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A depot-forming glucagon-like peptide-1 fusion protein reduces blood glucose for five days with a single injection

M. Amiram [a], K.M. Luginbuhl [a], X. Li [a], M.N. Feinglos [b], A. Chilkoti [a,*]

[a] Department of Biomedical Engineering, Duke University, Durham 27708, USA
[b] Division of Endocrinology, Metabolism, and Nutrition, Duke University Medical Center, Durham 27710, USA

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ABSTRACT

Peptide drugs are an exciting class of pharmaceuticals for the treatment of a variety of diseases; however, their short half-life dictates multiple and frequent injections causing undesirable side effects. Herein, we describe a novel peptide delivery system that seeks to combine the attractive features of prolonged circulation time with a prolonged release formulation. This system consists of glucagon-like peptide-1, a type-2 diabetes drug fused to a thermally responsive, elastin-like-polypeptide (ELP) that undergoes a soluble–insoluble phase transition between room temperature and body temperature, thereby forming an injectable depot. We synthesized a set of GLP-1-ELP fusions and verified their proteolytic stability and potency *in vitro*. Significantly, a single injection of depot forming GLP-1-ELP fusions reduced blood glucose levels in mice for up to 5 days, 120 times longer than an injection of the native peptide. These findings demonstrate the unique advantages of using ELPs to release peptide-ELP fusions from a depot combined with enhanced systemic circulation to create a tunable peptide delivery system.

1. Introduction

Type-2 diabetes accounts for 90 to 95% of all diagnosed cases of adult diabetes, and has been steadily increasing over the past few decades, with an estimated global prevalence of 300 million by 2025 [1]. Glucagon-like peptide-1 (GLP-1) is a 30 amino acid peptide released by gastrointestinal cells upon meal ingestion [2], and stimulates the release of insulin from pancreatic β-cells [3–5]. In addition, GLP-1 has also been shown to inhibit glucagon secretion, reduce appetite, slow gastric emptying, reduce hepatic glucose production [6,7] and enhance β-cell survival and growth in rodents [8]. Despite these attractive features, native GLP-1 has not been utilized for treatment of type-2 diabetes, as the peptide undergoes rapid deactivation *in vivo via* N-terminal truncation by dipeptidyl-peptidase IV (DPPIV), leading to a half-life of less than 2 min [9]. To circumvent this problem, DPPIV resistant GLP-1 analogs have been designed, but due to the small size of the peptide, renal clearance still limits their half-life to 4–5 min [9].

Significant effort has hence been expended to develop longer acting analogs of GLP-1 with the goal of generating a drug that will, with a single injection, provide a flat concentration *versus* time profile in the therapeutic range [10]. This will not only reduce the number of injections but also prevent "peak-and-valley" fluctuations associated with bolus injections of short-acting analogs that only provide a therapeutically effective drug concentration for a short time and cause undesired side effects because of their sub-optimal pharmacokinetics [11].

Currently, there are three FDA approved GLP-1R agonists to treat type 2 diabetes. Exenatide, a 39 amino acid peptide with 53% homology to GLP-1, which has been shown to be protease resistant with a modestly increased half-life compared with GLP-1 (2.4 h after *s.c.* injection in humans [12]), is administered *via* twice daily injections. Liraglutide is an acylated GLP-1 approved for once-a-day injection (11–15 h half-life after *s.c.* injection in humans [12]) and Bydureon, a sustained release formulation of exenatide in biodegradable poly(lactic glycolic acid) (PLGA) microspheres, is approved for once-weekly injection [13]. In addition, several groups have proposed the attachment of GLP-1 or exenatide to large carrier molecules such as PEG [9], serum albumin [14], Fc fragments [15] and other recombinant biopolymers [16] as a strategy to increase their *in vivo* half-life. However, recombinant fusion to Fc fragments or serum albumin requires eukaryotic expression systems that are expensive and difficult to scale-up, while site-specific covalent PEGylation has a low yield and high cost [17]. Similarly, a recent paper describes the generation of a recombinant exenatide-xten fusion wherein the xten moiety is a large recombinant polypeptide engineered to have an unstructured conformation that extends the half-life of exenatide from 0.17 h to 12 h in mice [16]. Nevertheless, to the best of our knowledge, to date GLP-1 delivery systems have been designed for *either* prolonged release from a drug depot [13,18] *or* prolonged circulation.

Herein we describe the design and characterization of an alternative system that seeks to combine the attractive features of a soluble fusion protein, with prolonged circulation time, and a prolonged release formulation, that allows for slow released from a subcutaneous (s.c.) depot. This system consists of a (GLP-1)-ELP fusion protein in which GLP-1 is fused to a thermally sensitive elastin-like polypeptide (ELP)

* Corresponding author. Tel.: +1 919 660 5373; fax: +1 919 660 5362.
E-mail address: chilkoti@duke.edu (A. Chilkoti).

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that is soluble at room temperature but forms a depot – a viscous coacervate – upon *s.c.* injection at body temperature [19]. The central hypothesis that underlies this design is that the ELP domain will drive a thermal transition upon *s.c.* injection of (GLP-1)-ELP fusions, thereby forming a stable drug depot. Over time, active (GLP-1)-ELP fusion will be released from the depot, providing prolonged release of high MW drug into circulation — which will also enhance the peptide's half-life. This design combines the ability to titrate the release of the drug from a depot, similar to degradable microsphere delivery systems, with the tunable half-life extension of macromolecular GLP-fusions.

We show that (GLP-1)-ELP fusions can be expressed in *Escherichia coli*, and that ELP fusion tags enable temperature triggered coacervation that is easily tuned by choice of the ELP fusion protein which also assists in facile, chromatography-free, purification. (GLP-1)-ELP fusions maintain secondary structure, are resistant to degradation by proteolysis, and are capable of activating the GLP-1 receptor. We demonstrate that a single injection of (GLP-1)-ELP forms a *s.c.* depot that provides superior glucose control as compared with native peptide injections. Finally, we discuss future work and the potential of this fusion technology for the delivery of other peptide therapeutics.

2. Materials and methods

2.1. Recombinant (GLP-1)-ELP synthesis and expression

The gene of modified [$G_8E_{22}A_{36}$]GLP (Table 1) with an alanine–alanine leader, and 5' NdeI and 3' HindIII restriction sites was amplified using a [G8E22]GLP synthetic gene template [18] and F primer: "GATA TACATATGGCAGCGCACGGTGAAGGCACCTTT" and R primer: "CATAG GAAGCTTGCCCGCACCTTTCACCAGCCACGCGAT".

The PCR reaction mixture consisted of 10 pmol of [$G_8E_{22}A_{36}$]GLP template, 10 pmol each of sense and antisense DNA primers, 50 μl GoTaq green master mix (Promega) and water for a final volume of 100 μl. The PCR reaction conditions were 95 °C for 2 min for initial denaturation, followed by 35 cycles at 95 °C for 30 s, 52 °C for 30 s and 72 °C for 40 s. The resulting PCR product was purified using a PCR purification kit (Qiagen) and visualized on a 1% agarose gel stained with SYBR® Safe DNA stain (Invitrogen). 2 μg PCR products was digested with 2 μl NdeI, 2 μl Hind III, 1× NEB 2 buffer and nuclease free water in total volume of 40 μl at 37 °C for 2 h and PCR purified to remove residual enzymes and buffer. A modified pET25b+ vector containing the ELP tag was modified to include a leader cassette with NdeI and Hind III restriction sites for in-frame ligation of GLP-1 to the ELP. 2 μg vector DNA was digested with 2 μl NdeI, 2 μl Hind III, 1× NEB 2 buffer and nuclease free water in total volume of 40 μl at 37 °C for 2 h and PCR purified to remove residual enzymes and buffer. The restriction product was ligated to the vector using 4 units of T4 DNA ligase, 1× ligation buffer, ~250 ng of the annealed product, ~250 ng of digested vector, and nuclease-free water in a total volume of 20 μl. The ligation mixture was incubated at room temperature for 1 h, and BL21(DE3) cells were then transformed with 7 μl of the ligation mixture for 15 min in an ice-water bath, heat-shocked at 42 °C for 30 s, and returned to the ice-water mixture for another 2 min. The cells were recovered in SOC media while horizontally shaking at 200 rpm at 37 °C for 40–60 min, and were then plated on TB agarose plates containing 1 mg/ml ampicillin. Correct clones were identified by colony PCR and verified by DNA sequencing. GLP-ELP constructs were expressed and purified using a modified inverse transition cycling (ITC) protocol [20] detailed in the Supplementary material section.

Table 1
Sequences used for generation of (GLP-1)-ELP fusion proteins. By convention, the first amino acid in the GLP-1 sequence (Histidine (H)) is numbered 7. Mutations in native GLP-1 sequence are underlined. The Alanine (A) to Glycine (G) mutation in the 8th position confers DPPIV resistance, The Glycine (G) to Glutamic acid (E) mutation in the 22nd position stabilizes the alpha helical structure of GLP-1 and the Arginine (R) to Alanine (A) mutation in the 36th position prevents cleavage by Arginine-targeting proteases in the *s.c.* space. ELPs were chosen to have a T_t that is lower (ELP_{Depot}) or higher (ELP_{Sol}) than body temperature which was modulated by the choice of guest residue composition. More hydrophobic amino acid residues (*e.g.*, Valine) reduce the T_t whereas more hydrophilic amino acids (Alanine and Glycine) increase it. A DPPIV cleavable Alanine-Alanine (AA) dipeptide was added to the N-terminus of each construct to facilitate removal of any extra Methionine residues and the linker between the GLP and ELP moieties was dictated by the choice of restriction endonucleases in the assembly of the fusion gene.

Native GLP-1	HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR
[$G_8E_{22}A_{36}$]GLP-1	AAHGEGTFTSDVSSYLEEQAAKEFIAWLVKGA
ELP_{Sol}	$(GAGVPGGGVP)_{60}GY$
ELP_{Depot}	$(GVGVP)_{120}GWP$
Leader	AA
Linker	KLAM

2.2. Phase transition and secondary structure analysis

The secondary structure was studied by circular dichroism (CD) using an Aviv Model 202 instrument and 1 mm quartz cells (Hellma) by scanning from 280 nm to 180 nm with 1 nm steps and a 3 second averaging time at 19 °C. Purified constructs were diluted to 7.5 μM in water. Data were considered for analysis whenever the Dynode voltage was below 500 V [21].

To characterize the inverse transition temperature of (GLP-1)-ELP constructs, the optical density of a 200 μM–10 μM solution in PBS was monitored at a wavelength of 350 nm as a function of temperature, with heating and cooling performed at a rate of 1 °C min^{-1} on a Cary 300 UV–visible spectrophotometer equipped with a multicell thermoelectric temperature controller (Varian Instruments, Walnut Creek, CA).

2.3. Degradation by neutral endopeptidase (NEP)

(GLP-1)-ELP_{Depot} and native GLP-1 (30 μM) were incubated at 20 or 37 °C for up to 18 h with 0.46 μg NEP protease (Enzo). Following incubation, fractions were separated on a 10–20% tris-tricine SDS gel (Bio-Rad) and stained with Coomassie brilliant blue.

2.4. In vitro assay for GLP-1 activity

The ability of (GLP-1)-ELP_{Depot} and (GLP-1)-ELP_{Sol} to activate the GLP-1 receptor (GLP-1R) *in vitro* after incubation for 20 h with 0.5 μg DPPIV (ProSpec) at 20 or 37 °C to remove the AA leader was assessed using baby hamster kidney (BHK) cells that are stably transfected with rat GLP-1R (a gift of Prof. Drucker, University of Toronto [22]). Intracellular cAMP concentrations were measured using a competitive binding assay according to the manufacturer's instructions (Assay Designs).

2.5. Animal studies

5–6 week old male C57BL/6 J mice [23] (stock number 000664) were purchased from Jackson Laboratories (Bar Harbor, Me., USA). All experimental procedures were approved by the Duke Institutional Animal Care & Use Committee.

2.6. In vivo near infrared fluorescence tomography

For *in vivo* tomography of depot formation, (GLP-1)-ELP_{Depot} and (GLP-1)-ELP_{Sol} were labeled with IRDye® 800CW NHS Ester (LICOR) by conjugation to lysine residues in GLP-1. All mice were anesthetized with 2% isoflurane throughout the imaging procedures. The torsos of mice (7 weeks old, n = 3 per group) were shaved, and labeled constructs were injected *s.c.* (175 or 700 nmol/kg). Images were collected immediately after injection and at 5, 24, 48, 72, 96, 120 and 144 h post-injection. Imaging was performed with an FMT2500LX fluorescence tomography *in vivo* imaging system (PerkinElmer) and the images were acquired and analyzed with TrueQuant 3D software (PerkinElmer).