are considered as viable therapeutic agents because of their superior specificity compared to small molecules and their smaller size relative to antibodies.[1–4] However, the discovery of peptide drugs has been hampered by their short half-life and limited bioavailability.[5] Many approaches have been explored to improve their pharmacokinetics including structural modifications, coadministration of competitive inhibitors, and various delivery systems.[6,7] Of these strategies, coupling a bioactive peptide to a plasma protein, or a protein domain that can associate with a plasma protein, shows promise for extending the peptide half-life.[8–14] Human serum albumin (HSA) is an endogenous molecule transporter with a half-life of 19 days,[15] and thereby HSA has unique advantages over other plasma proteins as a drug carrier for improving the half-life of therapeutic peptides.[9] However, coupling to HSA often reduces peptide bioactivity in the fusion form. To regain peptide activity, the coupled peptide should be liberated from its fusion form without modification.

Our intention is to design a small albumin-binding polypeptide that can associate with HSA and liberate the bioactive peptide. To achieve this goal, an albumin-binding polypeptide containing three function domains, an albumin-binding domain (ABD), a bioactive peptide (PEP), and a protease-cleavable linker (LK), was designed (Scheme 1 a). In vivo, the polypeptide can associate with HSA so that its circulation life can be enhanced (Scheme 1 b, step 1). When the polypeptide dissociates from HSA, it is scissile to protease hydrolysis and consequentially releases the coupled peptide (Scheme 1 b, step 2). We aim to utilize either human thrombin (TBN) or Factor Xa (FXa) as scissors because they are highly specific and regulated.[16–18] It is unlikely that the designed polypeptides will interfere with the physiological functions of the proteases. More importantly, the basal concentrations of these proteases in diabetes patients are at a stable level.[19–21] TBN is specific for the amino acid sequence Phe(F)-Asn(N)-Pro(P)-Arg(R)-Xxx(X)-Zzz(Z), where X and Z are small amino acid residues such as Gly (G) and Ser (S), respectively.[22] FXa is specific for the Ile(I)-Glu(E)-Gly(G)-Arg(R)-Yyy(Y) sequence, where Y can be any amino acid residue. If these sequences are used as the LK in albumin-binding polypeptides, FXa will mediate the release of the unmodified peptides because the N terminus of the peptide target can be integrated into the LK sequence. But TBN-mediated hydrolysis will generate an intermediate with an X-Z dipeptide attached to the N terminus of the peptide target.

To examine a proof of concept that TBN or FXa can control the release of a coupled peptide by modifying the LK sequence, this work primarily focuses on the selection of amino acid residues at positions P_1' and P_2' for fast cleavage (Scheme 1 a). We also consider employing DPP4, a plasma dipeptidase, to remove X-Z dipeptide from the intermediate generated by TBN (Scheme 1 b, step 3). DPP4 is specific for



Scheme 1. Plasma protease-based strategy for controlled release of therapeutic peptides. a) An albumin-binding polypeptide contains three functional domains. The specificity of proteases is presented by their substrate sequence, and their cleavage sites are marked by arrows. Note that human thrombin (TBN) (or human Factor Xa (FXa)) and plasma dipeptidase DPP4 hydrolyze the LK sequence sequentially. b) The half-life of ABD-LK-PEP in the bloodstream can be elongated through association with HSA (step 1). Stepwise release of bioactive peptide from dissociated fusion polypeptide is achieved through cleavage of the LK sequence by either TBN or FXa (step 2). Dipeptidase DPP4 can be employed (step 3) following TBN-mediated hydrolysis. K_d = dissociation constant.

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N-terminal dipeptide with Pro (P) or Ala (A) at position P_2'. Taken together, a consensus of the LK sequence, that is, $A_1A_2A_3$R-GA/P/S (where $A_1A_2A_3$ is F-N-P or I-E-G), should be a suitable linker for the fast release of the unmodified peptide from an albumin-binding polypeptide. Therefore, finding high-affinity ABDs and tuning the proteolysis rate of LK sequences by TBN, FXa, and DPP4 are key issues to make this strategy effective for the controlled release of bioactive peptides.

We used glucagon-like peptide-1 (GLP-1, a potential therapeutic peptide for Type 2 diabetes) and its clinically approved analogue (exendin-4, or Ex-4) as models to demonstrate that plasma proteases can function as scissors for the sustained release of the unmodified therapeutic peptides from albumin-binding polypeptides. The clinical application of GLP-1 and Ex-4 is limited by their short half-lives (2 and 40 min, respectively).[23–25] The improvement of their stability through structural modification has not yet been successful. Coupling of GLP-1 to HSA largely affects its ability to cross the blood–brain barrier and its biological activity.[8,26]

There are few ABDs available that either exist naturally or are selected from peptide libraries.[9,11,14] These ABDs either have large molecular sizes or contain cysteine residue(s), which could complicate their medical application. Thus, we aimed to select small cysteine-free ABDs by using phage libraries (Supporting Information, Figure S1). As a result, nine 12-mer ABD peptides were identified to exhibit high affinity for HSA (Figure 1a). Four of these ABDs contained a motif of CLP*X*WGCLW (where *X* is K, R, or Q).[9] Nevertheless, a few cysteine-free sequences were also found to exhibit high affinity to HSA (Figure 1a).

We selected two cysteine-free ABDs (ABD#6 and ABD#8) and one cysteine-containing ABD (ABD#3), which have higher binding affinity to HSA, to construct albumin-binding polypeptides for GLP-1 (Figure 1b). As a result, 12 polypeptides were constructed with linker sequences relevant to either FXa or TBN in combination with DPP4, and defined as GA31, GP32, GS33, GA61, GP62, GS63, GA81, GP82, GS83, IE3, IE6, and IE8 (Figure 1c). These 12 albumin-binding GLP-1 polypeptides were prepared by using a recombinant peptide expression system (Supporting Information, Figures S2 and S3). It was found that these polypeptides exhibit binding affinities for HSA similar to those of their cognate ABDs alone, as measured by surface plasmon resonance (Supporting Information, Figure S4).

Either TBN or FXa can function in vitro as scissors to release GLP-1 from albumin-binding GLP-1 polypeptides. To simulate the bloodstream environment in this study, the assay was conducted in phosphate-buffered saline containing polypeptide (60 μM), TBN (0.45 U mL^{-1}) or FXa (0.4 U mL^{-1}), and/or DPP4 (0.08 ng mL^{-1}). The reaction process was monitored by analytical HPLC, ELISA, and mass spectrometry for evaluation of polypeptide stability. Figure 2a compares the half-lives of the polypeptides against TBN or FXa. In the absence of HSA, polypeptides with the FNPR-GA/P linker (e.g., GA61 and GP62) exhibited more resistance to TBN than those with the FNPR-GS linker (e.g., GS63). Among the polypeptides containing cysteine-free ABDs,

a)

Seq #	ABD	K_d (μM)
ABD#1	NV**CLP**K**WGCLW**E	2.11
ABD#2	DV**CLP**Q**WGCLW**G	1.56
ABD#3	DI**CLP**R**WGCLW**E	1.12
ABD#4	NI**CLP**R**WGCLW**D	1.89
ABP#5	LPWHLKYREPPR	4.35
ABD#6	LPHSHRAHSLPP	1.67
ABD#7	SLFRHQHATPQI	3.68
ABD#8	SLLHWTHKIPAL	2.92
ABD#9	KYNHSHLYWQRP	3.54

b)

7 10 20 30 37

HAEG TFTSDVSSYL EGQAAKEFIA WLVKGRG

c)

Seq #	Albumin-binding GLP-1 peptide	K_d (μM)
GA31	ABD#3-FNPR-**GA**-GLP	1.27
GP32	ABD#3-FNPR-**GP**-GLP	1.34
GS33	ABD#3-FNPR-**GS**-GLP	1.03
GA61	ABD#6-FNPR-**GA**-GLP	1.65
GP62	ABD#6-FNPR-**GP**-GLP	1.52
GS63	ABD#6-FNPR-**GS**-GLP	1.71
GA81	ABD#8-FNPR-**GA**-GLP	2.76
GP82	ABD#8-FNPR-**GP**-GLP	2.89
GS83	ABD#8-FNPR-**GS**-GLP	2.86
IE3	ABD#3-IEGR----GLP	1.20
IE6	ABD#6-IEGR----GLP	1.39
IE8	ABD#8-IEGR----GLP	2.83

Figure 1. Construction of albumin-binding GLP-1 polypeptides. a) A pool of 12-mer ABD sequences with high affinity to HSA was selected by biopanning a phage library. b) The amino acid sequence of GLP-1 is annotated with its glucagon precursor sequence number. c) Three selected ABDs (ABP#3, ABP#6, and ABP#8) were used to construct albumin-binding GLP-1 polypeptides. For polypeptides with the FXa substrate sequence (IE3, IE6, and IE8), the amino acids in positions P_1' and P_2' can be default.

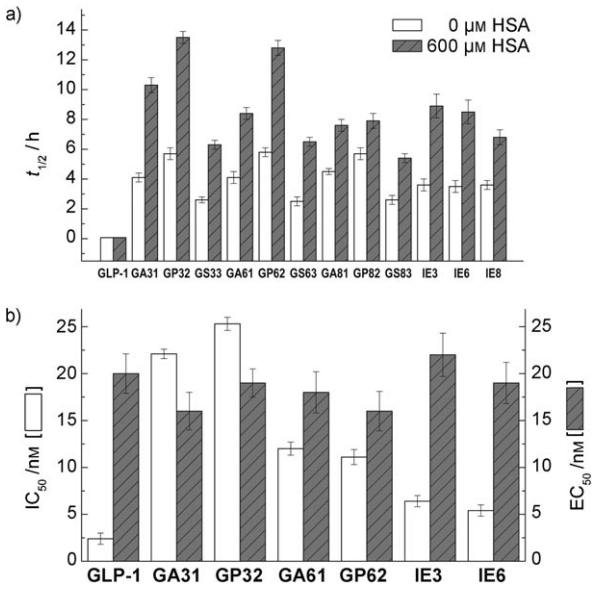

Figure 2. Comparison of in vitro stability and biological activity of albumin-binding GLP-1 polypeptides. a) The half-life values of the polypeptides were compared in the absence or presence of 600 μM HSA. b) Biological activities were compared for those polypeptides with half-lives longer than 8 h. IC_{50} and EC_{50} denote the concentration at 50% inhibitory effect and the concentration at 50% efficiency for cAMP production, respectively.

GP62 polypeptide exhibited the longest half-life. The presence of HSA in the assay solution significantly increased the resistance to TBN cleavage for all polypeptides. For instance, the half-life of GP62 polypeptide increased twofold when the reaction mixture contained 600 μM HSA, which was equivalent to the concentration in the human bloodstream.[15] Similar results were also obtained for FXa-mediated hydrolysis of polypeptides containing FXa-cleavable linker, that is, IE3, IE6, and IE8 (Figure 2a). Furthermore, the half-lives for the polypeptides containing TBN-cleavable linkers were examined against both TBN and DPP4 (Supporting Information, Table S1). As expected, the presence of DPP4 did not significantly affect the half-lives of the polypeptides, regardless of whether or not the reaction solution contained HSA.

In vitro, through TBN/DPP4-meditated hydrolysis, each polypeptide containing a TBN-cleavable linker generated three forms of GLP-1-related peptide products, namely, GA/P/S-GLP-1, GLP-1, and GLP-1(9-37). Using a monoclonal antibody specific for intact GLP-1, we were able to detect transient intact GLP-1 peptides in TBN/DPP4-catalyzed hydrolysis. In the presence of HSA in the reaction solution, the released GLP-1 from the polypeptides with FNPR-GA/P linkers reached the maximum level in 4.8 h (Supporting Information, Figure S5). Afterwards, the GLP-1 concentration started to attenuate, but was still maintained at a relatively high level for over 20 h.

The transient existence of intact GLP-1 peptide in these TBN/DPP4-catalyzed reactions was further confirmed by mass spectrometry (Supporting Information, Figure S5). It was presumed that transient GLP-1 would be destroyed promptly by DPP4. However, our data suggested that DPP4 had significant differential sensitivity to the three N-terminal dipeptides in the GA/P/S-GLP-1 products. We found that DPP4 could recognize GS dipeptide but the turnover was much slower than for the others. Mass spectrometric assays indicated that accumulated intermediate products were mainly GS-GLP-1. The cleavage rate for the three N-terminal dipeptides by DPP4 was in the order GP > GA > GS. It is likely that these differences play an important role in determining the lifetime of transient GLP-1.

In FXa-catalyzed hydrolysis in the presence of DPP4, only two GLP-1-related peptide products, GLP-1 and GLP-1(9-37), were generated because DPP4 removed the N-terminal dipeptide of GLP-1. Nevertheless, accumulation of intact GLP-1 peptide was observed in these reactions (Supporting Information, Figure S5). Taken together, it turned out that protection of GLP-1 by GA/P/S dipeptide was beneficial to the lifetime of transient GLP-1, as further supported by animal model tests (see below).

To evaluate the bioactivity of the designed albumin-binding polypeptides, the aforementioned hydrolysis mixtures were directly used to examine their binding affinities to human GLP-1 receptor (GLP-1R; IC_{50}) as well as their effects on intracellular cyclic adenosine 3′,5′-monophosphate (cAMP) production (EC_{50}). Figure 2b compares the bioactivities of the designed albumin-binding polypeptides, the half-lives of which were over 8 h, with those of GLP-1 and Ex-4. Although the binding affinity of each TBN/DPP4-mediated mixture to GLP-1R was lower than that of GLP-1, they exhibited a high efficiency similar to that of GLP-1 in producing intracellular cAMP. This similarity might result from the combined effect of the three forms of GLP-1 products, which is supported by recent work from Elahi et al.[27] On the other hand, it was found that products generated from FXa-mediated hydrolysis had biological activities compatible with GLP-1.

Furthermore, two albumin-binding polypeptides with longer half-lives (GP62 and IE6) were used in animal model tests using the Type 2 diabetes model (GK rat, 10 weeks old). A new albumin-binding polypeptide, in which the GLP-1 segment in GP62 was replaced by Ex-4 and defined as GPex4, was also introduced for these tests. After subcutaneous administration, GP62, IE6, and GPex4 reduced blood glucose levels in a similar manner to Ex-4 within 6 to 8 h (Figure 3a). Additionally, GP62 and GPex4 acted over 12 h in lowering the blood glucose level in the GK rats. Each polypeptide was found to significantly enhance the insulin-to-glucose ratios in acute insulin secretion in the control experiments using Wistar rats (Figure 3b), and their insulin-producing efficiencies accounted for 98 (IE6), 90 (GP62), and 86% (GPex4) that of Ex-4, respectively. Importantly, these polypeptides have a much longer lifetime in plasma than Ex-4 in GK rats

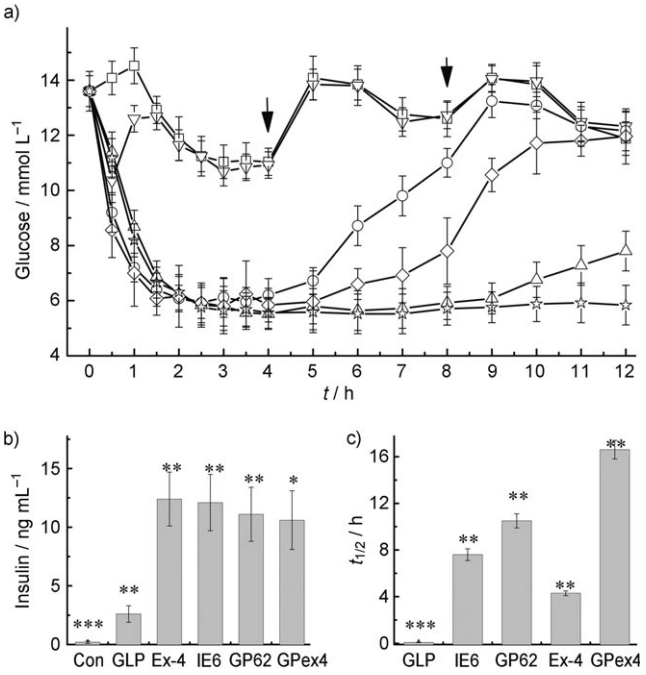

Figure 3. Antidiabetic activities of the designed albumin-binding GLP-1 polypeptides in a mouse model. a) Glucose levels in GK rats were measured following 30 min of administering vehicle (□), GLP-1 (▽), Ex-4 (○), GP62 (△), IE6 (◇), and PGex4 (star) (500 μg kg^{-1}, $n=6$, $p<0.01$). The rats were regularly fed (marked by arrows). b) Comparisons were made of the effect of GP62, IE6, and GPex4 on acute insulin secretion (average of 5, 10, and 20 min levels) in fasted Wistar rats. Con: control; GLP: GLP-1. c) The half-lives of GP62, IE6, and GPex4 were compared with those of GLP-1 and Ex-4 in vivo after they were subcutaneously administered to adult GK rats (30 weeks old). Values are mean ± standard error of the mean ($n=6$; *, $p<0.05$; **, $p<0.01$; and ***, $p<0.001$).

(Figure 3c), and their half-lives in rat plasma were 8 (IE6), 17 (GP62), and 20 times (GPex4) longer than that of Ex-4.

In summary, our work on the sustained release of GLP-1 or its analogue from albumin-binding polypeptides is not only just one example, but also provides promise for the prevention of Type 2 diabetes. Considering that Ex-4 is a clinically approved twice-daily injection therapeutic, our polypeptides have potential to develop long-lasting therapeutics against Type 2 diabetes. This strategy may also be applicable to other therapeutic peptides and proteins. As many diseases including cancers are relevant to aberrant protein–protein interactions in cellular signal transduction pathways, and these interactions have large interaction interfaces that are not suitable for small-molecule compounds, peptides are eagerly awaited as a potent drug source for these diseases.

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