Supporting Information

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SI Materials and Methods

GLP-1 Oligomer Gene Synthesis. Synthetic genes encoding glucagon-like peptide-1 (GLP-1) oligomers were assembled by Overlap Elongation Rolling Circle Amplification (OERCA), as previously described (1). BL21(DE3) competent cells (EdgeBio) were directly transformed with the OERCA reaction mixture. Following verification by DNA sequencing, colonies with oligomers of $(GLP\text{-}1)_{x6}$ were selected from OERCA-generated libraries. Cells were grown overnight and the plasmid DNA was purified by a plasmid miniprep kit (Qiagen). A total of 2 μg of the plasmid DNA were digested with 2 μL each of NdeI and HindIII and 1× NEB (New England Biolabs) buffer 2 for 2 h at 37 °C, enzymatically dephosphorylated with 1 μL calf intestinal phosphatase (CIP) for 15 min to 1 h at 37 °C, and then purified by a PCR purification kit. A similar digestion was performed on pET25b+ vectors containing an elastin-like-polypeptide (ELP) gene that was 3′- to the site of insertion of the GLP-1 oligomer gene. The GLP-1 oligomer genes (6 μL) were ligated to various ELP-containing vectors (3 μL) using four units of T4 DNA ligase (Fermentas), 1× ligation buffer, 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 super optimal broth (SOC) media while horizontally shaking at 200 rpm (on a symphony Incubating Microplate Shaker, VWR) at 37 °C for 40–60 min, and were then plated on terrific broth (TB) agarose plates containing 1 mg/mL ampicillin.

POD Expression and Purification. Before large-scale expression, starter cultures (4 mL) of TB media (Mo Bio) supplemented with 100 μg/mL ampicillin were inoculated with transformed cells from a fresh agar plate or from DMSO stocks stored at −80 °C, and incubated overnight at 37 °C while shaking at 250 rpm. Expression cultures (4 L flasks containing 1 L of TB media and 100 μg/mL ampicillin) were inoculated with the starter culture and incubated at 30 °C for 6 h and then 16 °C with shaking at 200 rpm. After 7 h of growth, expression was induced by the addition of IPTG to a final concentration of 0.5 mM.

Cells were harvested 24 h after inoculation by centrifugation at 3,200 *g* for 10 min at 4 °C. The cell pellet was resuspended by vortex in ∼25 mL PBS buffer and stored at −80 °C. For purification, resuspended pellets were thawed and lysed by ultrasonic disruption (10 cycles of 10 s sonication separated by 40 s intervals). Poly(ethyleneimine) [2.5 mL of 10% (vol/vol) solution, Fluka] was added to each lysed suspension before centrifugation at 14,000 *g* for 10 min at 4 °C to separate cell debris from the soluble cell lysate.

All GLP-1 Protease Operated Depots (PODs) were purified by a modified Inverse Transition Cycling (ITC) protocol consisting of multiple "hot" and "cold" spins (2) using NaCl to trigger the phase transition. For "hot" spins, the ELP phase transition was triggered by adding NaCl to a solution at ∼10 °C of the cell lysate or the product of a previous cycle of ITC at a final concentration of 1 M NaCl for ELP_{Low} or 3 M NaCl ELP_{High} fusion constructs. The solutions were then centrifuged at 14,000 *g* for 5 min at 10 °C, and the pellets were resuspended in PBS, followed by a 10 min "cold" spin at 4 °C without additional NaCl to remove denatured contaminant. Five rounds of ITC were carried out for each construct. The concentrations of the purified ELP fusions were measured on a NanoDrop ND-1000 (NanoDrop Technologies) UV-vis spectrophotometer at 280 nm using 50,420 $M^{-1}\cdot cm^{-1}$ as the extinction coefficient for all ELP_{Low} fusion proteins and 46,410 $M^{-1}\cdot cm^{-1}$ as the extinction coefficient for all ELP_{High} fusion proteins. Purified $([G8E22]GLP)_{x6}$-$ELP_{Low}240$ was submitted to the Duke proteomics facility for MS sequencing. For in vivo studies, peptide solutions were passed through a polymyxin B column (Pierce) at 4 °C, resuspended in D-PBS (D8662, Sigma), and sterile filtered through a 0.2 μM cellulose filter.

In Vitro Assay for GLP-1 Activity. $([G_8E_{22}]GLP)_{x6}$-$ELP_{Low}240$, $([G_8E_{22}]GLP)_{x6}$-$ELP_{Low}60$ PODs, and $([G_8E_{22}]GLP)_{x6}$-$ELP_{High}60$ PODs (50 μM) were incubated at 16 or 37 °C (below and above the POD transition temperature) for up to 24 h with 1.1 μg FXa protease (Thermo Scientific) and 0.5 μg dipeptidyl-peptidase IV (DPPIV) (ProSpec). In a second assay, $([G_8E_{22}]GLP)_{x6}$-$ELP_{Low}240$ (1,10,100 nM and 1μM) were incubated at 16 °C (below and above the POD transition temperature) for up to 24 h with 1.1 μg FXa protease (Thermo Scientific) and 0.5 μg DPPIV (ProSpec). Receptor activation was determined by measuring the intracellular cAMP concentration as a function of incubation time or protein concentration. Cells were seeded at 30,000 cells per well in 24-well plates and allowed to reach 70–80% confluence in media at 37 °C. Before beginning the assay, cells were incubated with 100 μM 3-isobutyl-1-methylxanthine for 1 h (3). Cells were then incubated for 10 min with 10 μL of the GLP-1 fractions. Intracellular cAMP concentrations were measured colorimetrically using a competitive binding assay following cAMP acetylation according to the manufacturer's instructions (Assay Designs).

Animal Studies. The 5–6-wk-old male C57BL/6J mice (stock number 000664) were purchased from Jackson Laboratories. C57BL/6J mice were used to test PODs since these mice are predisposed to diet-induced diabetes and mimic the prediabetic stage as they present elevated glucose and insulin levels while maintaining pancreatic β-cell function, thus enabling us to study the stimulatory effects of GLP-1 on insulin release (4). Mice were housed under controlled light (12 h light/12 h dark) cycle with free access to food (Labdiet 5001, Purina) and water. Mice were allowed at least 1 wk acclimation to the facility and blood sampling procedures before initiation of experiments. All experimental procedures were approved by the Duke Institutional Animal Care and Use Committee.

Fluorescent Labeling of GLP-1 PODs. The 0.5 mg Dylight dissolved in 50 μL anhydrous dimethylformamide were added to ∼0.5 mL of ∼200 μM POD or soluble control in 0.1 M sodium bicarbonate, pH 8.9, at room temperature and continuously rotated at 4 °C. The reaction was stopped after 1 h, and excess fluorophore was removed by separation of the fusion and unreacted fluorophore on a PD-10 gel-filtration column (GE Healthcare). Subsequently, a round of ITC was used to remove any residual unconjugated fluorophore by the addition of NaCl, and the protein pellet was resuspended in PBS to 150 μM. The protein concentration was measured by UV-vis spectroscopy (Nanodrop, Thermo Scientific) using the Dylight 488–adjusted measurement tool. The typical conjugation ratio ranged between 1.7 and 1.9 Dylight molecules per fusion.

Near Infrared Labeling of GLP-1 PODs. The 1 mg IRDye dissolved in 20 μL Dimethyl sulfoxide (DMSO) were added to ∼1 mL of ∼200 μM POD or soluble control in phosphate buffer pH 8.5, at room temperature, and continuously rotated at 4 °C. The reaction was quenched after 2 h, and excess fluorophore was removed by separation of the fusion and unreacted fluorophore on a PD-10 gel-filtration column (GE Healthcare). Subsequently, a round of

ITC was used to remove any residual unconjugated fluorophore by the addition of NaCl, and the protein pellet was resuspended in PBS to 150 μM. The protein concentration was measured by UV-vis spectroscopy (Nanodrop, Thermo Scientific).

1. Amiram M, Quiroz FG, Callahan DJ, Chilkoti A (2011) A highly parallel method for synthesizing DNA repeats enables the discovery of 'smart' protein polymers. *Nat Mater* 10(2):141–148.
2. Meyer DE, Chilkoti A (2002) Genetically encoded synthesis of protein-based polymers with precisely specified molecular weight and sequence by recursive directional ligation: Examples from the elastin-like polypeptide system. *Biomacromolecules* 3(2):357–367.
3. Baggio LL, Huang Q, Brown TJ, Drucker DJ (2004) A recombinant human glucagon-like peptide (GLP)-1-albumin protein (albugon) mimics peptidergic activation of GLP-1 receptor-dependent pathways coupled with satiety, gastrointestinal motility, and glucose homeostasis. *Diabetes* 53(9):2492–2500.
4. Surwit RS, Kuhn CM, Cochrane C, McCubbin JA, Feinglos MN (1988) Diet-induced type II diabetes in C57BL/6J mice. *Diabetes* 37(9):1163–1167.

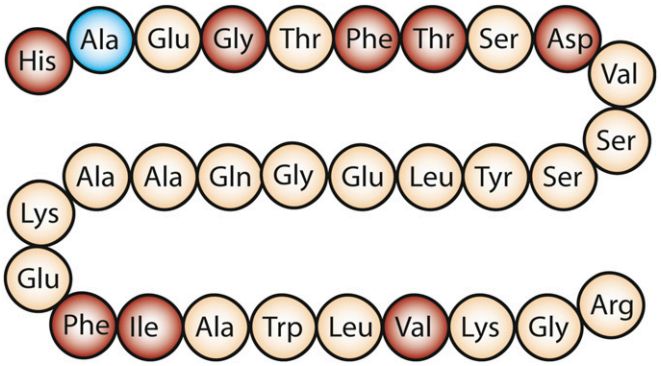

Fig. S1. Amino acid sequence of native GLP-1[7-36]. Amino acids highlighted in red are essential for GLP-1 activity; the second alanine (highlighted in blue) is the site of deactivation by DPP-IV. (modified from ref. 1).

1. Knudsen LB (2004) Glucagon-like peptide-1: The basis of a new class of treatment for type 2 diabetes. *J Med Chem* 47(17):4128–4134.

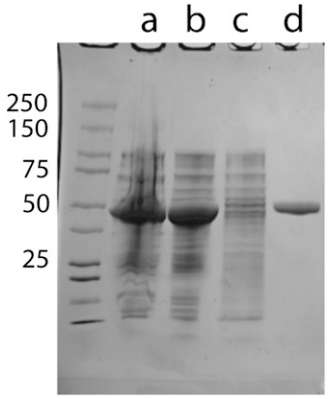

Fig. S2. Purification of GLP-1 PODs. ITC purification of $([G_8E_{22}]GLP)_{x6}$-$ELP_{Low}60$. Lanes, (*a*) sonicated total cell lysate, (*b*) soluble fraction of cell lysate, (*c*) resuspended cold spin pellet, and (*d*) purified $([G_8E_{22}]GLP)_{x6}$-$ELP_{Low}60$ construct after 2 ITC cycles.

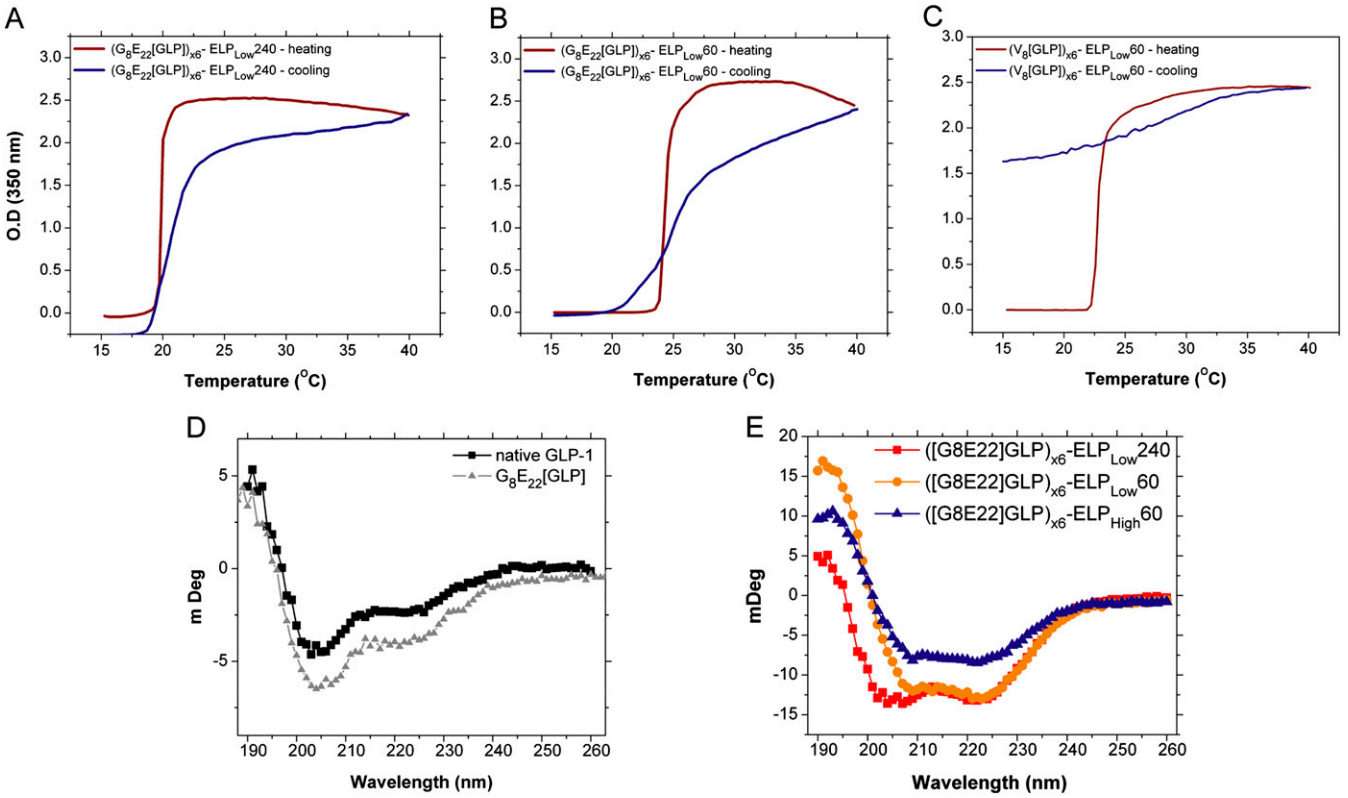

Fig. S3. Thermal phase behavior of GLP-1 PODs and CD spectra of GLP-1 monomer and PODs. Transition profile and reversibility of (*A*) ([G8E22]GLP)$_{x6}$-ELP$_{Low}$240, (*B*) ([G8E22]GLP)$_{x6}$-ELP$_{Low}$60, and (*C*) the irreversible ([V8]GLP)$_{x6}$-ELP$_{Low}$60 (all constructs at 150 μM in PBS). The turbidity is measured spectrophotometrically at 350 nm and is a measure of the aggregation of the POD as a function of solution temperature. (*D*) CD spectra of monomer [G8E22]GLP and native GLP-1 (15 μM each). (*E*) CD spectra of ([G8E22]GLP)$_{x6}$-ELP$_{Low}$240, ([G8E22]GLP)$_{x6}$-ELP$_{Low}$60, and ([G8E22]GLP)$_{x6}$-ELP$_{High}$60 (2.5 μM fusion protein or 15 μM GLP-1 equivalents).

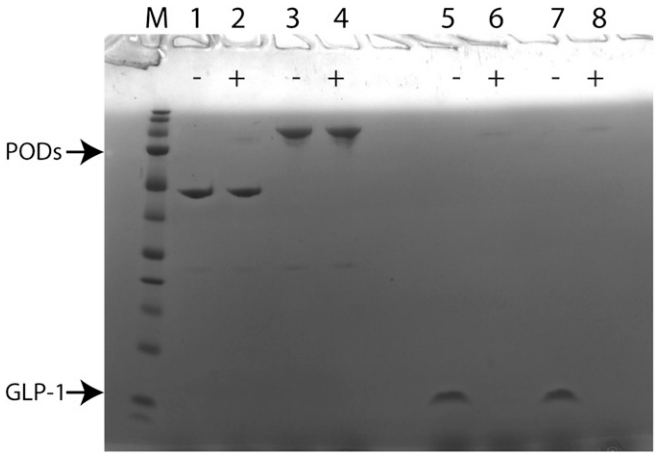

Fig. S4. NEP degradation of monomer GLP-1 and PODs at 16 °C. Degradation of PODs or GLP-1 by NEP after 18 h incubation. Lane 1, ([G$_8$E$_{22}$]GLP)$_{x6}$-ELP$_{Low}$60; lane 2, ([G$_8$E$_{22}$]GLP)$_{x6}$-ELP$_{Low}$60+NEP; lane 3, ([G$_8$E$_{22}$]GLP)$_{x6}$-ELP$_{Low}$240; lane 4, ([G$_8$E$_{22}$]GLP)$_{x6}$-ELP$_{Low}$240 +NEP; lane e5, [G$_8$E$_{22}$]GLP; lane 6, [G$_8$E$_{22}$]GLP +NEP; lane 7, native GLP-1; lane 8, native GLP-1+NEP. NEP may be seen as a faint band at ~85 kDa.

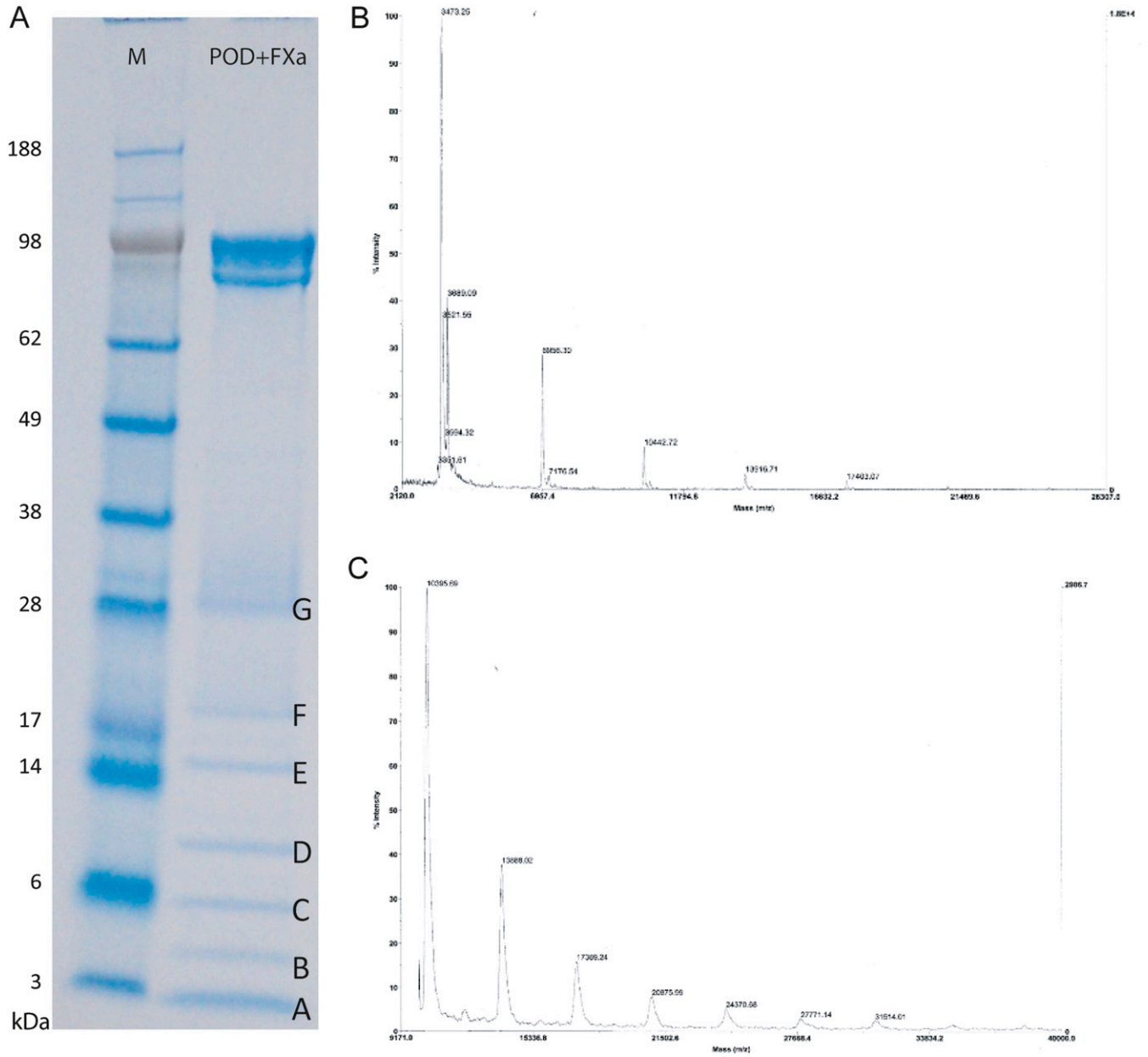

Fig. S5. Analysis of GLP-1 release from PODs by FXa. (*A*) $([G8E22]GLP)_{x6}$-$ELP_{Low}240$ was incubated with FXa for 24 h (to generate predominantly GLP-1 oligomers without the leader peptide) and labeled protein bands excised and sequenced confirming correct N terminus of GLP-1 in bands A–F; band G was attributed to FXa. The visualized sample was also analyzed by MALDI-TOF, showing a difference of ~3,480 ± 10 Da ([G8E22] GLP predicted M_r = 3,484 Da) between (*B*) low M_r fragments and (*C*) high M_r fragments. Very minor aggregation of GLP-1 oligomers is seen as higher M_r species in *C*.

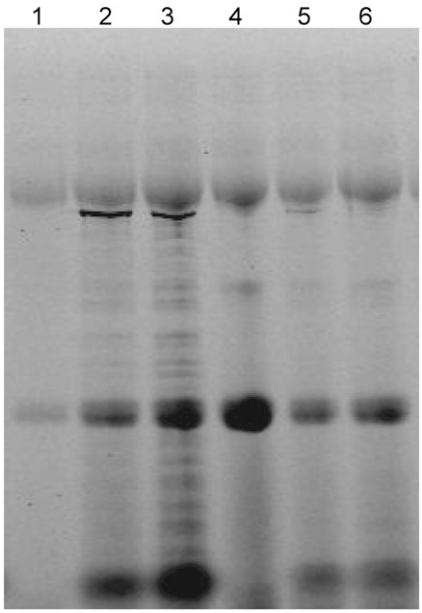

Fig. S6. In vivo release of GLP-1 from reversible and irreversible PODs. SDS/PAGE gel of Dylight 488–labeled PODs, injected s.c. and sampled from the tail vain at 0, 3, and 6 h postinjection. Reversible, $([G_8E_{22}]GLP)_{x6}$-$ELP_{Low}60$ 0, 3, and 6 h postinjection (lanes 1, 2, and 3, respectively), and irreversible, $([V_8]GLP)_{x6}$-$ELP_{Low}60$ (lanes 4, 5, and 6, respectively).

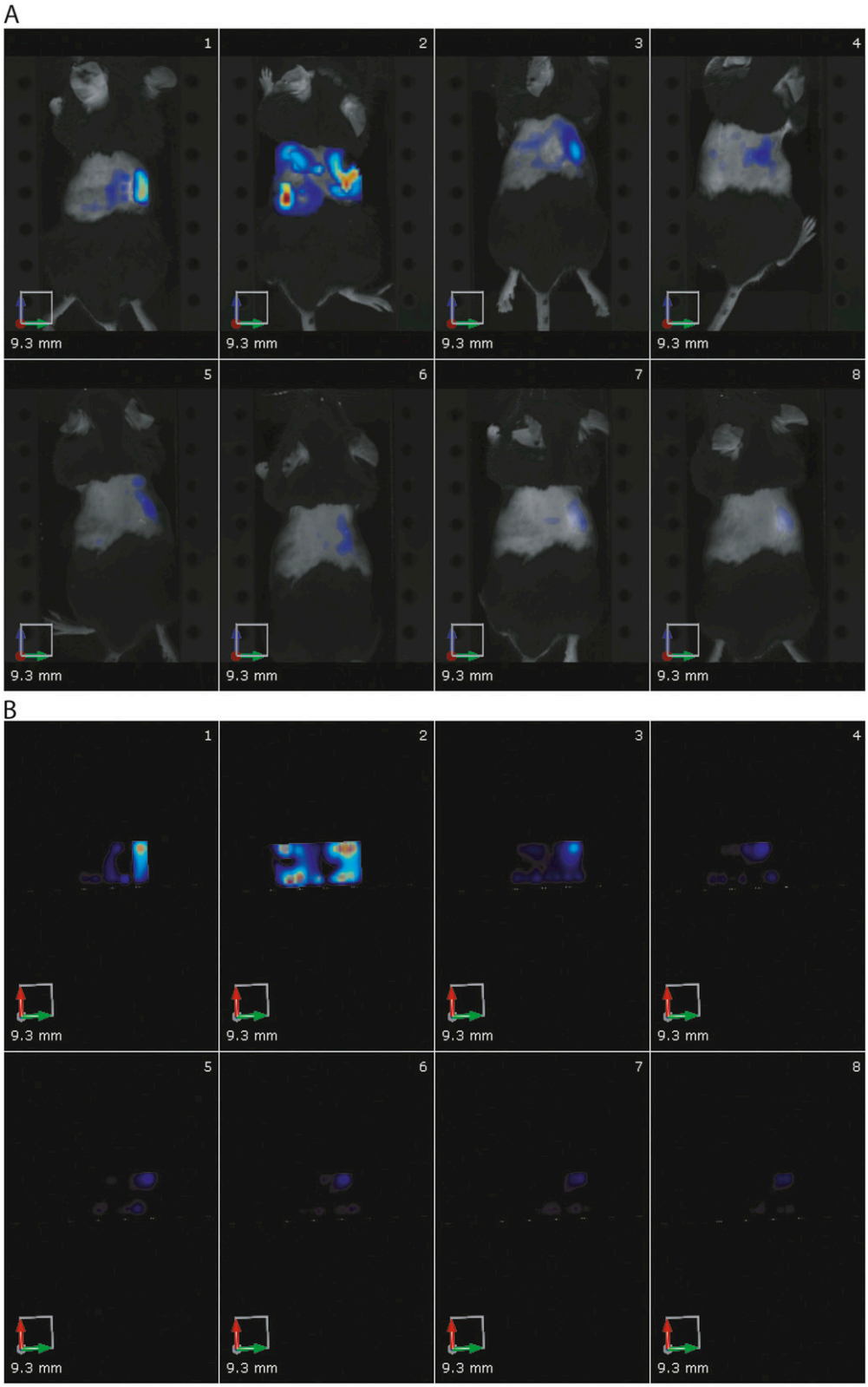

Fig. S7. In vivo near infrared fluorescence tomography of $([G8E22]GLP)_{x6}$-$ELP_{High}60$. NIR tomography images (*A*) and cross-sections (*B*) after a single s.c. injection of $([G8E22]GLP)_{x6}$-$ELP_{High}60$ at 0, 5, 24, 48, 72, 96, 120, and 144 h (panels 1–8, respectively).

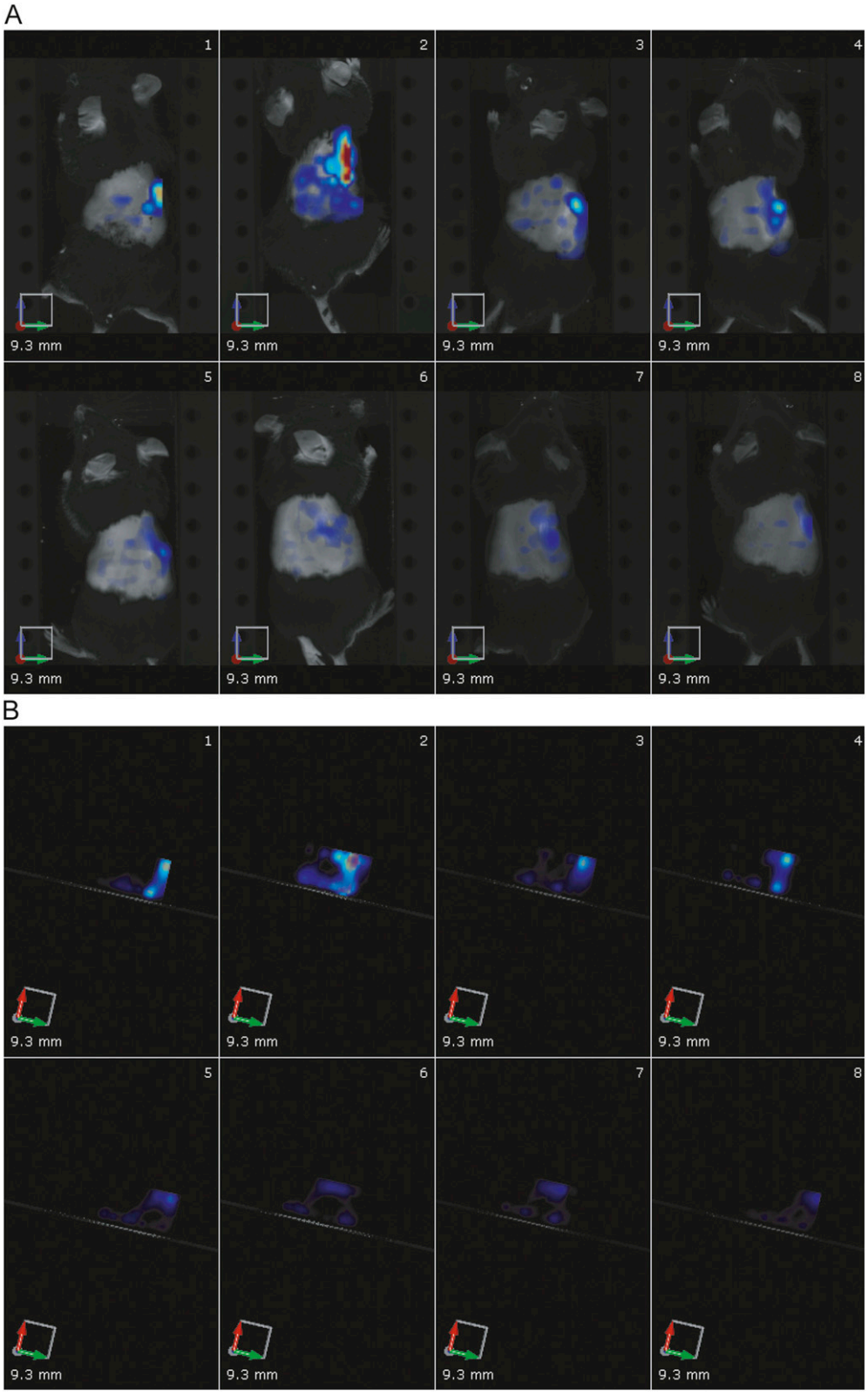

Fig. S8. In vivo near infrared fluorescence tomography of $([G8E22]GLP)_{x6}$-$ELP_{Low}60$. NIR tomography images (*A*) and cross-sections (*B*) after a single s.c. injection of $([G8E22]GLP)_{x6}$-$ELP_{Low}60$ at 0, 5, 24, 48, 72, 96, 120, and 144 h (panels 1–8, respectively).

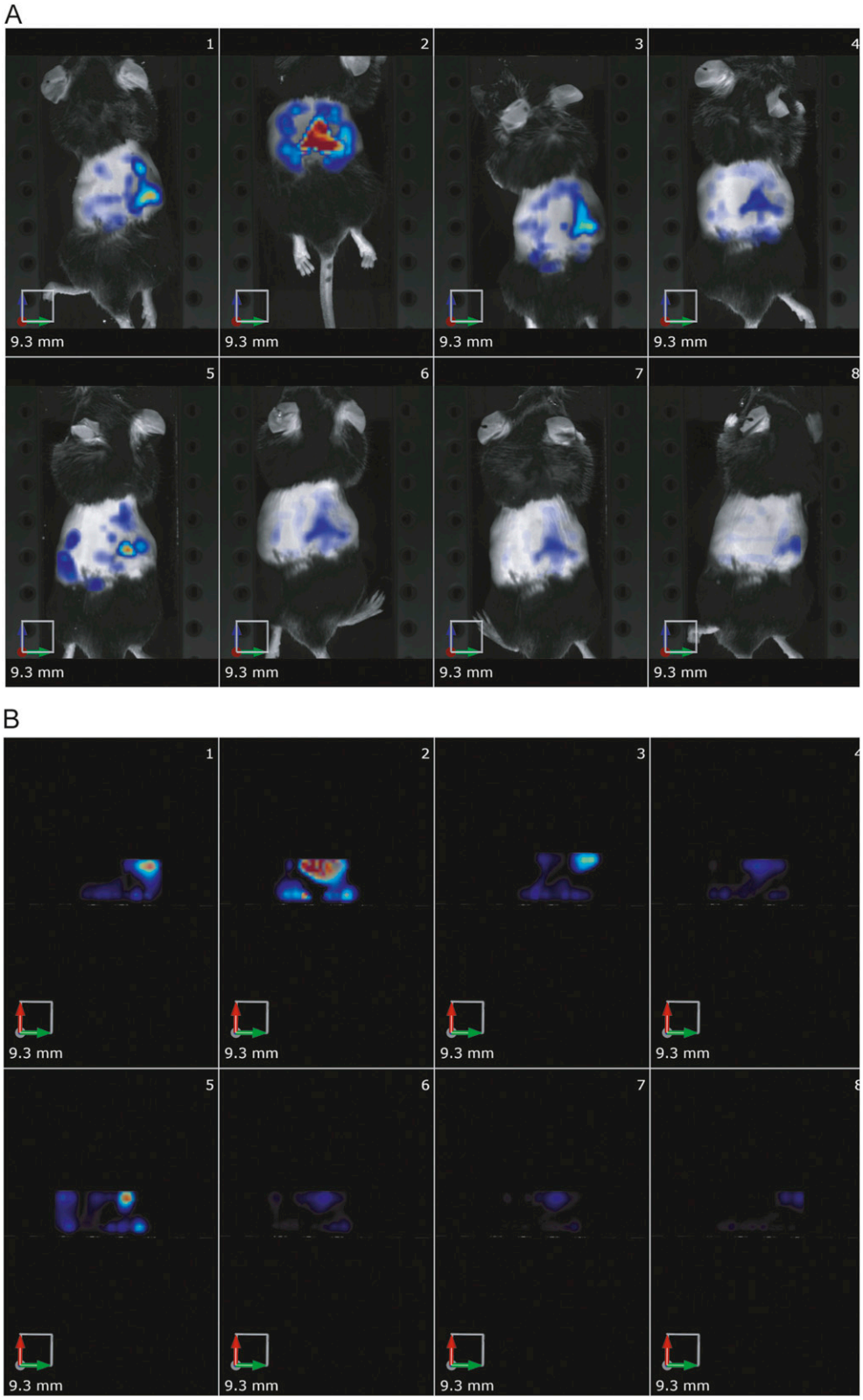

Fig. S9. In vivo near infrared fluorescence tomography of $([G8E22]GLP)_{x6}$-$ELP_{Low}240$. NIR tomography images (*A*) and cross-sections (*B*) after a single s.c. injection of $([G8E22]GLP)_{x6}$-$ELP_{Low}240$ at 0, 5, 24, 48, 72, 96, 120, and 144 h.

Table S1. GLP-1 sequences used for GLP-1 polymerization and ELP fusion sequences

Name	Sequence	Polymer properties	Reversible
$[G_8E_{22}]$GLP	GAH**G**EGTFTSDVSSYLE**E**QAAKEFIAWLVKGR	Helix stabilized, DPPIV resistant, cleavable by arginine proteases	Yes
$[G_8E_{22}A_{36}]$GLP	GAH**G**EGTFTSDVSSYLE**E**QAAKEFIAWLVKG**A**	Helix stabilized, DPPIV resistant, not cleavable by arginine proteases	No
$[V_8]$GLP	GAH**V**EGTFTSDVSSYLEGQAAKEFIAWLVKGR	DPPIV resistant, cleavable by arginine proteases, KGR|GA cleavage sequence (1)	No
$[V_8]$GLP/2	H**V**EGTFTSDVSSYLEGQAAKEFIAWLVVPR	DPPIV resistant, cleavable by arginine proteases, VPR|HV cleavage sequence (1)	No
$[V_8]$GLP/3	GAH**V**EGTFTSDVSSYLEGQAAKEFIAWLVVPR	DPPIV resistant, cleavable by arginine proteases, VPR|GA cleavage sequence (1)	No
$ELP_{LOW}60$	$(GVGVP)_{60}GWP$	$T_t < 37$ °C	
$ELP_{LOW}240$	$(GVGVP)_{240}GWP$	$T_t < 37$ °C	
$ELP_{HIGH}60$	$(GAGVPGGGVP)_{30}GY$	$T_t > 37$ °C	
Leader	MTMDPNYPR		
Linker	GSVENLYFQGAKLA		

Boldface letters indicate mutated residues (compared with native GLP-1). Underlined letters indicate DPPIV cleavable linkers.

1. Amiram M, Quiroz FG, Callahan DJ, Chilkoti A (2011) A highly parallel method for synthesizing DNA repeats enables the discovery of 'smart' protein polymers. *Nat Mater* 10(2):141–148.