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Prolonged half-life of small-sized therapeutic protein using serum albumin-specific protein binder

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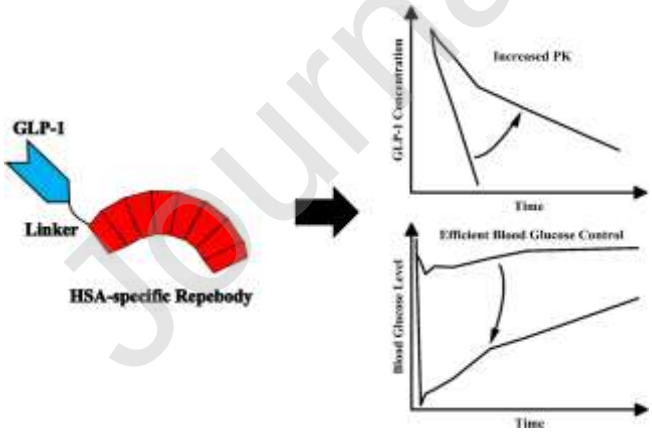
Graphical abstract



Highlights

- A novel protein binder, termed 'Repebody', that specifically binds to human serum albumin with high affinity was developed.
- The genetic fusion of GLP-1 to the repebody significantly improved the blood half-life of GLP-1.
- The repebody-fused GLP-1 showed enhanced therapeutic efficacy *in vivo*.
- The present approach can be effectively used for extending the blood half-life of small-sized therapeutic proteins.

Abstract

Many small-sized proteins and peptides, such as cytokines and hormones, are clinically used for the treatment of a variety of diseases. However, their short half-life in blood owing to fast renal clearance usually results in a low therapeutic efficacy and frequent dosing. Here we present the development of a human serum albumin (HSA)-specific protein binder with a binding affinity of 4.3 nM through a phage display selection and modular evolution approach to extend the blood half-life of a small-sized therapeutic protein. As a proof-of-concept, the protein binder composed of LRR (Leucine-rich repeat) modules was genetically fused to the N-terminus of Glucagon-like Peptide-1 (GLP-1). The fused GLP-1 was shown to have a significantly improved pharmacokinetic property: The terminal half-life of the fused GLP-1 increased to approximately 10 h, and the area under the curve was 5-times higher than that of the control. The utility and potential of our approach was demonstrated by the efficient control of the blood glucose level in type-2 diabetes mouse models using the HSA-specific protein binder-fused GLP-1 over a prolonged time period. The present approach can be effectively used in enhancing the efficacy of small-sized therapeutic proteins and peptides through an enhanced blood circulation time.

Key words

Half-life; therapeutic protein; human serum albumin; LRR protein; genetic fusion

1. Introduction

Over the past decades, a number of small-sized proteins and peptides, such as cytokines and hormones, have been developed and clinically used as therapeutic agents for various diseases [1-4]. However, their small size usually leads to a short half-life in the blood owing to fast renal clearance, resulting in a low therapeutic efficacy and frequent dosing [5-8]. Many methods have been developed to improve such pharmacokinetics profile [7, 9, 10]. The hydrodynamic radius of proteins was increased through multimerization or fusion using a non-serum protein [11-14] and chemical modification such as PEGylation, biotinylation, and fatty-acid conjugation [15-17]. FcRn-mediated recycling of proteins has been developed through either a genetic fusion or chemical conjugation to serum albumin or Fc of IgG based on the fact that serum albumin and Fc bind to FcRn (Neonatal Fc receptor) in endothelial cells and avoid lysosomal degradation [9, 18-22]. Despite their widespread use, however, such approaches still have certain drawbacks. Multimerization might generate a degree of heterogeneity, and cause changes in the original property of the protein in terms of the binding affinity for a target and the therapeutic efficacy. Chemical modifications are complex, resulting in a low yield with high heterogeneity, and might alter the property of the protein, thereby leading to undesirable

outcomes such as immunogenicity and toxicity. The genetic fusion of a protein with Fc or serum albumin requires a mammalian expression system, which is costly and complex, and the biological activity of a fused protein can be affected by the steric hindrance of Fc and serum albumin owing to their large size. In addition, the fusion protein might be recognized as a foreign protein, triggering an unwanted immunogenic response.

Several strategies to elongate the blood circulation time of GLP-1 have been developed [23]. In the case of fatty acid conjugation, a whole peptide was chemically synthesized to incorporate non-natural amino acid at specific position for fatty acid conjugation [17, 24]. This approach led to a longer half-life (~ 168 h), but required a complicated chemical peptide synthesis process, and is unlikely to be generally applicable. The albumin-binding domain with high binding affinity (~ 8 pM) for rat serum albumin was revealed to extend a terminal half-life of a fused exendin-4 to 16 h in rat [23, 25].

Herein, we present the development and use of a human serum albumin (HSA)-specific protein binder as a general platform for prolonging the blood circulation time of a small-sized therapeutic protein. In an effort to exploit the structural and functional features of leucine-rich repeat (LRR) proteins for biotechnological and medical applications, we previously developed a non-antibody protein scaffold composed of LRR modules, called “repebody” [26-28]. The HSA-specific repebody with a binding affinity of 4.3 nM was developed through phage display selection and a modular evolution approach. As a proof-of-concept, glucagon-like peptide-1 (GLP-1) was genetically fused to the HSA-specific repebody. GLP-1 is a peptide hormone composed of 30 amino acids and is known to lower the blood sugar level in a glucose-dependent manner by stimulating the secretion of insulin [29-31]. The biological activity of a repebody-fused GLP-1 was tested in an insulinoma cell line, and the pharmacokinetic profile of the repebody-fused GLP-1 was investigated *in vivo*. The utility of our approach was shown by the enhanced hypoglycemic activity of the repebody-fused GLP-1 in type-2 diabetic mouse

models over a prolonged time period. Details are reported herein.

2. Materials and methods

2.1 Materials

Each serum albumins (fatty acid free, globulin free, ≥ 99%) was purchased from a different commercial source: Human, rat, and rabbit serum albumins were from Sigma Aldrich (St. Louis, MO, USA), monkey serum albumin was purchased from Athens Research & Technology (Athens, GA, USA), mouse serum albumin came from Equitech-Bio (Kerrville, TX, USA), and bovine serum albumin was from GenDEPOT (Katy, TX, USA). Glucagon-like peptide 1 (7-36) amide and its derivative Exendin-4 were purchased from R&D Systems (Minneapolis, MN, USA). All the gene constructs were cloned using a pET21a vector (Novagen, Madison, WI, USA) with Nde I and Xho I restriction enzymes and T4 DNA Ligase (Takara Bio, Shiga, Japan). LB broth media and antibiotics were purchased from Duchefa (Haarlem, the Netherlands). Origami B (DE3) and BL21 (DE3) competent cells for the expression of the repebody and fusion proteins were obtained from Novagen. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was received from LPS Solution (Seoul, Korea). The Ni-NTA agarose resin was obtained from Qiagen (Germantown, MD, USA). The size exclusion chromatography columns were from GE Healthcare (Uppsala, Sweden). All other reagents including buffers were of analytical grade.

2.2 Phage display selection of a human serum albumin-specific repebody

A human serum albumin (HSA)-specific repebody was selected and its binding affinity was maturated through a phage display and modular evolution approach, as described in our

previous studies [26-28, 32-34]. Briefly, a phage display library was generated using primers containing NNK codons for the library site through a PCR with Ex Tag DNA Polymerase (Takara). The library was ligated into phagemid pBEL118N, and phages were earned using an XL1-Blue cell line and M13KO7 helper phage (New England Biolabs, Ipswich, MA, USA). For the selection of an HSA-specific repebody, a 1 mL of human serum albumin (100 μg/ml) was coated onto a 5 mL immunotube (Greiner, Frickenhausen, Germany) and kept overnight at 4 °C. On the following day, the immunotube was washed with 5 mL of PBS (pH 7.4) three times and blocked with 1% BSA in PBST (PBS pH 7.4, 0.05% Tween 20) for 2 h at 4 °C. A phage solution of 1 mL (1.0×10^{12} cfu/mL) was diluted in 1% BSA in PBST (PBS pH 7.4, 0.05% Tween 20). After 1h, the phage solution was added into the tube and incubated for 2 h. The tube was washed with 5 mL of PBST (PBS pH 7.4, 0.05% Tween 20) for 5 min a total of five times. Target-bound phages were eluted with a 0.2 M glycine-HCl solution (pH 2.2) for 15 min and neutralized with 100 μL of a 1M Tris solution (pH 9.0). The eluted phages were infected into XL1-Blue F' E. coli cells and grown overnight at 30 °C in a 2xYT agar plate containing 100 μg/mL of ampicillin, 10 μg/ml of tetracycline, and 1% glucose. Cells grown on the plate were collected and subjected to repeated rounds of panning. After the fifth round of the bio-panning process, each colony on the plate was seeded into a 96 deep-well plate (Axygen Scientific, Corning, NY, USA) containing 100 μL of 2xYT with ampicillin and tetracycline. Following helper phage infection, 100 μL of 2xYT with kanamycin was added into each well and incubated overnight at 30 °C. The plate was centrifuged for 10 min at 2000 rpm, and supernatants containing phages were subjected to a phage ELISA. For the phage ELISA, a 96-well MaxiSorp plate (Nunc, Waltham, MA USA) was coated with 100 μL of HSA (10 μg/mL) overnight at 4 °C. On the following day, the plates were washed with PBS (pH 7.4) three times and blocked with 1% bovine serum albumin (BSA) in PBST (PBS pH 7.4, 0.05% Tween 20) for 2 h. The phage solutions were also incubated with a blocking solution for 1 h and added to

an HSA-coated plate for 2 h. The plates were washed with PBST (pH 7.4, 0.05% Tween 20) five times and incubated with an anti-M13 antibody-HRP conjugate (1:5000 dilution, GE Healthcare) for 1 h. After washing with PBST five times and PBS three times, 100 μL of a 3,3',5,5'-Tetramethylbenzidine (TMB) solution (Sigma Aldrich) was added into each well for signal generation. After incubation for 1 min, 1N H_2SO_4 was added, and the signals were measured at 450 nm using an Infinite M200 microplate reader (Tecan GmbH, Crailsheim, Germany). Each step was conducted at room temperature unless otherwise noted.

2.3 Surface plasmon resonance (SPR)

The binding affinity of a repebody for human serum albumin was measured using a Biacore 3000 (GE Healthcare). Briefly, a CM5 chip (GE Healthcare) was coated with human serum albumin using a 10 mM sodium acetate buffer (pH 4.0) and an amine coupling kit (GE Healthcare). A R_{max} value of 665 was targeted. After immobilization, a serial-diluted repebody was injected into the chip. Surface regeneration was conducted using 10mM NaOH, and SPR experiments were carried out using PBS (pH 7.4). Kinetic constants were determined by fitting sensorgrams to a 1:1 Langmuir binding model using Biacore 3000 evaluation software (GE Healthcare).

2.4 Circular dichroism (CD)

The molar ellipticity of a protein was measured from 190 to 280 nm at 25 °C using circular dichroism spectropolarimetry (Jasco J-810, Easton, MD, USA). The melting temperature (Tm) of the protein was determined by measuring the molar ellipticity at 222 nm with a gradual increase of temperature from 20 °C to 90 °C at a rate of 1 °C/min using a Peltier temperature

controller.

2.5 Enzyme-linked immunosorbent assay (ELISA)

2.5.1 Neonatal Fc Receptor (FcRn) binding assay

Human serum albumin (10 μg/mL, 100 μL/well) was coated onto a 96-well plate overnight at 4 °C. On the following day, the wells were blocked with 1% BSA in PBST (pH 7.4, 0.05 % Tween 20) for 1 h. Human FcRn (hFcRn, 1 μg/mL, R&D Systems) or hFcRn (1 μg/mL) added with a HSA-specific repebody (10 μg/mL) was prepared in blocking buffers with a final pH of 6.0 and 7.4. After 1 h of incubation, the prepared proteins were added to each well and incubated for 1 h. Anti-FCGRT and B2M heterodimer (FcRn) antibody (1 μg/mL, 100 μL/well, Sino Biological, Beijing, China) was used with a goat anti-rabbit IgG antibody-HRP conjugate (1:3,000 dilution, 100 μL/well, Bio-Rad Laboratories, Hercules, CA, USA) to detect the level of bound FcRn. A TMB substrate was added for a signal generation, and 1N H_2SO_4 was provided to stop the reaction after 1 min. The signals were measured at 450 nm using a microplate reader. Each step was conducted at room temperature, and the wells were washed with PBST (pH 7.4, 0.05% Tween 20) five times between each step.

2.5.2 Binding specificity

The cross reactivity of a HSA-specific repebody was assessed for various serum albumins. Each albumin (10 μg/mL) was coated onto a 96-well plate at a concentration of 100 μL/well overnight at 4 °C. On the following day, the wells were blocked using a SuperBlock T20 (PBS) blocking buffer (Thermo Scientific). The human serum albumin-specific repebody was diluted into a blocking buffer (10 μg/mL, 100 μL/well) and added to each well. An anti-repebody

antibody conjugated with biotin (1 μg/mL, 100 μL/well, Abclon, Seoul, Korea) and Streptavidin-HRP (1:1000 dilution, 100 μL/well, BioLegend, San Diego, CA, USA) were used for repebody detection. A TMB substrate was added for signal generation, and 1N H_2SO_4 was added to stop the reaction after 1 min. The signals were measured at 450 nm using a microplate reader. Each step was carried out at room temperature, and the wells were washed with PBST (pH 7.4, 0.05% Tween 20) five times between each step.

2.5.3 GLP-1 receptor binding assay

A GLP-1 receptor (10 μg/mL, 100 μL/well, Sino Biological) was coated onto a 96-well plate overnight at 4 °C. The next day, the wells were blocked with 1% BSA in PBST (pH 7.4, 0.05% Tween 20) for 1 h. A HSA-specific repebody-fused GLP-1 with a myc-tag (amino acid sequence: EQKLISEEDL) at the C-terminus was added to each well by 10-fold serial dilution starting from 10 μM (100 μL/well). An anti-c-Myc antibody (1:1000 dilution, 100 μL/well, Santa Cruz Biotechnology, Dallas, TX, USA) and anti-mouse IgG-HRP conjugate (1:10000 dilution, 100 μL/well, Abcam, Cambridge, UK) were used to detect the signals. A TMB substrate was added for signal generation, and 1N H_2SO_4 was provided to stop the reaction after 1 min. The signals were measured at 450 nm using a microplate reader. Each step was conducted at room temperature, and the wells were washed with PBST (pH 7.4, 0.05% Tween 20) five times between each step.

2.6 Stability test of a repebody in complex with human serum albumin

The binding stability of the HSA-specific repebody in complex with the HSA was checked using size exclusion chromatography and a his-tag pull-down assay. The complex was formed

by mixing 200 μM of the HSA-specific repebody with 100 μM of HSA for 1 h at 4 °C followed by purification using size exclusion chromatography with a Hiload Superdex 200 column. The resulting complex was incubated at 37 °C, and size exclusion chromatography was carried out after 24, 48, 72, and 96 h. To test the stability in the serum, 10 μM of the repebody in complex with the HSA was mixed with 10% (v/v) mouse serum, and the final volume was adjusted to 2.5 mL using PBS (pH 7.4), and incubated at 37 °C. At various intervals, 200 μL of a protein solution was sampled and stored at -20 °C prior to analysis. To assess the stability, the samples were gently melted at room temperature, and 50 μL of Ni-NTA resin, which had been pre-equilibrated with PBS (pH 7.4), was added to each sample. After treatment with 1 mL of a washing buffer (PBS pH 7.4, 20mM Imidazole) three times, the protein was eluted with 100 μL of an elution buffer (PBS, pH 7.4, 250mM Imidazole). Each sample was denatured and subjected to SDS-PAGE analysis.

2.7. Construction and purification of the repebody-fused GLP-1

GLP-1 was genetically fused to the C-terminus of the HSA-specific repebody or off-target repebody through a α-helix rigid linker ($A(EAAAAK)_3A$). A peptidase DPP-VI resistance mutation (Ala8Gly) was introduced into the GLP-1 sequence. A 6-histidine tag was added to the C-terminus of GLP-1 for purification using Ni-NTA agarose resin. A myc-tag was incorporated into the C-terminus of GLP-1 for the receptor binding assay. The resulting genes were ligated into a pET21a vector and transformed into BL21 (DE3) competent cells. A single colony expressing the fusion protein was seeded into a LB medium containing ampicillin (100 μg/mL) and grown overnight in a shaking incubator at 37 °C. On the following day, the cells were inoculated into 1 L of a LB medium with ampicillin and incubated at 37 °C until OD_{600} reached ~0.8. The cells were induced with 0.5 M of isopropyl-b-D-thiogalactopyranoside (IPTG) and further incubated for 20 h at 18 °C. The cells were harvested through centrifugation

at 8000 rpm for 10 min. The cells were then re-suspended using a lysis buffer (20 mM Tris, 150 mM NaCl, 10 mM Imidazole, pH 8.0) for ultra-sonication followed by centrifugation at 16,000 rpm at 4 °C for 1 h. The supernatants were filtered through a 0.22 μm syringe filter (EMD Millipore, Bedford, MA, USA) and purified using a Ni-NTA agarose resin column (Qiagen, Hilden, Germany). The column was washed thoroughly using a washing buffer (20 mM Tris, 150 mM NaCl, 20 mM Imidazole, pH 8.0), and eluted using an elution buffer (20 mM Tris, 150 mM NaCl, 250 mM Imidazole, pH 8.0). The eluted proteins were subjected to size exclusion chromatography (Superdex 200) using PBS (pH 7.4). The protein concentration was measured using a NanoDrop 2000c (Thermo Fisher Scientific, Waltham, MA USA). The SDS-PAGE gel images were analyzed using a Gel Doc EZ Imager (Bio-Rad).

2.7 Pharmacokinetics

2.7.1 Animals

Male balb/c mice (4 weeks old, Orent, Seongnam, Korea) were used for the pharmacokinetics experiments. The mice were adapted to the animal facility for 1 week after quarantine and housed in a room maintained at 23 ± 2 °C with 50 ± 5% relative humidity under a 12 h light/dark cycle. The animals were fed a standard animal diet and water. All animal experiments were approved by the institutional animal care and use committee of the Korea Advanced Institute of Science and Technology (KAIST, KA2017-29) and Advanced Radiation Technology Institute (ARTI) of the Korea Atomic Energy Institute (KAERI, KAERI-IACUC-2019-016).

2.7.2 Pharmacokinetics profile using sandwich ELISA

Mice were randomly divided into control and experiment groups. After anesthetizing with

isoflurane (2.5 %), the HSA-specific repebody-fused GLP-1 in complex with HSA or an off-target repebody-fused GLP-1 was injected intravenously into mice at an equimolar concentration (100 μL, 25 nmol/kg), and the pharmacokinetic profiles were obtained using a sandwich ELISA. For comparison, the first value in the serum at 3 min was set to 100%. To evaluate the blood clearance of the repebody, mice were euthanized at predetermined time points (n = 5 mice each at 0.05, 0.5, 1, 3, 6, 12 and 24 h post-injection) to collect whole blood from the aorta abdominalis. The collected blood samples (in a heparin-coated tube) were centrifuged at 3000 rpm for 10 min to separate the blood plasma. A 96-well plate coated with an anti-repebody antibody (10 μg/mL, 100 μL/well, Abclon, Seoul, Korea) overnight at 4 °C was used for analysis. The wells were blocked with a SuperBlock T20 (PBS) blocking buffer (Thermo Scientific), and blood plasma samples were added into each well and incubated for 1 h. An anti-repebody antibody-biotin conjugate (1 μg/mL, 100 μL/well) diluted in the blocking buffer was used as the detection antibody and incubated for 1 h. Streptavidin-HRP (1:1000 dilution, 100 μL/well) was added for 1 h, and a signal was generated using a TMB substrate (100 μL/well). The reaction was stopped after 1 min using 1N H_2SO_4 (100 μL/well), and the signals were measured at 450 nm using a microplate reader. Each step was conducted at room temperature, and the wells were washed with PBST (pH 7.4, 0.05% Tween 20) five times between each step.

2.7.3 Radioisotope-based pharmacokinetics

2.7.3.1 Radiolabeling with radioisotope ^{111}In

Radiolabeling of the HSA-specific repebody-fused GLP-1 and off-target repebody-fused GLP-1 with ^{111}In ($t_{1/2}$ = 2.8 days) was followed as a previous report with slight modification [35]. Briefly, the HSA-specific repebody-fused GLP-1 and off-target repebody-fused GLP-1 were

first conjugated using a TCO-NHS ester (Click Chemistry Tools, Scottsdale, AZ, USA) at a 1:10 molar ratio for 2 h at 4 °C. The remaining chemicals were removed using a PD-10 de-salting column (GE Healthcare) as previously reported. For the HSA-specific repebody-fused GLP-1, its complex form with the HSA was generated and purified using size exclusion chromatography. Radiolabeling of the resulting proteins was accomplished with the simple click reaction between ^{111}In-DOTA-methyltetrazine (FutureChem, Seoul, Korea) and TCO-NHS conjugated proteins. Briefly, ^{111}In-DOTA-methyltetrazine (1 mCi / 5 nmol of DOTA-methyltetrazine in 400 μL) was incubated with TCO-conjugated proteins (10 nmol in 100 μL) for 30 min at 40 °C in aqueous conditions in order to prepare radiolabeled proteins. After the reaction, the radiolabeled proteins were purified with using a PD-10 de-salting column, and the radiolabeling purity was estimated using thin layer chromatography (TLC).

2.7.3.2 Pharmacokinetics

Mice were randomly divided into control and experimental groups. After anesthetizing with isoflurane (2.5 %), radioisotope-labeled proteins were injected into mice intravenously (I.V.) (1.85 MBq in sterile saline, 100 μL). The pharmacokinetic profiles were obtained by measuring the radioactivity in a serum from mice. For comparison, the level of radioactivity in serum at 3 min was set to 100%. To evaluate the blood clearance of the protein, mice were euthanized at predetermined time points (n = 10 mice each at 0.05, 0.5, 1, 3, 6, and 24 h post-injection) to collect whole blood from the aorta abdominalis. The collected blood samples (in a heparin-coated tube) were centrifuged at 3,000 rpm for 20 min to separate the blood plasma, and the radioactivity of the plasma samples (250 μL per 96-well plate) was measured using a gamma counter. The serum half-lives of each protein were estimated using Origin software.

2.7.4 *Pharmacokinetic parameters*

The pharmacokinetic parameters were determined using Origin software. The initial and terminal half-lives were calculated by fitting the HSA-specific repebody-fused GLP-1 into two-phase exponential decay model. In contrast, an off-target repebody-fused GLP-1 was fitted into one-phase exponential decay model. The areas under the curve (AUCs) were calculated by integrating each curve. Other pharmacokinetic parameters were calculated using a pharmacokinetic profile obtained with radioisotope-labelled fusion proteins [36].

2.8 Assay of cAMP in vitro

Wild-type matured GLP-1(7-36) and the HSA-specific repebody-fused GLP-1 were subjected to assessment of their ability to generate intracellular cAMP through binding to a GLP-1 receptor. Rat insulinoma cells (INS-1) of 5 x 10^4 were seeded into each well of a 24-well plate using RPMI media containing 10% FBS and cultured under 5% CO_2 at 37 °C for 3 days. The media were removed, and a 10 mM glucose Krebs-Ringer Bicarbonate buffer (pH 7.5, Sigma Aldrich) was supplemented with 10 mM HEPES and 0.1% BSA for 2 h. Each construct was diluted into a 10 mM glucose Krebs-Ringer Bicarbonate buffer (pH 7.5, Sigma Aldrich) supplemented with 10 mM HEPES, 0.1% BSA, and 500 μM 3-Isobutyl-1-methylxanthine (IBMX, Sigma Aldrich). The cells were treated for 5 min at 37 °C and lysed in 200 μL of 0.1 M HCl. The intracellular cAMP level was measured using a cAMP ELISA kit (Abcam) according to the manufacture's protocols.

2.9 Glucose tolerance test in vivo

2.9.1 Animals

Male C57BL/6 mice (4 weeks old, Orent, Seongnam, Korea) were used for an intraperitoneal glucose tolerance test. The mice were adapted to the animal facility for 1 week after quarantine and housed in a room maintained at 23 ± 2 °C with 50 ± 5% relative humidity under a 12 h light/dark cycle. The animals were fed a standard animal diet and water. All animal experiments were approved by the institutional animal care and use committee of the Korea Advanced Institute of Science and Technology (KAIST).

2.9.2 Intraperitoneal glucose tolerance test

Mice were randomly divided into control and experiment groups and fasted for 3 h before the start of the experiment. One hour before glucose injection, 100 μL of GLP-1 or the HSA-specific repebody-fused GLP-1 (25 nmol/kg) was injected into mice intraperitoneally (n = 6 per each group). PBS was used as a control. After 1 h, 1.5 g/kg of glucose dissolved in sterile water was injected intraperitoneally. At each time point, the blood glucose level was measured using an Accu-Check (Roche Diabetes Care, Indianapolis, IN, USA) until 4 h post-injection.

2.10 Hypoglycemic activity in diabetic mouse model

2.10.1 Animals

Male C57BLKS/J lar- $Lepr^{db}$/$Lepr^{db}$ mice (5 weeks old, Central Lab Animal, Seoul, Korea) were used as a model of type-2 diabetes for a hypoglycemic activity test. Mice were adapted to the animal facility for 1 week after quarantine and housed in a room maintained at 23 ± 2 °C with 50 ± 5% relative humidity under a 12 h light/dark cycle. The animals were fed a standard animal diet and water. All animal experiments were approved by the institutional animal care and use committee of the Korea Advanced Institute of Science and Technology (KAIST).

2.10.2 *Hypoglycemic activity test*

Mice were randomly divided into control and experiment groups, and 100 μL of either GLP-1(7-36), the HSA-specific repebody-fused GLP-1 in complex with the HSA, or an off-target repebody-fused GLP-1 were injected into mice intravenously at equal dose (25 nmol/kg, n = 5 per each group). PBS was used as a control. At each time point, the blood glucose level was measured using an Accu-Check until 24 h post-injection.

2.11 Statistical analysis

All experimental data were expressed as the mean ± standard deviation unless otherwise noted. A statistical analysis was conducted using a one-way analysis of variance (ANOVA) or a student's t-test for two independent samples, and inter-group comparisons were made using a Tukey's multiple comparison test. When the p value was below 0.05 ($p < 0.05$), data were considered statistically significant.

3. Results and discussion

3.1 Development of a human serum albumin-specific repebody

To enhance the blood half-life of a small-sized therapeutic protein, we intended to develop a HSA-specific repebody through a phage display selection and modular evolution approach as described in our previous studies [26-28, 32-34]. Briefly, a repebody library was constructed by randomizing six sites on the third and fourth modules of the concave region (**Supplementary Figure S1a, b**). Through six rounds of the panning and phage ELISA process,

a total of 73 colonies showing high ELISA signals were selected and sequenced. Among them, the aA1 clone was selected and subjected to affinity maturation through a modular evolution approach. Through an additional five rounds of the panning and phage ELISA process, 11 clones with high signals were selected and sequenced. The aA1C4 clone showing the highest signal was finally selected (**Supplementary Figure S1c**).

The binding affinity of the repebody (aA1C4) for HSA was determined through surface plasmon resonance (SPR) (**Fig. 1a**). The dissociation constant (k_{off}) and association constant (k_{on}) were determined to be 1.5 x 10^{-4} 1/s and 3.5 x 10^{4} 1/Ms, respectively, leading to a binding affinity (K_D) of 4.3 nM. The specificity of the repebody was examined using a direct ELISA for serum albumins from various species (**Fig. 1b**). The repebody was shown to have a binding affinity not only for human, but also for monkey and rabbit albumins, whereas a negligible affinity was observed for mouse, rat, and bovine serum albumins. Serum albumins are well known to share a high structural and sequence similarity with each other. Nonetheless, the repebody showed moderate cross-reactivity for various serum albumins, discriminating among mouse, rat and bovine albumins.

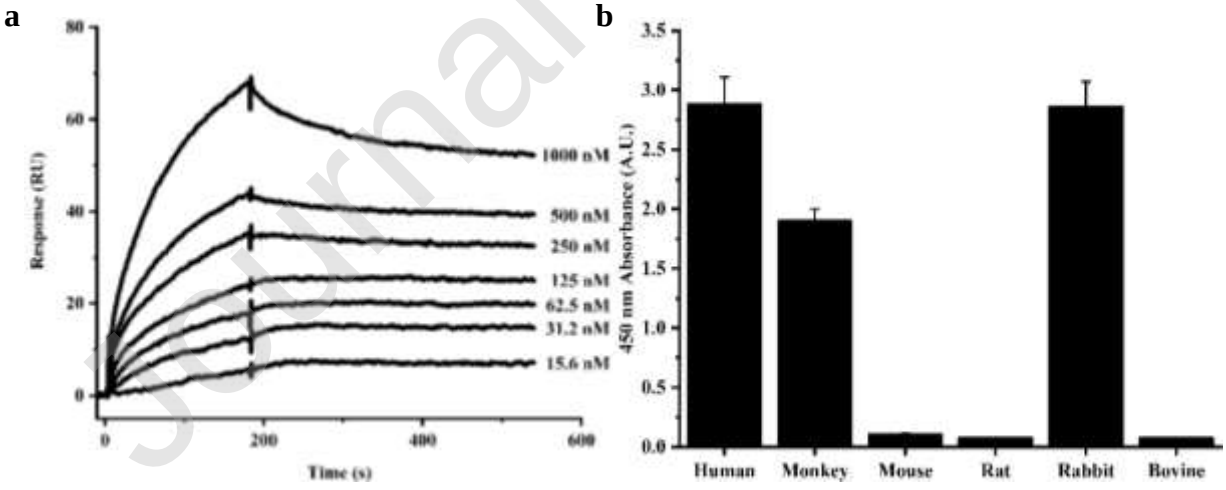


Fig. 1. Binding affinity and specificity of the HSA-specific repebody. (**a**) The binding affinity of the repebody was measured using SPR. 10 μg/mL of human serum albumin was immobilized on a CM5 chip using amine coupling, and a serially diluted repebody solution

was injected. The resulting sensorgrams were fitted and analyzed using an evaluation software using a 1:1 Langmuir binding model. The binding affinity (K_D) was estimated to be 4.3 nM. (**b**) The specificity of the repebody was tested toward various serum albumins using a direct ELISA. Error bars represent the average and standard deviation through the triplicated experiments.

3.2 Binding of HSA in complex with the repebody to neonatal Fc receptor

The blood half-life of HSA is known to be approximately 20 days, which results from its recycling through a neonatal Fc receptor (FcRn) at pH 6.0 [37]. We checked the binding ability of HSA in complex with the repebody toward human FcRn (hFcRn). To do so, the binding level of hFcRn to HSA was measured in the presence and absence of the repebody using direct ELISA at pH 6.0 and pH 7.4 (**Supplementary Figure S3**). The level of bound hFcRn to HSA in the presence of the repebody was almost the same as that in the absence of the repebody at pH 6.0. This result indicates that the repebody has a negligible effect on the binding of HSA to hFcRn. At pH 7.4, binding of the HSA to hFcRn was shown to be negligible regardless of the presence of the repebody as expected, which seems to be due to the fact HSA itself does not bind to hFcRn at a neutral pH. Based on the results, it is likely that the HSA-specific repebody has a negligible interference with the recycling of HSA through hFcRn on the epithelial cells.

3.3 Stability of a repebody in complex with human serum albumin

To use the HSA-specific repebody for increasing the blood circulation time of the repebody-fused protein, the repebody in complex with HSA is required to maintain its complex form as long as possible. We assessed the stability of the repebody in complex with HSA using size exclusion chromatography. The repebody was mixed with HSA at a molar ratio of 2:1 and

incubated at 4 °C for 1 h, followed by size exclusion chromatography to remove the free repebodies. The complex formation between the repebody and HSA was confirmed by analyzing the eluted fractions on SDS-PAGE (**Supplementary Figure S4a**). Two major bands, corresponding to HSA (66.5 kDa) and the repebody (30 kDa), respectively, were clearly observed. To examine the long-term stability of the repebody in complex with HSA, the eluted fractions were collected and stored at 37 °C. After 24, 36, 48, and 96 h, the protein solution was subjected to size exclusion chromatography (**Supplementary Figure S4b)**. The repebody in complex with HSA remained stable even after 96 h, indicating the formation of a highly stable complex between the repebody and HSA.

Next, we tested the stability of the repebody in complex with HSA in a mouse serum (**Supplementary Figure S4c**). The repebody in complex with HSA was mixed with 10% (v/v) mouse serum and stored at 37 °C. At various time intervals, 500 μL was sampled and subjected to a pull-down assay using a 6x-his tag at the C-terminus of the repebody. Because HSA has no his-tag, the repebody with a his-tag was used for the pull-down assay. Eluted samples were denatured and subjected to SDS-PAGE analysis. Two distinct bands, which correspond to the HSA and the repebody, respectively, were also observed even after 96 h. Based on the results, it seems that the HSA-specific repebody retained its binding ability for HSA for at least 96 h even in the mouse serum.

3.4 Construction and characterization of the repebody-fused GLP-1

To prove the utility of the HSA-specific repebody, a matured GLP-1(7-36) was chosen as a model therapeutic protein with a small size and fused to the HSA-specific repebody. Alanine at eighth position of GLP-1 was changed to glycine to provide resistance against DPP-IV peptidase. Of the many types of peptide linkers [38], we used a α-helix rigid linker ($A(EAAAAK)_3A$) to construct a fusion protein by taking into consideration the following

conditions: 1) a soluble expression of a fusion protein in *E. coli*, 2) a negligible inhibitory effect on the biological function of each protein, and 3) biophysical stability of the resulting fusion protein. GLP-1 was genetically fused to the C-terminal of the repebody using an α-helix rigid linker, and the resulting fusion protein was expressed in BL21 (DE3) cells. A his-tag was introduced to the C-terminus of the fusion protein for purification. For GLP-1 receptor binding assay, a myc-tag was additionally inserted into the C-terminus. The repebody-fused GLP-1 was expressed in a soluble form (> 20 mg/L). The fusion protein was eluted as a single peak using size exclusion chromatography, showing a single band with an expected molecular size (34.8 kDa) on SDS-PAGE (**Fig. 2a**). Size exclusion chromatography and SDS-PAGE analysis revealed that the fusion protein has high purity (> 98 %) and homogeneity. The bacterial expression of GLP-1 has been known to result in a low yield, making it difficult to purify using conventional chromatography. Interestingly, the repebody-fused GLP-1 was highly expressed in *E. coli* BL21 (DE3) cells and was easy to purify using conventional affinity and size exclusion chromatography.

We investigated the thermal stability of the repebody-fused GLP-1 by measuring the melting temperature using circular dichroism (**Fig. 2b, Supplementary Figure S5**). The melting temperature (Tm) of the fusion protein was estimated to be 60 °C. GLP-1 is known to have a relatively low stability, and tends to aggregate when stored at 4 °C. The fusion protein was observed to remain stable even for several weeks when stored at 4 °C. It is likely that the genetic fusion of the repebody to GLP-1 significantly improved the bacterial expression level and biophysical properties of GLP-1.

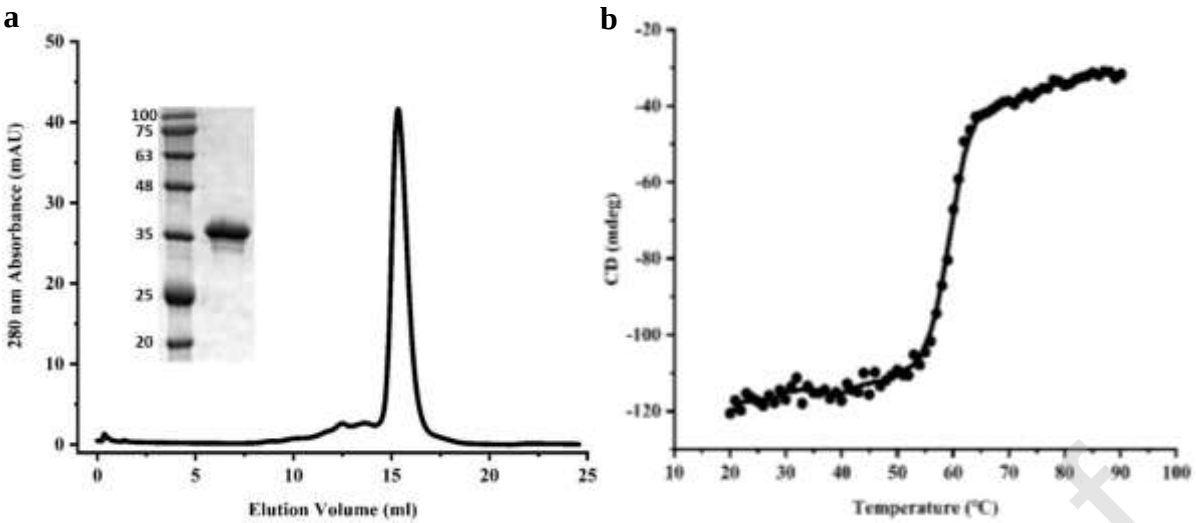


Fig. 2. Expression and biophysical stability of the repebody-fused GLP-1. (**a**) Size exclusion chromatography of the expressed fusion protein. The inset indicates the SDS-PAGE analysis of the eluted fraction. (**b**) Elliptical profile of the fusion protein based on circular dichroism at 222 nm.

3.5 Binding ability of the fusion protein toward HSA and GLP-1 receptor

To check whether each domain of the fusion protein retains its biological function, the binding ability of the fusion protein was examined toward both the HSA and extracellular domain of the GLP-1 receptor using a direct ELISA (**Fig. 3**). We first tested the binding ability of the fusion protein toward HSA. A serial diluted fusion protein was introduced into the wells coated with HSA, and the signals were detected using an anti-repebody antibody labeled with biotin and streptavidin-HRP conjugate. As shown in **Fig. 3a**, the fusion protein has almost the same binding ability as a naked repebody. We next examined the binding ability of the fusion protein toward the GLP-1 receptor through direct ELISA (**Fig. 3b**). The fusion protein showed a decreased binding affinity (EC_{50} = 9.8 nM) compared to free GLP-1, which has a binding affinity of 0.2 nM, and this seems to be due to possible steric hindrance by the repebody.

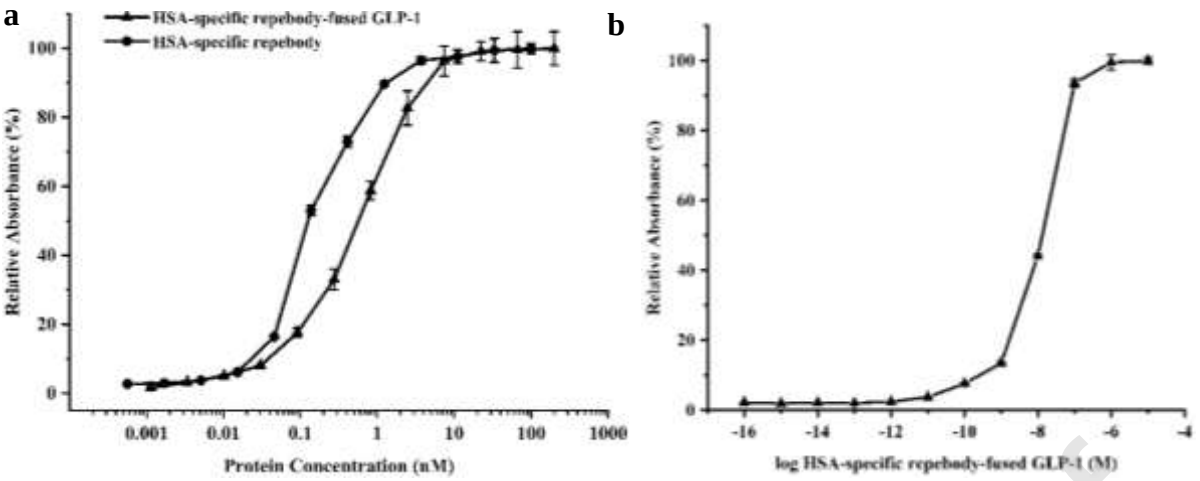


Fig. 3. Binding ability of the repebody-fused GLP-1 against HSA and GLP-1 receptor. (**a**) The binding ability of the repebody-fused GLP-1 was tested for HSA using a direct ELISA. A free repebody was used as a control. (**b**) The binding ability of the repebody-fused GLP-1 toward GLP-1 receptor was evaluated by direct ELISA. Bars represent the average and standard deviation in triplicated experiments.

3.6 Biological activity of the repebody-fused GLP-1 in vitro

The binding of the matured GLP-1 to its receptor is known to increase the intracellular level of cAMP, leading to the release of insulin into the blood and consequently reduction in the blood glucose level. We checked whether the repebody-fused GLP-1 can enhance the intracellular level of cAMP (**Fig. 4**). A serially diluted fusion protein was treated with INS-1 cells, and the intracellular cAMP level was determined. As a result, the EC_{50} values were calculated to be 0.4 and 15.3 nM for the native GLP-1 and the repebody-fused GLP-1, respectively. The repebody-fused GLP-1 retained its potency but exhibited a 38-fold decreased EC_{50} value than the native GLP-1 when the cells were used. This result also seems to be due to a steric hindrance when the repebody-fused GLP-1 binds to its receptor as observed in **Fig. 3b**. Similar results were reported when GLP-1 was conjugated with peptides or biopolymer [14, 39, 40].

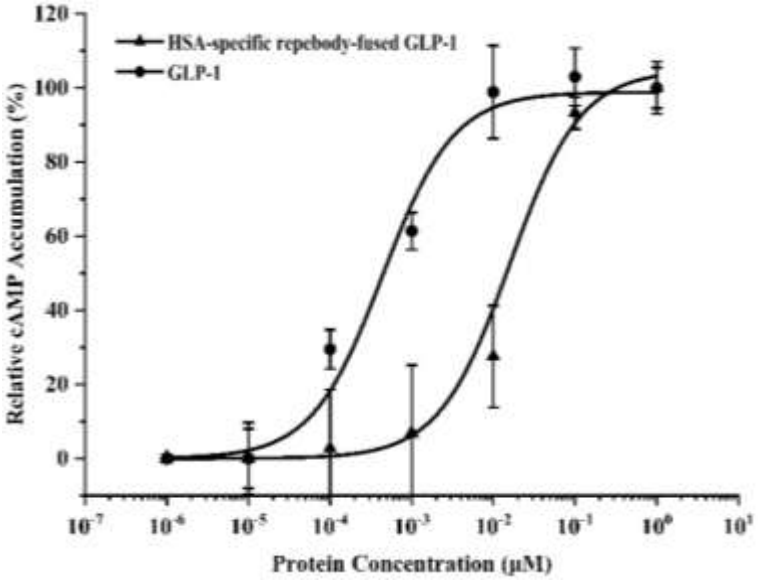


Fig. 4. Intracellular cAMP levels secreted by the repebody-fused GLP-1. The intracellular cAMP levels were analyzed after 5 min treatment with the serially diluted repebody-fused GLP-1 or free GLP-1 in rat insulinoma cell line INS-1 (n = 6 per each group). Error bars represent the average and standard deviation.

3.7 Pharmacokinetic profile of the repebody-fused GLP-1

The pharmacokinetic profile of the repebody-fused GLP-1 was analyzed using sandwich ELISA (**Fig. 5**). As a control, an off-target repebody-fused GLP-1 was used. The off-target repebody was shown to have a negligible binding to serum albumins (**Supplementary Figure S6)**. The HSA-specific repebody-fused GLP-1 in complex with HSA was purified through size exclusion chromatography and used for analysis of the pharmacokinetic profiles. The repebody-fused GLP-1 in complex with HSA was injected into mice intravenously, and the serum was collected at intervals followed by a sandwich ELISA. The off-target repebody-fused GLP-1 was shown to be completely cleared out in several hours. The initial half-life **($t_{1/2,\alpha}$)** was estimated to be 1.3 h, and the terminal half-life ($t_{1/2,\beta}$) was not measurable owing to a fast clearance. In contrast, the repebody-fused GLP-1 in complex with HSA exhibited a much longer pharmacokinetic profile. The initial and terminal half-life were estimated to be 4.2 h

and 10.7 h, respectively. The AUC was 746.6, which is 4.2-times higher than the off-target repebody-fused GLP-1. Similar pharmacokinetic profile and parameters were obtained when the In^{111}-labelled fusion protein was used (**Supplementary Figure S7, Supplementary Table S1**). The off-target repebody-fused GLP-1 exhibited an 11-fold higher clearance rate compared to the HSA-specific repebody-fused GLP-1. The fusion of the HSA-specific repebody to GLP-1 resulted in not only a prolonged blood circulation time, but also a significant increase in the serum concentration as compared to the control, effectively enhancing the relative drug exposure of GLP-1. This result indicates that the HSA-specific repebody can be effectively used as a platform for enhancing the blood circulation time of a small-sized protein through genetic fusion. In this study, the HSA-specific repebody-fused GLP-1 in complex with HSA was used for a pharmacokinetic profile in mice because the repebody has a negligible cross-reactivity for mouse serum albumin. Nonetheless, the fusion protein showed a significantly enhanced blood circulation time even in mice, implying that the half-life of the HSA-specific repebody-fused GLP-1 will be much longer when tested in humans. In such case, the repebody-GLP-1 is expected to form a complex with HSA in the blood. As the complex is in equilibrium between association and dissociation with HSA, free form of the repebody-GLP-1 will bind to GKP-1 receptor during blood circulation, triggering the signaling process. Accordingly, the repebody-fused GLP-1 alone can be used as a potential therapeutic agent.

The terminal half-life of the HSA-specific repebody-fused GLP-1 was estimated to be 10.7 h, even though it was injected in complex with HSA because the repebody has no binding affinity for mouse serum albumin. Pharmacokinetic study with mice expressing both HSA and hFcRn or rabbit will result in much prolonged half-life of GLP-1. The repebody was shown to have high stability against heat, pH, and protease even in serum condition [23], which is crucial to a long half-life in the blood. Our approach enables easy construction and production of homogeneous fusion proteins by *E. coil*. In addition, genetic fusion of the repebody can lead

to overexpression of a fused protein as a soluble form, since the repebody tends to facilitate the folding of a fused protein.

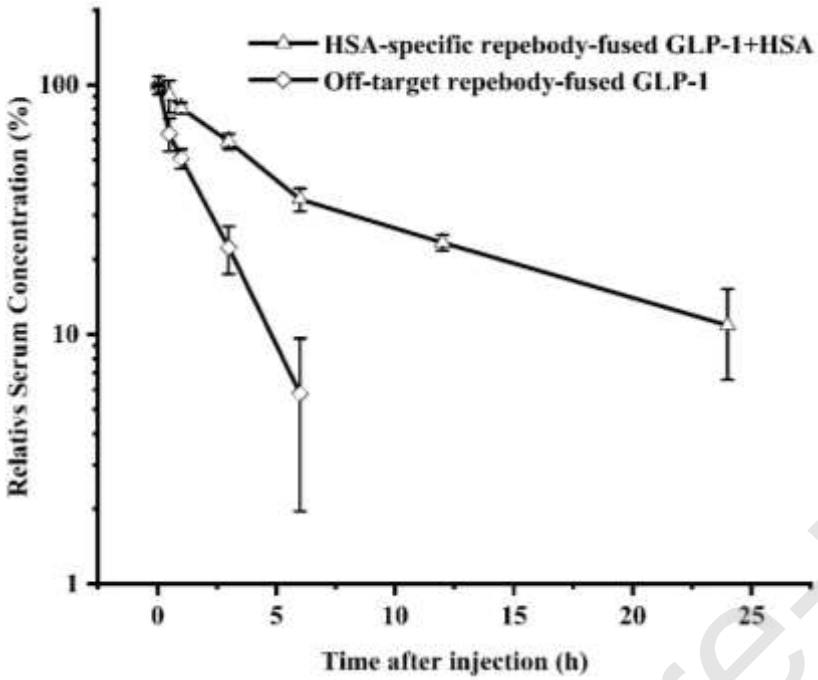


Fig. 5. Pharmacokinetic profile of the repebody-fused GLP-1. The HSA-specific repebody-fused GLP-1 in complex with HSA and an off-target repebody-fused GLP-1 were injected into mice intravenously (n = 5 per each group). Mice plasma were sampled at each time point and subjected to a sandwich ELISA, and the fusion protein levels were determined. Based on the pharmacokinetic profiles, the initial half-life ($t_{1/2,\alpha}$) and terminal half-life ($t_{1/2,\beta}$) were determined. The terminal half-life was undetectable for the off-target repebody-fused GLP-1 owing to a fast clearance. Error bars represent the average and standard deviation.

3.8 In vivo *hypoglycemic activity of the repebody-fused GLP-1*

The *in vivo* hypoglycemic activity of the HSA-specific repebody-fused GLP-1 was evaluated through an intraperitoneal glucose tolerance test in fasted normal mice (**Fig. 6a**). Mice were randomly divided into three groups (repebody-fused-GLP-1, GLP-1, and PBS) and fasted for 3 h prior to the experiments. At -1 h, each group was injected with respective proteins (25 nmol/kg, 100 μL), followed by intraperitoneal injection of glucose (1.5 g/kg, 100 μL) at 0 h.

The blood glucose levels were measured at each time point until 3 h, where the blood glucose levels returned to normal. Mice injected with an equal dose of either the repebody-fused GLP-1 or GLP-1 showed a tolerance against injected glucose. It is noteworthy that the repebody-fused GLP-1 gave rise to a more significant tolerance over injected glucose. The blood glucose level started to decrease after the injection of the fusion protein and maintained a normal glucose level throughout the entire experiment time, even after intraperitoneal injection of glucose. In the case of PBS, the blood glucose level immediately peaked at over 400 mg/dl when glucose was injected. This result indicates that the repebody-fused GLP-1 shows highly effective hypoglycemic activity *in vivo*.

We next investigated the hypoglycemic activity of the HSA-specific repebody-fused GLP-1 in type-2 diabetes mouse model (**Fig. 6b**). To assess the effects of a prolonged half-life, the repebody-fused GLP-1 in complex with HSA and the off-target repebody-fused GLP-1 were both used. Each group of mice was intravenously injected with either the repebody-fused GLP-1 in complex with HSA or the off-target repebody-fused GLP-1 at an equimolar dose (25 nmol/kg, 100 μL). Free GLP-1 and PBS were used as a control. At each time point, the blood glucose levels were measured until 24 h post injection. After 1 h, the glucose level was significantly decreased to below 200 mg/dl by the repebody-fused GLP-1 in complex with HSA, which is a similar level as that observed in a normal mouse. The low glucose level was maintained for more than 24 h after injection and was at least 100 mg/dl lower than that of the PBS control group. In the case of the off-target repebody-fused GLP-1, a moderate decrease in glucose level was observed compared to free GLP-1, resulting in a lower glucose level by 100 mg/dl after 1 h, which might be attributed to an increase in size and resistance over DPP-IV. However, the glucose level soon returned to the initial state at near 400 mg/dl. This implies that simple increase in size has a limited effect on the elongation of the blood circulation time. In comparison with the off-target repebody-fused GLP-1, it is clear that the HSA-specific

repebody plays a distinct role in significantly improving the pharmacokinetic profile and consequently the hypoglycemic activity of GLP-1. In contrast, GLP-1 showed no viable effect on a decrease in the glucose level compared to PBS. It is well known that the serum half-life of GLP-1 is less than 2 min owing to the peptidase degradation and renal filtration. It is thus likely that native GLP-1 was cleared out of the blood immediately after injection. Based on the results, it is noteworthy that the HSA-specific repebody can be used as a platform to enhance the blood circulation time of small-sized therapeutic proteins, consequently improving their therapeutic efficacy.

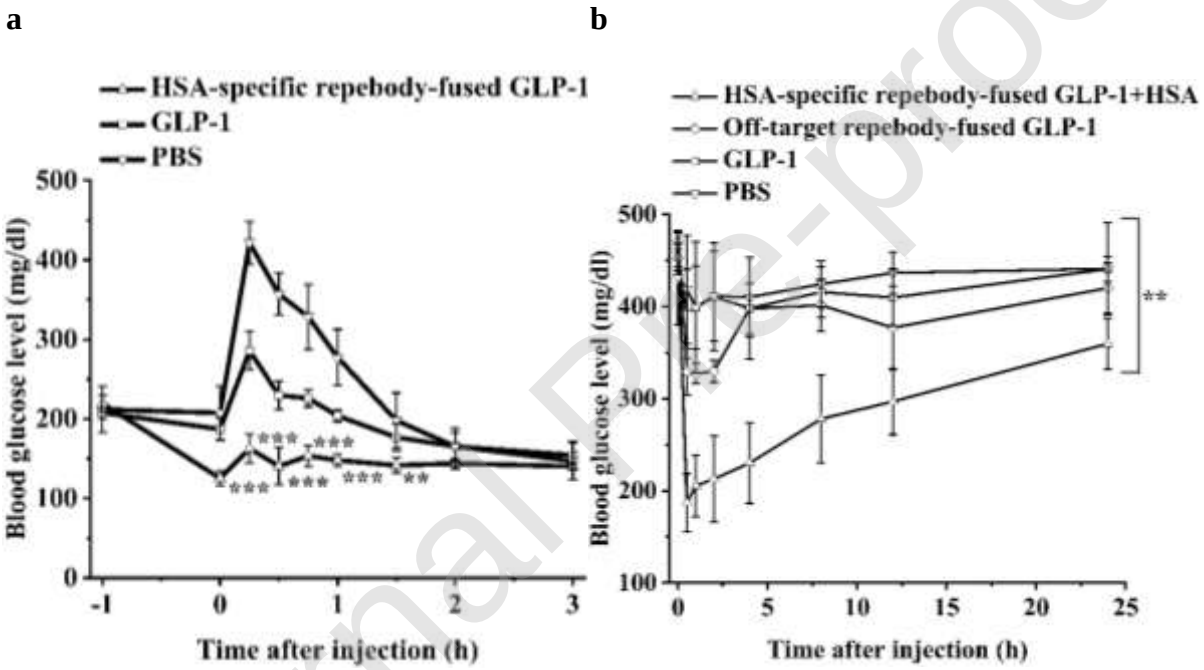


Fig 6. Hypoglycemic activity of the repebody-fused GLP-1 *in vivo*. (**a**) Intraperitoneal glucose tolerance by the HSA-specific repebody-fused GLP-1 was tested**.** C57BL/6 mice (n = 6 per group, 4 weeks) were fasted for 3 h before injection of each protein. At t = -1, the HSA-specific repebody-fused GLP-1 and free GLP-1 (25 nmol/kg) were injected intraperitoneally. PBS was used as a control. After 1 h, 1.5 g/kg of glucose was injected intraperitoneally, and the blood glucose levels were analyzed. The repebody-fused with GLP-1 showed significant tolerance (***$p < 0.001$, **$p < 0.005$) against injected glucose compared to free GLP-1 and PBS. Error bars represent the average and standard deviation in triplicate experiments. (**b**) Hypoglycemic activity of the HSA-specific repebody-fused GLP-1 was tested in type-2

diabetes mouse model. C57BLKS/J lar- $Lepr^{db}/Lepr^{db}$ mice (n = 5 per group, 6 weeks) were intravenously injected with the repebody-fused GLP-1 in complex with HSA, an off-target repebody-fused GLP-1, native GLP-1, and PBS. The blood glucose levels were analyzed at each time point for 24 h. The HSA-specific repebody-fused GLP-1 showed a significant decrease (**$p < 0.005$) in blood glucose level compared to other cases. The error bars represent the average and standard deviation in triplicate experiments.

4. Conclusions

We demonstrated that the HSA-specific repebody can significantly extend a half-life of a small-sized therapeutic protein through a simple genetic fusion, consequently improving the therapeutic efficacy. The HSA-specific repebody was shown to stably maintain the complex with HSA for a long period of time even in mouse serum. The repebody-fused GLP-1 retained its binding ability toward both HSA and GLP-1 receptor. Analysis of the pharmacokinetic profile strongly supported that the HSA-specific repebody can effectively extend a half-life of GLP-1, resulting in a terminal half-life of 10.7 h and a 4.2-fold higher AUC compared to an off-target repebody. In addition, the HSA-specific repebody was observed to reduce the clearance rate of GLP-1 by 11-fold. The repebody-fused GLP-1 was able to bind to its receptor, stimulating the cAMP accumulation in insulinoma cells. Consequently, the repebody-fused GLP-1 was shown to effectively lower the blood glucose level in a glucose tolerance test in fasted normal mice. More importantly, the blood glucose level was recovered very slowly for more than 24 h after injection of the repebody-fused GLP-1 in complex with HSA in diabetic model mice, indicating the enhanced therapeutic efficacy of GLP-1 owing to a prolonged half-life in the blood. Immunogenicity of the repebody-fused GLP-1 will be critical to its clinical application. Our previous study revealed a negligible immunogenicity of the repebody itself in mice [27]. A similar result was observed when tested with peripheral blood mononuclear cells (PBMC) from human donors, supporting the potential therapeutic use of the repebody-fused

GLP-1.

Taken together, the present approach can be effectively used as a platform to extend the pharmacokinetic profile of small-sized therapeutic proteins and consequently their therapeutic efficacy.

Declaration of interest

The authors declare no competing financial interests.

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