Injectable non-immunogenic PEG-like conjugate that forms a subcutaneous depot and enables sustained delivery of a peptide drug

Imran Ozer

Duke University

Anna Slezak

Duke University

Jeffrey Everitt

Duke University <https://orcid.org/0000-0003-0273-6284>

Xinghai Li

Duke University

Nikita Zakharov

Duke University

Joel Collier

Duke University <https://orcid.org/0000-0003-3536-4827>

Jonathan Campbell

<https://orcid.org/0000-0003-4358-6331>

David D'Alessio

Duke Molecular Physiology Institute <https://orcid.org/0000-0003-4155-4870>

Ashutosh Chilkoti (✉ chilkoti@duke.edu)

Duke University <https://orcid.org/0000-0003-1301-1836>

Article

Keywords: polyethylene glycol (PEG), biologics, drug delivery

Posted Date: July 28th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-737658/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Injectable non-immunogenic PEG-like conjugate that forms a subcutaneous depot and enables sustained delivery of a peptide drug

Imran Ozer¹, Anna Slezak^{2†}, Jeffrey I. Everitt³, Xinghai Li¹, Nikita Zakharov¹, Joel H. Collier¹, Jonathan E. Campbell^{4,5,6}, David A. D'Alessio^{4,5}, and Ashutosh Chilkoti^{1*}

¹Department of Biomedical Engineering, Duke University, Durham, North Carolina 27708, USA.

²Department of Chemistry, Duke University, Durham, North Carolina 27708, USA.

³Department of Pathology, Duke University Medical Center, Durham, North Carolina 27701, USA.

⁴Duke Molecular Physiology Institute, Duke University, Durham, North Carolina 27701, USA

⁵Division of Endocrinology, Duke University Medical Center, Durham, North Carolina, 27701, USA.

⁶Department of Pharmacology and Cancer Biology, Duke University, Durham, NC, USA.

[†]Present Address: Pritzker School of Molecular Engineering, University of Chicago, Chicago, Illinois, 60637, USA.

*e-mail: chilkoti@duke.edu

Abstract

Many biologics have a short plasma half-life, and their conjugation to polyethylene glycol (PEG) is commonly used to solve this problem. Unfortunately, PEG is immunogenic and forms vacuoles, and improvement in PEGylated drugs' half-life is at an asymptote. Here, we developed a PEG-like, non-immunogenic, and injectable conjugate technology for sustained delivery of biologics. An optimal poly[oligo(ethylene glycol) methyl ether methacrylate] (POEGMA) depot of exendin, a peptide drug used in the clinic in treating type 2 diabetes, outperformed PEG, non-depot-forming POEGMA, and a clinical sustained-release exendin-4 formulation in efficacy and pharmacokinetics. Notably, POEGMA was non-immunoreactive, while PEG induced a persistent anti-PEG immune response, leading to its subsequent doses' early clearance and loss of efficacy. POEGMA did not induce vacuolization. Solving these

22 problems of PEG and improving upon its half-life benefits by creating injectable POEGMA conjugates that form a
23 drug depot under the skin and provide sustained efficacy breathe new life into an established and valuable drug
24 delivery technology that is facing an impasse.

25

26 Introduction

27 Biologics are potent, highly specific, and well-tolerated and have become an important class of drugs.¹
28 Despite their promise, most of them have a short half-life due to rapid renal elimination and *in vivo* degradation.²
29 Their short *in vivo* half-life hence necessitates frequent injections, resulting in a peak-and-valley profile in drug
30 concentration that is pharmacologically suboptimal. It also incurs a high treatment cost and suboptimal patient
31 compliance because of the need for frequent drug administration and the side effects associate with a peak-and-
32 valley drug concentration profile.^{3,4} One of the most common approaches to overcome these challenges is the
33 covalent attachment of biologics to polyethylene glycol (PEG), a technology that is colloquially termed
34 PEGylation.⁵ PEGylated drug conjugates have a much longer plasma half-life than the native drug due to their
35 larger size leading to slower renal clearance, reduced opsonization that reduces clearance by the
36 reticuloendothelial system (RES), and improved solubility.⁶ Unfortunately, PEGylation has several significant
37 limitations, some of which have come to the forefront recently. First, PEG was initially believed to be non-
38 immunogenic. However, it is now well accepted that PEGylated therapeutics induce PEG antibodies upon
39 treatment.^{7,8} Pre-existing PEG antibodies have also been reported in up to 70% of the population who are naïve
40 to PEGylated therapeutics, possibly due to chronic exposure to PEG in consumer products and because PEG is
41 used as an excipient in many drug formulations.⁹⁻¹¹ Both induced and pre-existing PEG antibodies have caused a
42 severe allergic reaction and accelerated clearance in some patients, reducing the drugs' clinical efficacy.^{7,8,12,13}
43 These issues have collectively led to the early termination of several clinical trials of PEGylated drug candidates
44 and the withdrawal of several PEGylated therapeutics from the market.^{12,14,15} Second, attempts to improve the
45 pharmacokinetics (PK) of PEG have focused on synthesizing branched and star-shaped PEGs. However, these
46 architectures have a modest effect on PK¹⁶ and have antigenic and immunogenic profiles similar to linear PEG.¹²
47 Third, PEG forms vacuoles in major organs due to its non-biodegradable structure and clearance by the RES.¹⁷
48 Clearly, optimization of PEG is now at an asymptote, and new architectures that radically depart from linear PEG
49 are needed to address these limitations.

50 Motivated by these needs, we have developed a “next-gen” PEG-like polymer, poly[oligo(ethylene glycol)
51 methyl ether methacrylate] (POEGMA). POEGMA is an amphiphilic, hyperbranched polymer that breaks up the
52 long ethylene glycol sequences in PEG and presents them as much shorter oligomeric ethylene glycol (OEG) side-
53 chains along a hydrophobic backbone. It has been previously shown that POEGMA with three EG unit long side
54 chains improves the PK of exendin-4, a peptide drug used in the clinic to treat type 2 diabetes (T2D) and
55 subsequently referred to as exendin.¹⁸ The POEGMA conjugate of exendin (Ex-POEGMA) also showed no *in vitro*
56 binding to human-derived PEG antibodies, presumably because the short OEG side-chains in POEGMA lack the
57 anti-PEG epitope.^{18,19} Although these results were encouraging, the soluble Ex-POEGMA conjugate reported
58 previously only showed a modest improvement in the PK of the drug. This study also did not compare the PK and
59 efficacy of POEGMA conjugates with PEG conjugates, nor did it report the intrinsic immunogenicity of POEGMA
60 that could occur upon repeated administration of POEGMA-drug conjugates, which are critical for its clinical
61 translation.

62 This paper has two goals. The first goal was to devise a strategy to further enhance the half-life of exendin
63 beyond the four days of therapeutic action of a soluble POEGMA conjugate in mice¹⁸ and do so without
64 compromising its lack of reactivity to pre-existing PEG antibodies. Improving the PK of POEGMA beyond the
65 duration exhibited by soluble POEGMA conjugates is important because recent long-acting delivery technologies
66 such as Fc fusions have been shown to work for 4-6 days in mice^{20,21} and 7 days in humans²¹ so that a next-
67 generation PEGylation technology must match—at a minimum— the performance of Fc conjugates. To address
68 this goal, we report on the design and optimization of a gel-like injectable depot of an Ex-POEGMA conjugate that
69 achieves sustained release from the depot into the bloodstream while retaining its lack of reactivity towards PEG
70 antibodies. We identify an optimal Ex-POEGMA depot that maximizes blood glucose control in hyperglycemic mice
71 and investigate its PK and efficacy benefits. The second goal was to investigate the intrinsic immunogenicity and
72 toxicity of a POEGMA-drug conjugate and compare it to PEG. We report the remarkable result that Ex-POEGMA
73 conjugates have very little if any humoral immunogenicity in mice, as seen by the lack of an immunoglobulin (Ig)

M and IgG response to repeated doses of Ex-POEGMA. Furthermore, this lack of immunogenicity is highly robust, as confirmed by the lack of IgG and IgM response against POEGMA observed with a POEGMA conjugate of the highly immunogenic ovalbumin (OVA) protein, even with co-administered with an adjuvant. Finally, the optimized POEGMA conjugate did not cause the cellular vacuolization reported with conventional PEG conjugates.

Results

Injectable and depot forming POEGMA library. POEGMA exhibits lower critical solution temperature (LCST) phase behavior, allowing it to transition between an aqueous soluble and insoluble coacervate phase in a temperature- and concentration-dependent manner.²²⁻²⁴ We exploited this feature to design a drug depot under the skin and achieve sustained drug release from the POEGMA depot into circulation. To do so, we first identified POEGMAs that would be suitable as an injectable depot by creating a set of POEGMAs with azide-end groups (**Supplementary Fig. 1-2**) that phase transition between room temperature (25°C) and subcutaneous (s.c.) temperature of mice (34°C)²⁵ using activator-regenerated by electron transfer atom transfer radical polymerization (ARGET-ATRP).²⁶ We restricted the EG side-chain length to ≤ 3 (**Supplementary Fig. 3-6**) because previous studies showed that POEGMA conjugates with a side-chain length of >3 had a low, though measurable level of binding with PEG antibodies.^{18,19} We also fortuitously found that the phase transition temperature (T_t) of POEGMA was sensitive to the OEG side chain length, with shorter side-chains having a lower T_t due to the decreased hydrogen bonding. The T_t of a homopolymer POEGMA with 3EG-long side chains (EG3) was too high to form an s.c. depot, while the T_t of an EG2 homopolymer was too low, preventing it from being injected in a soluble form at room temperature.

These observations led us to hypothesize that a random copolymer of POEGMA with EG3 and EG2 units should have a T_t that is between room temperature and body temperature across a range of concentrations relevant for drug delivery, which would be ideal for the design of a conjugate that is injectable, but phase transitions into an insoluble coacervate upon s.c. injection, thereby forming a drug depot. To identify a copolymer with an optimal T_t for an injectable s.c. depot, we synthesized a set of copolymers using EG2 and EG3 monomers at various ratios in which the molar percentage of EG2 ranged from 58-100%, as determined by Nuclear Magnetic

98 Resonance (NMR) spectroscopy (**Supplementary Fig. 7-8**).²⁷ We also synthesized an EG3 POEGMA homopolymer
99 as a non-depot forming —soluble— control. The monomer composition of POEGMA was defined as the
100 percentage of EG2 (or EG3) monomer content in the total polymer. We used the nomenclature of EGX%, where X
101 is the length of the EG chain (2 or 3), and the subscript % is the percentage of the monomer in the total polymer.
102 All synthesized polymers were relatively monodisperse, as measured by gel permeation chromatography-multi-
103 angle light scattering (GPC-MALS), with a polydispersity ($\mathcal{D}$) of <1.2 (**Fig. 1a**; **Supplementary Fig. 9**). Their degree
104 of polymerization (DP) ranged from 100 to 400, translating to a weight-averaged molecular weight (M_w) range of
105 20-80 kDa. Each polymer's monomer composition, M_w , number-averaged molecular weight (M_n), $\mathcal{D}$, DP,
106 hydrodynamic size (R_h), and T_t are shown in **Supplementary Fig. 7-10** and summarized in **Supplementary Table 1**.

107 All polymers showed sharp and thermally reversible phase behavior, as seen by the sharp increase in
108 optical density as the temperature was increased and by the fact that their optical density decreased back to
109 baseline as the temperature was decreased below the T_t (**Fig. 1b**). Their phase behavior also showed no thermal
110 hysteresis, as seen by the overlapping turbidity curves for the heating and cooling cycle. The EG3_{100%} POEGMA
111 had a T_t of ~48°C at 25 μ M, consistent with a previous report,²⁸ confirming that it cannot form an s.c. depot. The
112 T_t of the POEGMA copolymers was a function of the EG2 content, as the T_t decreased with the increasing molar
113 ratio of the more hydrophobic EG2 monomer. All copolymers phase transitioned between 25 and 34°C, suggesting
114 that all copolymers were potential depot-forming constructs. The T_t of POEGMA also showed an inverse
115 concentration dependence. The T_t increased with POEGMA dilution (**Fig. 1c**), suggesting that sustained release of
116 a POEGMA conjugate from the s.c. depot should be possible *in vivo*, in response to continuous dilution at the
117 periphery of the depot. The T_t of POEGMA was also a function of its M_w , as the T_t decreased with increasing DP—
118 and M_w (**Fig. 1d**). These data clearly showed that the key molecular variables that control the phase behavior of
119 POEGMA copolymers—namely its T_t — are the composition —the ratio of EG2 to EG3 units— and M_w .

Figure 1. Well-defined POEGMAs of varying M_w and monomer composition phase-transition near body temperature in a concentration- and M_w -dependent manner and remain soluble at room temperature. (A) GPC chromatogram of EG2_{66%} copolymers at varied M_w . (B) The optical density of POEGMAs with constant DP but varying monomer composition was monitored as temperature increased (solid line) and decreased (dashed line) to demonstrate the reversibility of phase behavior. Data were shown for POEGMAs at DP 200 and 25μM. (C) At varying concentrations, the optical density of POEGMAs demonstrates concentration dependence of T_t . Data were shown for POEGMAs at DP 300 in phosphate-buffered saline (PBS). (D) The optical density of POEGMAs with varying DP and monomer composition was monitored to demonstrate T_t 's DP dependence. Data were shown for POEGMAs at 25μM in PBS.

Design and purification of a site-specific 1:1 conjugate. Conventional conjugation methods typically provide limited control over the conjugation site and stoichiometry, resulting in a heterogeneous mixture of conjugates with non-uniform PK and pharmacodynamics (PD).²⁹ To circumvent this issue, we site-specifically conjugated POEGMA to exendin at its C-terminus using bio-orthogonal click chemistry^{18,29-31} We chose the C-terminus as the

132 POEGMA conjugation site, as the N-terminus of exendin is critical to its function.¹⁸ To do so, we first covalently
133 conjugated a bio-orthogonal triglycine dibenzocyclooctyne (DBCO) group to the C-terminus of exendin via sortase
134 A-mediated native peptide ligation,^{18,30} yielding exendin-DBCO. To ensure that the conjugation is both
135 bioorthogonal—to avoid side-reactions of POEGMA with any internal residue in exendin—and site-specific, we
136 chose to synthesize azide-terminated POEGMA copolymers. The azide moiety readily reacts with the DBCO group
137 and does not react with any other chemical groups in exendin, yielding a site-specific conjugate with a 1:1
138 stoichiometry.³⁰ (**Supplementary Fig. 11**).

139 Purification of POEGMA conjugates from unreacted agents using chromatography techniques is labor-
140 intensive and has a low yield. To circumvent these problems, we exploited the LCST behavior of depot-forming
141 POEGMA conjugates. The conjugates were purified in a chromatography-free manner at room temperature by
142 simply triggering the phase transition of the conjugate by adding ammonium sulfate salt that lowers the T_t below
143 room temperature,³² leading to phase separation of Ex-POEGMA conjugates into an insoluble coacervate.
144 Unreacted exendin remained in the supernatant. The Ex-POEGMA coacervates were isolated by centrifugation
145 and were then resolubilized by adding PBS at room temperature. Importantly, this purification technique does not
146 apply to PEG as it does not show an LCST phase behavior.

147 **Identifying an optimal Ex-POEGMA conjugate.** We identified an optimal Ex-POEGMA conjugate that maximized
148 fed blood glucose control in hyperglycemic mice by first optimizing the T_t , then the M_w , and finally the dose of the
149 conjugate. POEGMA copolymers were synthesized for the T_t optimization study are shown in **Table 1** under the
150 heading —Index: T_t and the copolymers with varied M_w that were synthesized for the M_w optimization study are
151 listed under the heading —Index: M_w .

152 **Table 1. Characterization of exendin variants.** EG2% was calculated using NMR spectroscopy. M_n , M_w , and $\mathcal{D}$ values were determined by
153 size exclusion chromatography multi-angle light scattering (SEC-MALS). R_h was measured by dynamic light scattering (DLS) ($n=10$). DP was
154 calculated by dividing polymer M_w by the monomer M_w . The EC_{50} of exendin variants was determined from the dose-response curves in
155 Fig. 2c, Fig. 2i, and Fig. 3c ($n=10$). Data are reported as mean $\pm$ standard error of the mean (SEM). T_t was measured at 500 μ M. *Calculated

156 from the amino acid sequence. †Default value due to the monodisperse nature of the peptide. The indices in column 1 of the table — T_t ,
 157 M_w , and polymer type are explained in the text.

Compound	EG2 %	DP	T_t (°C)	M_w (kDa)	$\mathcal{D} (M_w/M_n)$	R_h (nm)	EC_{50} (nM)
Exendin	N/A	N/A	N/A	4.2*	1.00†	2.2 ± 0.1	0.04 ± 0.01
Index: T_t							
Ex-POEGMA _{31.9°C}	48.5	247	31.9	57.7	1.15	4.8 ± 0.7	4.7 ± 0.8
Ex-POEGMA _{29.9°C}	60.5	253	29.9	57.6	1.15	4.4 ± 0.9	3.0 ± 0.7
Ex-POEGMA _{28.4°C}	68.8	254	28.4	56.9	1.13	4.5 ± 0.7	4.5 ± 1.1
Index: M_w							
Ex-POEGMA _{18.9kDa}	100	71	32.3	18.9	1.03	2.6 ± 0.9	1.2 ± 0.4
Ex-POEGMA _{54.3kDa}	62.5	239	29.6	54.3	1.14	4.2 ± 0.5	2.8 ± 0.7
Ex-POEGMA _{99.4kDa}	56.8	454	29.0	99.4	1.13	4.5 ± 1.1	1.4 ± 0.3
Ex-POEGMA _{171.4kDa}	50.8	792	29.4	171.4	1.16	5.1 ± 1.0	1.8 ± 0.2
Index: Polymer type							
Ex-POEGMA _{opt}	62.3	241	29.9	54.7	1.09	4.1 ± 0.5	2.8 ± 0.7
Ex-POEGMA _{sol}	N/A	221	40.1	56.6	1.09	4.3 ± 0.3	3.6 ± 0.9
Ex-PEG _{Rh}	N/A	454	N/A	27.7	1.07	4.4 ± 1.1	1.3 ± 0.2
Ex-PEG _{Mw}	N/A	909	N/A	46.3	1.10	5.2 ± 1.0	2.7 ± 0.5

158
 159 In the first phase of optimization, to identify an Ex-POEGMA conjugate with an optimal T_t , we first
 160 conjugated exendin to POEGMAs that have a varied monomer composition and hence T_t , but all of which had a
 161 near-constant M_w of ~57 kDa (**Table 1; index T_t**). We chose an M_w of ~57 kDa, as it was previously shown to be
 162 the M_w that maximized the PK of a soluble Ex-POEGMA conjugate (**Table 1; Supplementary Fig. 12**).¹⁸ All
 163 conjugates had a low polydispersity and had a similar R_h (**Table 1; Supplementary Fig. 13**). In addition, they showed
 164 reversible phase behavior (**Fig. 2a**), and the T_t of all conjugates were between 28-32°C, allowing the conjugates to
 165 remain in solution at room temperature and transition into an insoluble coacervate when injected s.c. at an
 166 injection concentration of 500 μ M (see circled data in **Fig. 2b**). The T_t of the conjugates also showed an inverse
 167 concentration-dependence (**Fig. 2b**), indicating that they should release from the depot into the blood in response
 168 to dilution at the depot's margins. The conjugates with a greater EG2 content had a lower T_t , as the EG2 unit is
 169 more hydrophobic than the EG3 unit. (**Table 1; Supplementary Fig. 14**). Finally, the conjugates were tested for
 170 their ability to activate exendin's endogenous receptor, termed glucagon-like peptide 1 receptor (GLP1R), in an *in*
 171 *vitro* cell-based assay using unconjugated exendin and PBS as controls. The conjugates showed high potency in

activating GLP1R (**Fig. 2c**) but had a lower half-maximal effective concentration (EC_{50}) than exendin due to the steric hindrance imparted by the conjugated POEGMA, consistent with the literature.^{18,25,30} No significant difference was observed in the EC_{50} of depot-forming conjugates.

Figure 2. Identifying an optimal Ex-POEGMA conjugate that maximizes fed blood glucose control in hyperglycemic mice. (A) The optical density of Ex-POEGMA conjugates with similar M_w but varied T_t as temperature increased (solid line) and decreased (dashed line), demonstrating the reversibility of phase behavior at 25μM and **(B)** at varying concentrations to demonstrate inverse concentration-dependence of T_t . Circled data shows T_t at the injection concentration of 500μM. **(C)** *In vitro* activity of the conjugates ($n=6$). **(D)** Blood glucose normalized to $t=0$, **(E)** AUC of blood glucose, and **(F)** percent weight change relative to weight at $t=0$ after treating DIO C57BL/6J mice ($n=6$) with a single s.c. injection of the treatments. **(G)** The optical density of Ex-POEGMA conjugates with optimal T_t but varied M_w as

182 temperature increased, demonstrating phase behavior at 25 μ M and **(H)** at varying concentrations to demonstrate inverse concentration-
183 dependence of T_t . **(I)** *In vitro* activity of the conjugates ($n=6$). **(J)** Blood glucose normalized to $t=0$, **(K)** AUC of blood glucose, and **(L)** percent
184 weight change relative to weight at $t=0$ after treating DIO C57BL/6J mice ($n=6$) with a single s.c. injection of the treatments. Data were
185 analyzed by two-way repeated-measures ANOVA followed by *post hoc* Dunnett's multiple comparison test. *The last time point that blood
186 glucose for treatment is significantly lower than that of PBS treated mice. The AUC for glucose exposure was analyzed using two-way
187 ANOVA, followed by *post-hoc* Tukey's multiple comparison test. #The conjugate with the lowest AUC for glucose exposure. Data showed
188 the mean $\pm$ SEM and were considered statistically significant when $p<0.05$.

189 We next examined the fed blood glucose control provided by the Ex-POEGMA conjugates by administering
190 them s.c. into 11-week-old male diet-induced obese (DIO)³³ C57BL/6J mice. The equivalent dose of exendin and
191 equivalent volume of PBS were used as positive and negative controls, respectively. Mice treated with exendin
192 and the conjugates had lower blood glucose levels (**Fig. 2d; Supplementary Fig. 15**) and body weight (**Fig. 2f**) than
193 the PBS-treated group. Exendin provided a shorter temporal duration of control than the conjugates, likely due to
194 its short half-life.³⁴ The most hydrophobic conjugate tested—Ex-POEGMA_{28.4}—provided only modest control,
195 possibly because it released only a small amount of drug from the depot. The Ex-POEGMA_{29.9} conjugate
196 outperformed others by providing six days of glucose control and had the lowest area under the curve (AUC) for
197 glucose exposure (**Fig. 2e**), which accounts for both magnitude and duration of blood glucose reduction. This result
198 was consistent with the literature, where the optimal T_t of an injectable elastin-like polypeptide (ELP) drug depot
199 was also reported to be $\sim 30^\circ\text{C}$.²⁵

200 Having identified an Ex-POEGMA conjugate with an optimal T_t , we next identified an optimal M_w . Our
201 strategy was to synthesize Ex-POEGMA conjugates with the optimal T_t of $\sim 30^\circ\text{C}$ but with varying M_w (**Table 1; Index**
202 **M_w**)—18.9 kDa, 54.3 kDa, 99.3 kDa, and 171.4 kDa (**Supplementary Fig. 16**). As the T_t is a function of the M_w , we
203 carried out an iterative set of polymer syntheses to create POEGMA copolymers that have the similar T_t —close
204 to $\sim 30^\circ\text{C}$ across a range of concentrations— but varied M_w by titrating the ratio of EG2 to EG3 in the monomer
205 feed of the synthesis, leading to the set of polymers shown in **Table 1** and listed under Index: M_w . The polymers
206 with a lower M_w had a greater mole fraction of the more hydrophobic EG2 monomer in the POEGMA copolymers

207 and vice versa (**Supplementary Fig. 17**). These polymers were then conjugated to exendin. The resulting Ex-
208 POEGMA conjugates had varied R_h (**Supplementary Fig. 18**) consistent with their different M_w , but all showed
209 thermally reversible phase behavior (**Fig. 2g**). However, the T_t of the conjugates had a slightly different inverse
210 concentration dependence, as seen by the different slopes of the $\log(\text{concentration})$ versus T_t plots (**Fig. 2h**).
211 Importantly, these plots intersected at 500 μM and $\sim 30^\circ\text{C}$ (see circled data in **Fig. 2h**), a temperature that
212 fortuitously corresponds to the previously identified optimal T_t . This concentration of 500 μM was hence chosen
213 as the injection concentration for all *in vivo* studies to identify the optimal M_w with constant T_t of $\sim 30^\circ\text{C}$. No
214 significant correlation was observed between *in vitro* activity of the conjugates as seen by their EC_{50} and the M_w
215 of conjugates (**Fig. 2i**).

216 The conjugates were next administrated into 11-week-old male DIO mice ($n=6$), with PBS as a negative
217 control. Mice treated with the conjugates had lower blood glucose (**Fig. 2j**; **Supplementary Fig. 19**) and body
218 weight (**Fig. 2l**) than the PBS-treated group. The conjugate with the lowest M_w tested—Ex-POEGMA_{18.9}—
219 controlled blood glucose only one day and had a significantly higher glucose exposure as seen by the AUC (**Fig. 2k**)
220 than all other treatment groups. This data suggests that Ex-POEGMA_{18.9kDa} was cleared from the circulation much
221 faster than the other conjugates because of its significantly smaller size ($R_h \sim 2.6$ vs. $\sim 4\text{--}5$ nm for the other
222 conjugates) that is lower than the renal excretion threshold, defined as the size of serum albumin (~ 3.6 nm).^{18,25}
223 This data is consistent with the literature, where PEG and ELP conjugates show a prolonged half-life at an M_w
224 threshold of 30 kDa^{35,36} and 35.8 kDa²⁵, respectively. In addition, the higher M_w Ex-POEGMA conjugates differed
225 in their duration of glucose control. Ex-POEGMA_{99.4} and Ex-POEGMA_{171.4} showed modest glucose control for three
226 days, while Ex-POEGMA_{54.3} outperformed the rest with six days of glucose control and had the lowest AUC for
227 glucose exposure. This conjugate —Ex-POEGMA_{54.3}— was defined as the T_t - and M_w - optimized conjugate and is
228 subsequently referred to as Ex-POEGMA_{opt} in this paper. Finally, the optimal injection dose of this conjugate was
229 determined to be 1000 nmol kg^{-1} in a dose-escalation study, which completed the optimization process of the
230 conjugate (**Supplementary Fig. 20**).

231 **Short-term efficacy.** We next investigated the short-term efficacy of Ex-POEGMA_{opt} compared to its soluble
232 POEGMA and PEG counterparts. We synthesized the soluble counterpart of Ex-POEGMA_{opt}—termed Ex-
233 POEGMA_{sol}—using POEGMA consisting of only EG3 monomers at the near-identical M_w of ~57 kDa
234 (**Supplementary Fig. 21**). In preliminary studies, we found that linear PEG has a much larger hydrodynamic size
235 than hyperbranched POEGMA of the same M_w , resulting in conjugates with a much larger R_h at the same M_w .
236 Because this difference could complicate side-by-side efficacy comparison of the conjugates by affecting their
237 kidney clearance rates, we synthesized both M_w -matched and R_h -matched exendin-PEG conjugates—termed Ex-
238 PEG_{Mw} and Ex-PEG_{Rh} (**Table 1; Supplementary Fig. 22-23**). Ex-POEGMA_{opt} reversibly phase transitioned below body
239 temperature (**Fig. 3a**), allowing it to remain as a solution in a syringe at room temperature but to transition into
240 an insoluble coacervate phase when injected s.c., as tested at a concentration of 500 μ M (see circled data in **Fig.**
241 **3b**). In contrast, Ex-POEGMA_{sol} phase-transitioned well above body temperature at all concentrations, indicating
242 that it cannot form a depot and would remain soluble when injected into the s.c. space. Neither Ex-PEG_{Mw} nor Ex-
243 PEG_{Rh} showed phase behavior (**Fig. 3a**). The conjugates showed no difference in their EC₅₀ in activating the GLP1R
244 in an *in vitro*, cell-based assay (**Fig. 3c**).

245 The conjugates were next administered s.c. into 11-week-old male DIO mice ($n=6$) at the equivalent,
246 optimal dose, with PBS included as a control. Mice treated with the conjugates had lower fed blood glucose levels
247 than the control (**Fig. 3d; Supplementary Fig. 24**). Ex-PEG_{Rh} and Ex-PEG_{Mw} controlled fed blood glucose for three
248 and four days, respectively (**Fig. 3d**), consistent with the literature.³⁷ The one-day longer glucose control provided
249 by Ex-PEG_{Mw} could be attributed to its larger size, and hence slower clearance. In accord with the literature, Ex-
250 POEGMA_{sol} also provided four days of blood glucose control.¹⁸ In contrast, Ex-POEGMA_{opt} outperformed its soluble
251 POEGMA counterpart and the two PEGylated exendin controls by providing six days of fed blood glucose control
252 and had the lowest glucose exposure of all conjugates (**Fig. 3e**), showing the enhanced efficacy provided by
253 sustained release of the conjugate from an injectable s.c. depot. All soluble conjugates resulted in a much sharper
254 and more significant weight loss than Ex-POEGMA_{opt} (**Fig. 3f**), possibly because a sudden increase in drug

255 concentration of soluble conjugates can induce nausea and trigger transient weight loss, which is consistent with
256 common side effects of exendin.³⁸

257 Motivated by these results, we next tested the short-term efficacy of Ex-POEGMA_{opt} in a second mouse
258 model—the *db/db* line—that displays more severe hyperglycemia than DIO mice. *db/db* mice carry mutant leptin
259 receptor gene and have a progressive course of declining insulin secretion and hyperglycemia, similar to many
260 humans with T2D.³³ In this study, we also compared Ex-POEGMA_{opt} with Bydureon, a once-weekly FDA-approved
261 and clinically used sustained-release formulation of exendin encapsulated in poly-lactic-co-glycolic acid (PLGA)
262 microspheres.³⁹ All treatment groups (*n*=5) resulted in lower glucose levels than the PBS control (**Fig. 3g**;
263 **Supplementary Fig. 25**). Ex-POEGMA_{opt}, however, stood out from the competition by lowering fed blood glucose
264 for six days. A clear trend in glucose exposure (**Fig. 3h**) was also observed, with Ex-POEGMA_{opt} having the lowest
265 AUC. Soluble Ex-PEG_{Rh} induced a more significant weight loss than sustained-release formulations Ex-POEGMA_{opt}
266 and Bydureon (**Fig. 3i**), consistent with previous results.

267 To confirm these results, we next tested if Ex-POEGMA_{opt} also provided glycemic regulation by performing
268 an intraperitoneal (*i.p.*) glucose tolerance test (IPGTT) in *db/db* mice on days 1, 3, and 5 post-injection of the
269 treatments. Exendin-treated mice displayed similar glucose intolerance compared to the PBS-treated mice,
270 indicated by hyperglycemia, presumably because it cleared from circulation before IPGTTs were performed due
271 to its short half-life (**Fig. 3j-l**).^{34,40} There was a clear trend in glucose exposure on days 1 and 3 (**Supplementary**
272 **Fig. 26**), with Ex-POEGMA_{opt} regulating blood glucose more efficiently than Bydureon. Ex-PEG_{Rh} had a progressive
273 loss of glycemic lowering by Day 3 due to its faster clearance. Notably, Ex-POEGMA_{opt} showed superior glycemic
274 regulation to Ex-PEG_{Rh} throughout the study by providing the lowest glucose exposure among the treatments. This
275 difference was also observed when the experiment was repeated using DIO mice (**Supplementary Fig. 27**).
276 However, the magnitude of the difference was not as great, possibly due to the milder display of T2D allowing
277 lower circulating Ex-PEG_{Rh} concentrations to show therapeutic effect. The test time did not affect glucose

278 exposure in the Ex-POEGMA_{opt} treatment group ($p>0.99$) (**Fig. 3I**), indicating that Ex-POEGMA_{opt} maintained its
 279 superior glycemic regulation ability throughout the study.

280
 281 **Figure 3. Ex-POEGMA_{opt} outperforms its soluble POEGMA and PEG counterparts and a clinical sustained-release exendin formulation,**
 282 **Bydureon, in fed blood glucose and glycemic control.** The optical density of treatments (A) as temperature increased and decreased,
 283 demonstrating reversible phase behavior for Ex-POEGMA_{opt} and Ex-POEGMA_{sol} at 25μM and (B) at varying concentrations to demonstrate
 284 inverse concentration-dependence of T_t . Circled data show T_t at the injection concentration of 500μM. (C) *In vitro* activity of the conjugates
 285 ($n=6$). (D) Blood glucose normalized to $t=0$, (E) AUC of blood glucose, (F) and percent weight change relative to weight at $t=0$ after treating

286 DIO mice ($n=6$) with a single s.c. injection of the treatments. **(G)** Blood glucose normalized to $t=0$, **(H)** AUC of blood glucose, **(F)** and percent
287 weight change relative to weight at $t=0$ after treating six-week-old *db/db* mice ($n=6$) with a single s.c. injection of the treatments. An IPGTT
288 was performed on **(J)** day 1 and **(K)** day 5 post-injection of treatments into *db/db* mice ($n=5$), blood glucose was monitored, and **(L)** AUC of
289 blood glucose was quantified. Data were analyzed by two-way repeated-measures ANOVA, followed by *post hoc* Dunnett's multiple
290 comparison test. *The last time point that blood glucose for treatment is significantly lower than that of PBS treated mice. The AUC for
291 glucose exposure was analyzed using two-way ANOVA, followed by *post-hoc* Tukey's multiple comparison test. #The conjugate with the
292 lowest AUC for glucose exposure. Data showed the mean $\pm$ SEM and were considered statistically significant when $p<0.05$.

293 **Pharmacokinetics.** We next investigated the PK of Ex-POEGMA_{opt} and the PEGylated controls to better understand
294 the differences in the short-term efficacy profiles of the treatments. We fluorescently labeled exendin, Ex-
295 POEGMA_{opt}, Ex-PEG_{Rh}, and Ex-PEG_{Mw} (**Supplementary Fig. 28**), followed by s.c. administration into naïve DIO mice
296 ($n=4$). The PK parameters were determined from the drug's plasma concentration (**Fig. 4a-b**) and are shown
297 in **Table 2**. All conjugates had much slower absorption kinetics than exendin, suggesting that the conjugates were
298 primarily taken up from the s.c. space into the blood through the lymphatic system due to their large size.⁴¹ Ex-
299 POEGMA_{opt} had the slowest absorption ($t_{1/2 \text{ absorption}}=9.0 \pm 1.8\text{h}$) among the treatments, possibly due to its
300 amphiphilic structure and sustained release. Exendin had a fast elimination half-life ($t_{1/2 \text{ elimination}}=2.1 \pm 0.5\text{h}$),
301 defined as the time needed to reach half-maximal blood concentration, consistent with its small size (**Fig. 4a**).⁴⁰
302 The conjugates had much longer elimination half-lives due to their slower renal clearance (**Fig. 4b**). Ex-PEG_{Rh} ($t_{1/2}$
303 $\text{elimination}=23.1 \pm 6.4\text{h}$) and Ex-PEG_{Mw} ($t_{1/2 \text{ elimination}}=34.9 \pm 9.1\text{h}$) prolonged exendin's circulation by ~11- and ~17-fold,
304 respectively. The 55.6 kDa soluble Ex-POEGMA conjugate with a matching M_w as the optimal depot-forming
305 POEGMA conjugate had a $t_{1/2 \text{ elimination}}$ of $61.2 \pm 5.0\text{h}$.¹⁸ In contrast, Ex-POEGMA_{opt} outperformed all conjugates by
306 prolonging exendin's circulation by ~46-fold with a $t_{1/2 \text{ elimination}}$ of $97.3 \pm 5.6\text{h}$, and it provided the highest drug
307 exposure (AUC) among the treatments. These results clearly demonstrate the PK benefits of sustained release
308 from a depot. This study also allowed us to infer that the minimal effective concentration for Ex-POEGMA_{opt} is 5.1
309 nM, which corresponds to the concentration of Ex-POEGMA_{opt} after 6 days post-injection.

Figure 4. Pharmacokinetics of Ex-POEGMA_{opt}. Fluorescently labeled (A) exendin and (B) Ex-PEG_{Mw}, Ex-PEG_{Rh}, and Ex-POEGMA_{opt} were s.c. administrated into naïve male DIO C57BL/6J mice ($n=4$) at 1000 nmol kg⁻¹, followed by blood collection at specified time points for 168 hours to calculate drug concentration. The treatments were administrated four more times to immunize the mice and induce ADAs. At the last injection, concentrations of fluorescently labeled (C) exendin, (D) Ex-PEG_{Rh}, and (E) Ex-POEGMA_{opt} were tracked (dotted lines). P values were shown for AUC comparison between naïve (solid line) and immunized mice for each treatment. Fluorophore concentration was 45 nmol kg⁻¹. Data represent the mean $\pm$ SEM.

Long-term efficacy. We next investigated the long-term efficacy of Ex-POEGMA_{opt} to understand better how the differences in PK and short-term efficacy translated into long-term management of T2D. We hypothesized that Ex-POEGMA_{opt} should outperform the other exendin formulations because of its superior fed blood glucose and glycemic control and longer PK. We tested this hypothesis by administering sterile and endotoxin-free Ex-POEGMA_{opt}, Ex-PEG_{Rh}, Bydureon, exendin, and PBS (- control) s.c. into naïve six-week-old male *db/db* mice ($n=5$) every week for eight weeks, followed by monitoring their blood glucose, changes in body weight, and glycated hemoglobin (HbA1c%) levels. We chose to monitor HbA1c%, as it is a measure of long-term T2D management because it is insensitive to daily blood glucose fluctuations and only changes as red blood cells (RBC) turn over every 40-60 days in rodents.²⁵ All treatments resulted in lower fed blood glucose levels (Fig. 5a; Supplementary

326 **Fig. 29)** and glucose exposure (**Fig. 5b**) than the PBS control. Exendin only had a modest and short-lived effect due
 327 to its poor circulation. Long-term treatment with Bydureon resulted in glycemic improvement (**Fig. 5a-b**). Ex-PEG_{Rh}
 328 controlled fed blood glucose at varying degrees, with later injections providing less glycemic reduction, and
 329 resulted in a HbA1c% level that was not significantly different from PBS-treated mice ($p>0.05$). In contrast to the
 330 other treatments, Ex-POEGMA_{opt} consistently lowered fed blood glucose control after each injection and provided
 331 the lowest glucose exposure among all treatments. This profile was reflected by a lower HbA1c% in Ex-POEGMA_{opt}-
 332 treated mice than the levels attained with the other treatments. The sustained release formulations Bydureon
 333 and Ex-POEGMA_{opt} resulted in a more moderate weight loss profile than Ex-PEG_{Rh} (**Supplementary Fig. 30**),
 334 consistent with the results after a single injection of the treatments.

335 **Table 2. Pharmacokinetic parameters of Ex-POEGMA_{opt}.** Pharmacokinetic parameters were calculated from the data given in Figure 4.
 336 Data were fitted to a one-phase exponential decay curve and analyzed via non-compartmental pharmacokinetic analysis using GraphPad
 337 Prism 8 software. Absorption phase pharmacokinetics of Ex-PEG_{Rh} in immunized mice were not calculated (N/C) due to its low goodness-
 338 of-fit values. Data showed the mean $\pm$ SEM.

	Treatment	$t_{1/2}$ absorption (h^{-1})	$t_{1/2}$ elimination (h^{-1})	t_{max} (h)*	C_{max} (nM)*	AUC (h x nM)
Naïve Mice	Exendin	0.6 $\pm$ 0.1	2.1 $\pm$ 0.5	1.6 $\pm$ 0.2	31.3 $\pm$ 15	188 $\pm$ 18
	Ex-PEG _{Rh}	6.6 $\pm$ 0.5	23.1 $\pm$ 6.4	16.6 $\pm$ 2.2	34.6 $\pm$ 2.4	1585 $\pm$ 112
	Ex-PEG _{Mw}	6.6 $\pm$ 1.7	34.9 $\pm$ 9.1	19.5 $\pm$ 4.9	35.3 $\pm$ 4.5	2206 $\pm$ 84
	Ex-POEGMA _{opt}	9.0 $\pm$ 1.8	97.3 $\pm$ 5.6	32.1 $\pm$ 5.8	35.8 $\pm$ 2.6	3664 $\pm$ 167
Immunized Mice	Exendin	0.8 $\pm$ 0.1	1.9 $\pm$ 0.5	1.7 $\pm$ 0.3	36.9 $\pm$ 6.8	159 $\pm$ 18
	Ex-PEG _{Rh}	N/C	11.4 $\pm$ 5.4	N/C	N/C	424 $\pm$ 35
	Ex-POEGMA _{opt}	12.1 $\pm$ 2.0	105.7 $\pm$ 12	42.6 $\pm$ 6.4	30.3 $\pm$ 4.0	3664 $\pm$ 167

339

Figure 5. Long-term efficacy of Ex-POEGMA_{opt}. (A) Blood glucose normalized to t=0, and (B) AUC of blood glucose level was monitored after treating *db/db* ($n=5$) with weekly *s.c.* injections of exendin, Ex-PEG_{Rh}, Ex-POEGMA_{opt}, Bydureon, or PBS for eight weeks. (C) Percent glycated hemoglobin (Hb1Ac%). Data represent the mean $\pm$ SEM. The AUC for glucose exposure and HbA1c% were analyzed using two-way ANOVA, followed by *post-hoc* Tukey's multiple comparison test. Data were considered statistically significant when $p<0.05$.

Immunogenicity. We next tested the immunogenicity of POEGMA and compared it to PEG. PEG crosslinks the B-cell receptors (BCR) because of its linear repetitive structure, resulting primarily in a T-cell independent B-cell immune response. This response is characterized by a persistent and predominantly IgM response.⁴² In humans, IgG responses are also observed in some individuals, though the mechanism of class switching is not well understood.⁴³ We hypothesized that POEGMA should not be immunogenic because of its hyperbranched structure and tested this hypothesis by assessing the induction of anti-drug antibodies (ADA) toward the full immunizing material (*i.e.*, Ex-POEGMA_{opt}). We administrated sterile and endotoxin-free Ex-POEGMA_{opt}, Ex-PEG_{Mw}, exendin, and PBS (- control) *s.c.* into naïve DIO mice ($n=10$) (see dosing and blood sampling regimen in **Fig. 6a**) and assessed ADAs in terms of their titer, specificity (*i.e.*, anti-conjugate, anti-exendin, anti-PEG, or anti-POEGMA), and subtype (*i.e.*, IgM or IgG) using a Luminex multiplexed immunoassay. Briefly, the Luminex immunoassay used drug-conjugated, fluorescently barcoded magnetic beads to capture ADAs. We coupled exendin, Ex-PEG, and Ex-POEGMA onto separate sets of beads to determine ADAs induced by the treatments. OVA versions of these beads—OVA, OVA-PEG, and OVA-POEGMA—were also coupled to separate sets of beads to use as a cross-reactivity control and to determine PEG- and POEGMA-specific ADAs, respectively. We eliminated the possibility of detecting anti-linker antibodies by synthesizing OVA-PEG and OVA-POEGMA conjugates using a different

360 conjugation chemistry than Ex-PEG_{MW} and Ex-POEGMA_{opt} (urethane linkage in OVA-based conjugates vs. strained
361 alkyne-azide linkage in exendin-based conjugates) (**Supplementary Figure 31; Supplementary Table 2**). Each drug-
362 conjugated bead set had a different fluorescent barcode, allowing us to measure the signal detected by each of
363 them when multiplexed. Mouse IgG- or IgM- coupled beads in diluent were used as positive controls, while the
364 multiplexed beads in diluent served as a negative control. The immunoassay's optimization and validation are
365 summarized in the **Supplementary Information**, and supplementary data are shown in **Supplementary Fig. 31-32**
366 and **Supplementary Tables 2-8**.

367 Ex-POEGMA_{opt}, Ex-PEG_{MW}, and exendin treatments induced a transient IgM response against exendin (**Fig.**
368 **6b-c**). No anti-exendin IgG response was detected (**Supplementary Fig. 33**). This data is consistent with the
369 literature⁴⁴, where only mild induction of anti-exendin antibodies was reported in clinical trials. In contrast, Ex-
370 PEG_{MW} induced a significant and persistent anti-PEG IgM response (**Fig. 6b-c**), consistent with the literature.^{7,8,42}
371 PEG specificity was indicated by the significant signal measured with both Ex-PEG and OVA-PEG beads. Anti-PEG
372 antibodies were strictly IgM, and no class switching to IgG was observed (**Supplementary Fig. 33**). PEG-specific
373 IgM response increased with the number of Ex-PEG_{MW} injections (**Supplementary Fig. 34**). Notably, the induced
374 PEG antibodies did not bind POEGMA conjugated bead sets, indicating that POEGMA eliminates PEG antigenicity
375 in agreement with the literature.^{18,19} Strikingly, Ex-POEGMA_{opt} did not induce anti-POEGMA antibodies, indicating
376 that it is non-immunogenic.

377 We further tested the immunogenicity of POEGMA and compared it to PEG using highly immunogenic
378 OVA as its conjugation partner. Briefly, OVA, OVA-PEG, OVA-POEGMA, and PBS (negative control) were repeatedly
379 injected s.c. into naïve C57BL6/J mice (*n*=10) in accordance with the timeline in **Fig. 6a**. The Luminex immunoassay
380 was used to quantify the ADAs induced by the treatments with minor changes from the previous experiment.
381 Briefly, OVA, OVA-PEG, and OVA-POEGMA coupled beads were used to quantify ADA responses to the full
382 immunizing material, while Ex-PEG and Ex-POEGMA coupled beads were employed as irrelevant protein
383 conjugates to measure responses to the polymer domains only. The exendin-coupled bead was used as a cross-

384 reactivity control. Free, PEG-conjugated, and POEGMA-conjugated OVA induced significant anti-OVA IgG
385 responses by Day 44 (**Supplementary Fig. 35a-b**). The OVA-PEG conjugate induced lower titers of anti-OVA IgG
386 than unmodified OVA (**Supplementary Fig. 35b**). This result was attributed to the steric hindrance imparted by
387 the PEG,⁶ allowing the conjugate to expose fewer OVA epitopes to the immune system. The differences in anti-
388 OVA antibody titers were not as drastic when POEGMA was used. We speculate that this is because POEGMA
389 provides less steric hindrance than PEG to its conjugation partners due to its more compact architecture.⁴⁵ PEG
390 was highly immunogenic, as seen by the high titers of PEG-specific ADAs detected in OVA-PEG-immunized mice
391 plasma with Ex-PEG beads (**Supplementary Fig. 35c-d**). Similar to when exendin was the conjugation partner, PEG-
392 specific ADAs were restricted to the IgM class (**Supplementary Fig. 35c-d**), possibly due to the lack of T-cell help
393 for class switching, as reported in the literature.⁴² The PEG-specific IgM titer was ~60-fold higher with OVA-PEG
394 than Ex-PEG, possibly due to the higher immunogenicity of OVA. In contrast, only a mild anti-POEGMA IgM
395 response was detected after the first drug injection. The anti-POEGMA response was not detectable at later time
396 points (**Supplementary Fig. 36d**) and did not mature into an IgG response (**Supplementary Fig. 36h**), further
397 supporting our conclusions that POEGMA appears to be significantly less immunogenic than PEG.

Figure 6. Ex-POEGMA_{opt} does not induce anti-POEGMA antibodies. (A) Timeline of PBS, exendin, Ex-PEG_{MW}, or Ex-POEGMA_{opt} administration into DIO C57BL/6J mice ($n=10$) and blood collection. IgM subtype ADA response on (B) Day 10 and (C) Day 44. Blood samples were collected from mice repeatedly treated with PBS, exendin, Ex-PEG_{MW}, or Ex-POEGMA_{opt}. ADA response was measured by a multiplexed Luminex immunoassay. Ex-PEG- and Ex-POEGMA-coupled beads were used to determine ADAs induced towards the entire conjugate (*i.e.*, anti-exendin and anti-polymer — PEG or POEGMA). OVA-PEG- and OVA-POEGMA-coupled beads were used to determine ADAs induced towards PEG or POEGMA, respectively. The OVA-coupled beads were used as a negative control for cross-reactivity towards OVA. Data represent the mean ADA response induced in each mouse $\pm$ SEM. Data were analyzed by two-way repeated-measures ANOVA, followed by *post-hoc* Tukey's multiple comparison test. Data were considered statistically significant when $p < 0.05$.

Finally, we tested POEGMA's immunogenicity in an extreme case by immunizing mice with OVA-POEGMA emulsified with complete Freund's adjuvant (CFA), boosting the immune response with OVA-POEGMA emulsified with incomplete Freund's adjuvant (IFA), and comparing with OVA-PEG that administered in CFA/IFA emulsions.⁴⁶

410 Freund's adjuvants (FA) significantly enhance the titer and affinity of the immune response induced towards co-
411 administered immunogens by extending their presentation, stimulating the innate immune system, and
412 facilitating T-cell help, commonly allowing immunogens to reveal their most immunogenic state.⁴⁷ We
413 hypothesized that POEGMA should at most induce a weak IgM immune response. We tested this hypothesis by
414 administering PBS (- control), OVA, OVA-PEG, and OVA-POEGMA s.c. into naïve C57BL/6J mice ($n=10$) in FA
415 emulsions. Blood was collected by following the timeline in **Fig. 6a**, processed into plasma, and tested by a
416 Luminex immunoassay. Strikingly, a POEGMA-specific IgM response was observed transiently and disappeared
417 after the first injection (**Supplementary Fig. 37-38**). In contrast, CFA/IFA emulsions magnified the anti-OVA and
418 anti-PEG responses by ~2-fold. Notably, no change in the specificity and subtype of the immune response
419 developed towards the treatments was detected (**Supplementary Fig. 37**). In sum, this data confirmed the
420 immunogenicity benefits of POEGMA over PEG even delivered with a potent adjuvant.

421 **Effect of ADAs on long-term efficacy.** When immunogenicity and long-term efficacy data were evaluated
422 together, they suggested that the inferior blood glucose control with the increasing number of injections and poor
423 HbA1c% control by Ex-PEG_{Rh} could result from anti-PEG antibody interference. To test this hypothesis, we next
424 measured the ADA levels in the plasma of long-term treated mice. To do so, plasma was collected on day 66 after
425 8 weekly treatments and assayed for ADAs by the Luminex immunoassay. Both exendin and Bydureon induced a
426 mild anti-exendin IgM response. Notably, Ex-POEGMA_{opt} did not induce anti-POEGMA antibodies and resulted in
427 a much weaker anti-exendin IgM response, possibly due to the non-immunogenic nature of POEGMA and the
428 shielding of exendin's immunogenic epitopes by POEGMA (**Supplementary Fig. 39a**). Notably, anti-exendin IgM
429 titers were higher in Ex-PEG_{Rh} treated mice plasma, and an anti-exendin IgG response was detected
430 (**Supplementary Fig. 39b**). Ex-PEG_{Rh} also induced both PEG-specific IgM and IgG responses. Given the absence of
431 exendin- and PEG-specific IgGs in the prior immunogenicity experiment, the observation of IgGs towards both
432 exendin and PEG in mice treated with Ex-PEG_{Rh} was attributed to the greater number of injections (3 in the

immunogenicity study vs. 8 here), more frequent dosing (17 vs. 7 days), and the longer time of the treatment (44 vs. 66 days) that are known to affect the immune response.⁴²

We next investigated if the ADAs could neutralize the treatments. We analyzed long-term treated mice plasma for neutralizing antibodies (NABs) using the cell-based assay developed to test the GLP1R-binding by exendin variants with minor modifications.⁴⁴ Assay design and optimization are summarized in the **Supplementary Information** and given in **Supplementary Table 9** and **Supplementary Fig. 40**. The GLP1R-binding by the treatments did not change when incubated with the mice plasma, indicating that the ADAs did not neutralize the treatments (**Supplementary Fig. 41**). This result was consistent with the results from the clinical trials of exendin, where anti-exendin ADAs did not affect its PD. We also tested if PEG-specific ADAs neutralized Ex-PEG_{Mw} by incubating the conjugate with the plasma samples of the mice immunized with FA emulsion of OVA-PEG. Ex-PEG_{Mw} showed no change in activity (**Supplementary Fig. 41e**), indicating that even high titers of anti-PEG antibodies did not have neutralizing activity.

We finally tested the effect of ADAs on the PK of the treatments. We hypothesized that the loss of efficacy in the Ex-PEG_{Mw} long-term treatment was due to the binding of the anti-PEG antibodies to the circulating drug, resulting in accelerated blood clearance and preventing it from showing efficacy.^{42,48} We also hypothesized that Ex-POEGMA_{opt} did not show a difference in PK among injections due to the lack of ADAs. To test these hypotheses, we s.c. administered sterile, endotoxin-free, and fluorescently-labeled exendin, Ex-PEG_{Rh}, and Ex-POEGMA_{opt} into DIO C57BL/6J (*n*=4) and tracked their PK after the first (naïve mice) and fifth injection (immunized mice). Exendin did not show any difference in PK parameters between naïve and immunized mice (**Fig. 4c; Table 2**), indicating that anti-exendin antibodies were not PK-altering, consistent with the literature.⁴⁴ Importantly, Ex-PEG_{Rh} showed significantly different PK profiles in naïve and immunized mice (**Fig. 4d; Table 2**). Its absorption was significantly lower in the immunized mice, suggesting local lymph nodes' involvement in its elimination.⁴⁹ Ex-PEG_{Rh}'s elimination half-life was ~2-fold shorter in the immunized mice ($t_{1/2 \text{ elimination}}=11.4 \pm 5.4\text{h}$ vs. $23.1 \pm 6.4\text{h}$), indicating the PK-altering nature of anti-PEG antibodies. Together, the ~4-fold lower drug exposure in immunized mice

(AUC=424 ± 35 vs. 1585 ± 112) indicated that anti-PEG immune response was responsible for the loss of efficacy in the long-term Ex-PEG_{Rh} treatment. Notably, Ex-POEGMA_{opt} preserved its PK benefits after repeated administrations (**Fig. 4e; Table 2**), owing to its non-immunogenic structure.

Histopathological effects. We next investigated if treatment with Ex-POEGMA_{opt} had any histopathological effects and compared it to Ex-PEG_{Rh}, which induces significant vacuolization in major organs, and with Bydureon, exendin, and PBS (- control).⁵⁰ We collected kidney, pancreas, spleen, and thyroid tissues from the *db/db* mice (*n*=3) treated in the long-term study. These tissues were fixed in 10% neutral buffered formalin (NBF), processed, embedded into paraffin, sectioned, and hematoxylin and eosin (H&E) stained. The resulting tissue slides were evaluated by a veterinary pathologist experienced in toxicologic pathology. Exendin and Bydureon treatment did not induce any histopathological effects on the kidney (**Supplementary Fig. 42a-c**), whereas significant vacuolization was noted in the Ex-PEG_{Rh} group (**Supplementary Fig. 42d**). These vacuolar lesions were prominent primarily within epithelial cells of the renal proximal tubules in the outer cortical region. Similarly, a low level of vacuolar change was also observed in pancreatic acinar cells of all Ex-PEG_{Rh}-treated mice (**Supplementary Fig. 43d**), possibly because of GLP1R targeting by exendin⁵¹ and vacuole formation by the conjugated PEG. No changes were noted within the splenic or thyroid tissues treated with Ex-PEG_{Rh} (**Supplementary Fig. 44**). Importantly, Ex-POEGMA_{opt} treatment induced no histopathological changes in the kidney (**Supplementary Fig. 42e**) or pancreas (**Supplementary Fig. 43e**).

Discussion

We have developed a PEG-like, minimally immunogenic, and injectable conjugate for sustained delivery of biologics from a s.c. depot. This next-generation POEGMA technology platform has five key advantages over traditional PEGylation. First, to date, PEG has been conjugated with biologics to create only soluble drug formulation. The injectable, depot-forming POEGMA adds a new dimension to PEGylation by creating a drug depot under the skin that achieves sustained drug release into the circulation. Synthesizing a POEGMA conjugate of exendin that is injectable but undergoes an LCST phase transition into an insoluble coacervate phase that provides

the maximal duration of release from the s.c. depot with a plasma concentration that remains above the therapeutic threshold required a step-wise optimization of the polymer composition and M_w . In the first stage of optimization, we fixed the M_w of the POEGMA but varied the ratio of the EG2 and EG3 side chains in the monomer to create a set of copolymers that exhibit a range of T_t . Upon s.c. injection of these Ex-POEGMA conjugates in mice, we found that the T_t of these copolymers determined the depot's stability, with depots with a lower T_t leading to more stable depots, but a slower rate of release, while depots with a higher T_t had a higher rate of release with a shorter duration of release. We identified $\sim 30^\circ\text{C}$ as the optimal T_t that balances the release rate of the conjugate (and thereby steady-state plasma concentration) with the duration of release, so that a depot with a T_t of $\sim 30^\circ\text{C}$ provides the maximum efficacy, as by the blood glucose AUC. In the next optimization step, we fixed the T_t of the conjugate at $\sim 30^\circ\text{C}$ while varying their M_w from ~ 20 to ~ 170 kDa. The rationale for this second stage of polymer optimization was the recognition that once the conjugate is released from the s.c. depot, the plasma half-life is controlled by renal clearance, which is a function of the M_w of the conjugate. *In vivo* efficacy studies revealed that a conjugate M_w of ~ 55 Da yielded the maximum reduction in glucose AUC compared to the PBS control. Together, these experiments identified an optimal Ex-POEGMA conjugate that has the highest efficacy, with a T_t of $\sim 30^\circ\text{C}$ and an M_w of ~ 55 kDa.

Second, by tuning the molecular architecture of POEGMA, we also eliminated its immunogenicity while preserving its favorable PK and PD benefits. The hyperbranched structure of POEGMA does not induce a POEGMA-specific immune response, presumably because its short OEG side chains do not crosslink B-cell receptors as PEG does, which is a prerequisite for T-cell independent B-cell immune responses.⁴² Unlike PEG, which is unstructured in aqueous solvents, POEGMA self-organizes into nanoparticle-like chains due to the interfacial energy balance driven by its amphiphilicity and the repulsion forces among hydrated OEG side-chains.⁴⁵ We speculate that POEGMA's compact molecular conformation in water may effectively reduce the number of epitopes exposed to the immune system, allowing it to remain non-immunoreactive. Third, in addition to its lack of immunogenicity, POEGMA does not react with PEG antibodies, suggesting that it can be safely used in the pre-existing PEG antibody

505 positive population, which is now rampant because of chronic exposure to PEG through excipients in drugs and
506 consumer products. These findings are highly topical because up to 70% of the human population has pre-existing
507 antibodies to PEG,¹¹ and billions of individuals are in the process of being immunized with a PEG-containing
508 liposome formulation of the Pfizer and Moderna vaccines for COVID-19. There is also a growing consensus that
509 PEG is responsible for the immediate-type hypersensitivity reactions^{52,53} seen with the Moderna and Pfizer COVID-
510 19⁵⁴ and other vaccines.⁵⁵ Fourth, another favorable feature of POEGMA is that it does not exhibit the PEG-induced
511 vacuolization problem, presumably due to its partially biodegradable and non-immunogenic structure. We
512 speculate that cleavage of the OEG side chains from the polymethacrylate backbone through the ester bonds by
513 esterase enzymes in the blood⁵⁶ reduces overall hydration, thereby conjugate size, allowing POEGMA to clear by
514 the kidneys. Finally, injectable and depot-forming POEGMA technology allows chromatography-free purification
515 of conjugated drugs, eliminating a significant time-, labor-, and cost-intensive step in manufacturing PEGylated
516 drugs.

517 Collectively, these advantages allowed an optimal Ex-POEGMA depot to provide T2D management
518 superior to PEG and a clinical sustained-release exendin formulation—Bydureon. Importantly, Ex-PEG lost its
519 efficacy after repeated administrations due to the induction of PEG-specific PK-altering antibodies, preventing it
520 from being used as a long-term treatment strategy. In contrast, the optimal Ex-POEGMA conjugate preserved its
521 efficacy and PK benefits through repeated injections over 56 days, indicating that optimizing the molecular
522 architecture —as we have done with POEGMA— is a powerful approach to solve the mounting problems of
523 PEGylated therapeutics.

524 The POEGMA depot technology has several advantages over other drug delivery technologies. First,
525 POEGMA combines sustained-release and extended circulation in one molecule. Most exendin formulations, such
526 as Bydureon, release the unmodified exendin peptide into the bloodstream that, after release, circulates only for
527 a few hours, while the POEGMA depot releases exendin as a POEGMA conjugate that has ~46-fold longer half-life
528 in the circulation than the unmodified peptide. The molecular design of the POEGMA conjugate also eliminates

529 the variable drug loading seen with PLGA⁵⁷- and nanoparticle-based formulations⁵⁸ as exendin is covalently
530 conjugated with POEGMA at a known—equimolar—stoichiometry. In recent years, the Fc fusion technology—
531 wherein a peptide or protein drug is presented on an IgG Fc scaffold— has become increasingly popular because
532 it provides a long plasma half-life due to the Fc component of the fusion⁵⁹ and because its manufacturing leverages
533 the established expertise of the bioprocessing industry in overexpression of antibody drugs in engineered
534 mammalian cell lines. However, the Fc fusion technology is not a panacea for all peptide and protein drugs that
535 have a short circulation time, as not all drugs can be expressed as Fc fusions because even subtle changes in the
536 Fc structure can dramatically alter the potency and function of the fused drug.⁵⁹ In contrast to Fc fusions restricted
537 to genetically encodable peptides and proteins, the injectable POEGMA drug depot technology applies to all
538 biologics—peptide, protein, and oligonucleotide drugs that have suboptimal PK. The fact that the injectable
539 POEGMA depot technology is competitive with Fc fusion can be inferred by comparing the results presented
540 herein exendin with a preclinical study on Trulicity —a once-weekly FDA-approved Fc fusion of an exendin analog
541 GLP-1. Trulicity required twice-weekly injections in *db/db* mice to adequately control blood glucose, whereas a
542 single injection of Ex-POEGMA_{opt} provided six days of blood glucose control in the same animal model. Allometric
543 scaling suggests that Ex-POEGMA_{opt} can be administered twice-monthly to T2D patients,⁶⁰ necessitating less
544 frequent injections and thereby promoting better patient adherence, currently a major problem in diabetes
545 management. Third, a POEGMA depot releases a steady amount of drug into circulation to achieve a desired
546 therapeutic effect. In contrast, PLGA⁵⁷- and nanoparticle-based sustained-release exendin formulations⁵⁸ have a
547 burst release profile, characterized by a peak-and-valley PK.⁶¹ Fourth, Ex-POEGMA conjugate eliminates the need
548 for surgical implantation, replacement, and removal seen with an exendin pump —TCA 650 (Intarcia),⁶² thereby
549 patient discomfort. Finally, POEGMA shields exendin's immunogenic epitopes, while free exendin-releasing
550 technologies do not have a mechanism for reduced immunogenicity, resulting in higher ADA titers and potentially
551 altered drug efficacy. Our results supported this assertion, where no anti-exendin ADAs were detected after long-
552 term treatment with Ex-POEGMA_{opt}, while exendin and Bydureon treated mice had exendin-specific ADAs.

In conclusion, the next-generation POEGMA technology offers a new and innovative approach to combat the growing antigenicity and immunogenicity problems of PEG with a bonus of enhanced PK and PD benefits over existing PEG conjugates. Future plans include investigating the biodistribution of POEGMA, identifying the molecular variables that endow low immunogenicity to POEGMA conjugates, and testing the immunogenicity and efficacy of POEGMA-drug conjugates in a large animal preclinical model as a prelude to clinical translation.

Methods

Synthesis and purification of POEGMA. All materials were purchased from Millipore Sigma. Triethylene glycol methyl ether methacrylate (EG3) and diethylene glycol methyl ether methacrylate (EG2) were passed through basic alumina columns to remove inhibitors. Other materials were used as received. A catalytic complex was prepared by mixing tris(2-pyridylmethyl) amine (TPMA) and copper (II) bromide (CuBr_2) in ultrapure water with 18.2 MOhm resistivity at a final concentration of 0.8 M and 0.1 M, respectively. In a typical copolymerization, a Schlenk flask contained EG3 (3.5 mmol; 701.57 μl), EG2 (6.5 mmol; 1199.44 μl), azide functional polymerization initiator (0.2 M in methanol; 125 μl ; see **Supplementary Information** for synthesis), the catalytic complex (62.5 μl), methanol (5.875 ml) and 100 mM NaCl (11.946 ml). The polymerization flask was sealed and cooled to 0°C in an ice bath. A separate Schlenk flask contained 64 mM ascorbic acid in ultrapure water. Both flasks were purged with argon for 45 min on ice to remove oxygen. After deoxygenation, the ascorbic acid solution was continuously injected into the polymerization flask at a rate of $1\ \mu\text{l}\ \text{min}^{-1}$ using a syringe pump under an inert atmosphere. The resulting solution was kept under vacuum to remove methanol and freeze-dried overnight. The resulting POEGMA was dissolved in acetonitrile and passed through a neutral alumina column to remove the catalytic complex. POEGMA was purified from unreacted monomer by precipitation in cold diethyl ether, followed by overnight evaporation of excess diethyl ether under vacuum.

Physical characterization of POEGMA. The M_n , M_w , and $\bar{D}$ of POEGMA were assessed by GPC-MALS. POEGMA was solubilized in tetrahydrofuran (THF) at $2\ \text{mg}\ \text{ml}^{-1}$, followed by filtration through a $0.22\ \mu\text{m}$ Teflon syringe filter. 50 μl of the solution was separated on an Agilent PLgel mixed-C column ($105\ \text{\AA}$, 7.5 mm internal diameter x 300 mm

length, and 5 μm particle size) using an Agilent 1100 analytical high-pressure liquid chromatography (HPLC). The HPLC was equipped with a UV detector operating at 254 nm (Agilent), a Dawn EOS MALS detector (Wyatt Technology), and an Optilab DSP refractometer (Wyatt Technology). The mobile phase consisted of 100 ppm butylated hydroxytoluene (BHT) in THF as a stabilizer at a 1 ml min⁻¹ flow rate. Before each analysis, the MALS detector was normalized with 30 kDa polystyrene (Wyatt Technology). Refractive index increment (dn/dc) of POEGMA was calculated using ASTRA software provided with the HPLC (v. 6.0, Wyatt Technology) based on injections of known concentrations and mass, followed by data analysis for M_n , M_w , and $\bar{D}$.

Structural characterization of POEGMA. The POEGMA structure was characterized by H-NMR spectroscopy using a 400 MHz Varian INOVA spectrometer and ACD/NMR software (ACD Labs). The monomer composition was defined as the molar percentage of EG2 (or EG3) content in the copolymer. The monomer composition was calculated from the integral value that corresponds to the average number of hydrogens (H) present in the OEG side-chain (b; 3.4-4.4 ppm; 6H for EG2_{100%} homopolymer; 10H for EG3_{100%} homopolymer) except the chain end-group (c; 3.5-3.3 ppm; 3H) and methylene protons (a; 4.0-4.4 ppm; 2H). The DP were calculated by subtracting the polymerization initiator's M_w from POEGMA's M_w and dividing the resulting mass by the average M_w of a monomeric unit.

Hydrodynamic size characterization. The R_h of POEGMA and Ex-POEGMA conjugates were characterized by dynamic light scattering (DLS) in PBS at 1 mg ml⁻¹ using a temperature-controlled DynaPro Plate Reader (Wyatt Technology). Samples were filtered through a 100 nm syringe filter (Whatman) prior to measurement. Ten repeat measurements of 10-second acquisitions were made at 15°C. Data were analyzed for Raleigh spheres by applying a regularization fit using Dynamics 6.12.0.3 software (Wyatt Technology). The laser wavelength and scattering angle of the instrument were 831.95 nm and 90°, respectively.

Phase behavior characterization. The optical density of POEGMA and Ex-POEGMA conjugates were monitored at 600 nm in PBS at pH 7.4, as the temperature of the cuvet containing the samples was increased at a rate of 1°C min⁻¹ on a temperature-controlled UV-vis spectrophotometer (Cary 300 Bio, Varian Instruments). A sharp increase

601 in the optical density with temperature indicates the occurrence of the phase transition. The T_t was defined as the
602 temperature at the inflection point of the optical density versus the temperature curve and is calculated as the
603 maximum of the first derivative using GraphPad Prism 8.0 software. The reversibility of the phase behavior was
604 investigated by monitoring the optical density as the temperature was gradually decreased from a temperature
605 above the T_t back to 15°C.

606 **Protein expression and purification.** Exendin was recombinantly expressed in *E. coli* from a synthetic gene in a
607 pMA-T expression vector as an ELP fusion protein with a sortase-A recognition site (LPETG) and a polyhistidine
608 tag, yielding exendin-LPETG-His₆-ELP (ESE). The ELP tag enables rapid non-chromatographic purification of ESE,
609 while the LPETG peptide acts as the sortase ligation site for the ATRP initiator. Both ESE and His₆-Sortase A were
610 available from a previous study and expressed and purified to >95% purity, as previously described.¹⁸ The purity
611 of all proteins was visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 4-
612 20% precast Tris-HCl gels (Bio-Rad), followed by staining with Simply Blue Safe Stain (Thermo Scientific) and gel
613 densitometry analysis using Image Lab software (Bio-Rad). The ESE concentration was measured using a
614 Bicinchoninic Acid (BCA) assay (Pierce) according to the manufacturer's instructions. The His₆-Sortase A
615 concentration was measured by UV-visible spectroscopy on an ND-1000 Nanodrop spectrophotometer (Thermo
616 Scientific) and the known extinction coefficient of the protein (14440 M⁻¹ cm⁻¹).

617 **Synthesis, purification, and characterization of exendin-DBCO.** A bio-orthogonal DBCO group was installed on
618 the C-terminus of exendin by sortase A-mediated native peptide ligation, yielding exendin-DBCO. Briefly, ESE (100
619 μM) and His₆-Sortase A (50 μM) were reacted in the presence of triglycine-DBCO (Gly₃-DBCO) (5 mM; Click
620 Chemistry Tools) in ligation buffer (50 mM Tris, 150 mM NaCl, and 10 mM CaCl₂; pH 7.5) at room temperature for
621 16 hours. Exendin-DBCO was purified from the reaction mixture by reverse immobilized metal affinity
622 chromatography using an AKTA Purifier (GE Healthcare) equipped with a photodiode array operating at 220 and
623 280 nm and a HisTrap HP (GE Healthcare) column. Exendin-DBCO was collected in the flow-through as it was the
624 only species that did not carry an oligohistidine tag, and hence did not bind to the resin. Exendin-DBCO was

625 concentrated by ultrafiltration using Centricon 70 (Millipore Sigma) filters with a 3,000 Da molecular weight cut-
626 off (MWCO), followed by dialysis into cold water and lyophilization. Stoichiometric (1:1) attachment of DBCO to
627 exendin was confirmed by Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight mass spectroscopy (MALDI-
628 TOF-MS).

629 ***Synthesis and purification of exendin conjugates.*** Exendin-DBCO was conjugated to azide-functional POEGMA or
630 PEG via the strain-promoted alkyne-azide click reaction. Exendin-DBCO and azide-functional POEGMA or PEG were
631 dissolved in PBS at a 1.05:1 ratio and reacted overnight at 4°C. Depot-forming Ex-POEGMA conjugates were
632 purified by triggering their LCST phase transition by the addition of ammonium sulfate to a final concentration of
633 0.1 M. The dense phase that contained the conjugate was recovered by centrifugation at 21,000 g for 15 minutes
634 at room temperature, the supernatant was removed, and the conjugate was dissolved in PBS at 4°C. The last two
635 steps were repeated twice to obtain conjugates with > 99% purity, as verified by SDS-PAGE and HPLC. In the final
636 step, the conjugate was dissolved in ultra-pure water and lyophilized. Soluble Ex-POEGMA and Ex-PEG conjugates
637 were purified by a single round of size exclusion chromatography (SEC) using an AKTA purifier equipped with a
638 photodiode array detector operating at 220 and 280 nm and a HiLoad 16/600 Superdex 75 pg column (GE
639 Healthcare) at 4°C using PBS as the mobile phase. The purified conjugates were concentrated by ultrafiltration
640 using Amicon filters (Millipore Sigma) with a 3,000 Da MWCO, followed by dialysis into the water at 4°C overnight
641 and lyophilization.

642 ***Physical characterization of exendin conjugates.*** The conjugates were characterized in terms of their M_n , M_w ,
643 and $\bar{D}$ by SEC-MALS using an Agilent 1260 analytical HPLC equipped with a UV detector operating at 280 nm
644 (Agilent), a DAWN HELEOS II MALS detector (Wyatt Technology), and an Optilab T-rEX refractive index detector
645 (Wyatt Technology). The DAWN HELEOS II MALS detector was annually calibrated in toluene and normalized with
646 2 mg ml⁻¹ bovine serum albumin (Pierce) before each analysis. The exendin conjugates were dissolved in 10 mM
647 phosphate buffer at pH 7.4, followed by filtration through a 100 nm syringe filter (Whatman). 50 µl of the
648 conjugate was separated on a Shodex KW-803 column (8 mm internal diameter x 300 mm length, and 5 µm particle

size) at a flow rate of 0.5 ml min⁻¹. The mobile phase was 30% (v/v) methanol in 10 mM phosphate buffer at pH 7.4. Data were analyzed for M_n , M_w , and $\bar{D}$ using ASTRA software (v. 7.0, Wyatt Technology).

In vitro activity of exendin variants. The activity of exendin and its conjugates with POEGMA and PEG were measured by GLP1R activation in a cell-based assay, which increases intracellular cyclic adenosine monophosphate (cAMP) levels upon activation of the GLP1R by exendin or its conjugates. Intracellular cAMP concentrations were quantified by treating Human Embryonic Kidney 293 cells recombinantly expressing GLP1R and luciferase fused cAMP (HEK293/CRE-Luc/GLP1R) with exendin variants. This recombinant cell line was a generous gift from Timothy Kieffer (University of British Columbia) and authenticated via short tandem repeat (STR) analysis by the American Type Culture Collection (ATCC).

HEK293/CRE-Luc/GLP1R cells were cultured in high glucose Dulbecco's Minimal Essential Medium (DMEM) (Gibco), supplemented with 10% fetal bovine serum (Hyclone), 400 µg ml⁻¹ G418 (Thermo Fisher), and 200 µg ml⁻¹ Hygromycin B (Invitrogen). Cells were subcultured at least once before the assay at approximately 80% confluency. One day before performing the assay, cells were seeded without antibiotics in phenol red-free DMEM (Gibco) on 96-well plates at 25,000 cells per well in 90 µl media followed by incubation at 37°C under 5% CO₂ atmosphere overnight. Exendin conjugates (20 µM in PBS) were incubated with dipeptidyl peptidase IV (DPP-IV, Prospeco Bio) to expose an active N-terminus for 16 h at room temperature. DPP-IV amount was 2.5 mass% of exendin present in the conjugates. On the day of the assay, exendin (Santa Cruz Biotechnology) was dissolved to a final concentration of 20 µM in PBS. Logarithmic serial dilutions were made for exendin variants in PBS using unmodified exendin as a positive control. 10 µl of each dilution was transferred to wells ($n=6$), yielding a concentration range of 10⁻¹³–10⁻⁶ M. The plates were then incubated at 37°C for 5 hours, followed by equilibration with room temperature for 1 hour. Next, 100 µl Bright-Glo™ reagent (Promega) was added to the wells and incubated for 2 minutes, followed by measuring luminescence using a Victor plate reader (Perkin Elmer). Data were analyzed for net luminescence by subtracting PBS-treated wells' mean luminescence (negative control) from

672 exendin variants. The effective half-maximal dose (EC_{50}) of each exendin variant was determined by fitting the
673 dose-response curve to a four-parameter logistic, nonlinear regression model using GraphPad Prism 8 software.

674 ***In vivo studies.*** *In vivo* studies were conducted under protocols approved by Duke Institutional Animal Care and
675 Use Committee (IACUC) by employing six-week-old male C57BL/6J (Jackson Laboratories; stock no. 000664) or
676 B6.BKS(D)-*LepR^{db}*/J mice (*db/db*; Jackson Laboratories; stock no: 000697). C57BL/6J mice were kept on a 60
677 kilocalorie (kcal) % fat diet (Research Diets Inc.; #D12492i) for at least five weeks before and during the
678 experiments unless otherwise noted, yielding DIO mice. Six-week-old male *db/db* mice were fed a standard rodent
679 diet (LabDiet 5001) and acclimatized to facilities for one week before the experiments. In immunogenicity
680 experiments, mice treated with OVA variants were kept on a standard rodent diet. Mice were group-housed under
681 controlled photoperiod with 12 h light and 12 h dark cycles and acclimated to the facility for a week before the
682 start of experiments. Mice had ad libitum access to water and food unless otherwise noted.

683 All samples were endotoxin purified using endotoxin removal columns (Pierce) and sterilized using a 0.22
684 μ m Acrodisc filter with a Mustang E membrane (Pall Corporation). The final endotoxin amount was tested below
685 5 EU per kg mouse body weight using the Endosafe nexgen-PTS instrument and cartridges (Charles River). For the
686 samples used in the immunogenicity and the long-term efficacy experiments, a more stringent endotoxin limit of
687 a maximum of 0.2 EU per kg mouse body weight was used.

688 ***Fed blood glucose measurements.*** In the short-term efficacy experiments, the fed blood glucose was measured
689 after a single s.c. injection of the treatments. On the day of injection, the tail was sterilized with alcohol pads (BD).
690 The first drop of blood collected from a tiny incision on the tail vein was wiped off. The second drop of blood was
691 used to measure fed blood glucose using a hand-held glucometer (AlphaTrack, Abbott). The treatments were
692 solubilized in PBS and kept on ice before injection to prevent phase transition, followed by s.c. administration into
693 mice. Bydureon was prepared for injection according to the manufacturer's instructions. Fed blood glucose levels
694 were measured 24 h and immediately before injection, at 1-, 4-, and 8-h post-injection, and every 24 h after that

695 until no significant effect of treatments on fed blood glucose was observed. Body weight was tracked daily. In the
696 long-term efficacy experiment, fed blood glucose and body weight were tracked every three days.

697 **IPGTT.** The glycemic regulation ability of treatments was assessed by performing three IPGTTs after a single s.c.
698 injection. *Db/db* mice were used during the first week of the long-term pharmacodynamics study. On day 0, the
699 treatments were administrated into the mice at an equivalent dose (1000 nmol per kg bodyweight) and
700 concentration (500 μ M) using the equivalent injection volume of PBS as a negative control. Bydureon was
701 prepared for injection according to the manufacturer's instructions. On the day of the IPGTT (Day 1, 3, and 5),
702 mice were fasted six hours before the glucose challenge by an *i.p* injection of 1.5 g kg⁻¹ of glucose (Sigma) followed
703 by blood glucose monitoring at 5-, 15-, 30-, 60-, 90-, 120-, 180- and 240 min.

704 **HbA1c% measurement.** In long-term pharmacodynamics experiments, *db/db* mice (*n*=5) were repeatedly injected
705 with the treatments at an equivalent dose (1000 nmol per kg body weight in PBS) and concentration (500 μ M)
706 every seven days over 56 days using an equivalent injection volume of PBS as a negative control. Bydureon was
707 prepared for injection according to the manufacturer's instructions. HbA1c% was measured on Day 0 before
708 injection, Day 28, and Day 56 using DCA Vantage Analyzer and DCA HbA1c reagent kit (Siemens).

709 **Pharmacokinetics.** Exendin variants were labeled with a fluorophore to track their pharmacokinetics. Briefly,
710 Alexa Fluor 488 NHS ester (Pierce) was reacted with exendin variants (5 mg ml⁻¹) at a 5:1 molar ratio in PBS for
711 one hour at room temperature. Unreacted excess fluorophore was removed by dialysis into the water at 4°C using
712 membranes with a 3,000 Da MWCO (Pierce), verified by HPLC. The labeling efficiency was calculated from UV-vis
713 spectroscopy using an ND-1000 Nanodrop spectrophotometer (Thermo Scientific).

714 The fluorophore-labeled treatments were administered into DIO C57BL/6J mice via a single s.c. injection
715 at 1000 nmol kg⁻¹ (45 nmol kg⁻¹ fluorophore). Ten μ l of blood was collected from a tiny incision on the tail vein into
716 tubes containing 90 μ l of 1,000 U ml⁻¹ heparin (Sigma) at 5-min, 1-, 2-, 4-, 8-, 24-, 48-, 72-, 96-, 120-, 144- and 168-
717 h. Blood samples were centrifugated at 1600 g at 4°C for 15 minutes for plasma. Fluorophore concentration in

718 plasma samples was detected by a Victor plate reader (Perkin Elmer) at 485 nm (excitation) and 535 nm
 719 (emission). PK parameters were derived by plotting the drug's plasma concentration as a function of time and
 720 fitting it to a non-compartmental PK model for the absorption and elimination phases using GraphPad Prism
 721 software. The absorption phase described the time between injection at 0-h and t_{max} , where the drug
 722 concentration was maximum. The elimination phase described the time after t_{max} . t_{max} was calculated from
 723 equation $t_{max} = \left(\frac{2.303}{k_a - k_e}\right) * \log \frac{k_a}{k_e}$, where k_a and k_e were the apparent absorption and elimination rate constants.
 724 The rate constants were determined from the linear regression slope of the log (drug concentration) versus time
 725 graph using equation $k = -2.303 * slope$. Half-lives of treatments in each phase were determined from the
 726 equation $t_{1/2} = \frac{0.693}{k}$. The maximum drug concentration (C_{max}) was calculated at t_{max} . Minimal effective
 727 conjugate concentration was calculated by triangulating the concentration values based on the duration of blood
 728 glucose control determined in short-term efficacy experiments in DIO mice.

729 ADAs' effect on the PK was investigated by weekly administering the fluorophore-labeled treatments into
 730 the DIO C57BL/6J mice five times. Blood samples were collected after the first and last injection, and PK
 731 parameters were determined as described above.

732 **Immunogenicity.** We tested the immunogenicity of POEGMA and compared it to that of PEG in three sets of
 733 immunogenicity experiments. In the first set, sterile and endotoxin-free exendin, Ex-PEG_{Mw}, and Ex-POEGMA_{opt}
 734 were s.c. administrated into 7-week-old C57BL/6J mice ($n=10$) at an equivalent dose (1000 nmol kg⁻¹) and
 735 concentration (500 μM). In the second set of experiments, mice were injected with OVA (Invivogen), OVA-PEG_{10K},
 736 and OVA-POEGMA_{10K} at 9.6 nmol kg⁻¹ (100 μl). In the final set, OVA, OVA-PEG_{10K}, and OVA-POEGMA_{10K} were
 737 emulsified in an equal volume of sterile CFA/IFA (Invivogen) and then administrated into 7-week-old C57BL/6J
 738 mice ($n=10$) at 9.6 nmol kg⁻¹ (100 μl). CFA was used for the first injections, while IFA was used for the subsequent
 739 injections. An equal injection volume of PBS (or PBS emulsified in CFA or IFA) was used as a negative control in all
 740 experimental sets. All drugs were administrated into mice every 17 days three times (Day 0, 17, and 34). The

741 injection site was the loose skin over the shoulders and was kept the same across the injections. Blood samples
742 were collected seven days before the first injection (Day -7) and ten days after each injection (Day 10, 27, and 44),
743 followed by plasma isolation via centrifugation at 4°C at 1600 g for 15 minutes, aliquoting on ice, and storage at -
744 80°C until analysis.

745 **Analysis of ADA response.** ADAs were analyzed using a Luminex multiplex immunoassay according to the
746 manufacturer's instructions with minor modifications. Assay design, optimization, and validation were given in
747 **Supplementary Information.** The plasma samples of mice treated with exendin variants were diluted 200-fold in
748 0.2% (w/v) I-Block protein-based blocking reagent (Thermo Scientific) in PBS (Hyclone), defined as the assay buffer.
749 The plasma samples of mice treated with OVA variants ($n=10$) were diluted 500- and 10000-fold in the assay buffer
750 for IgM and IgG analysis, respectively.

751 In a typical LMI assay, 50µl of the diluted plasma samples ($n=3$) were transferred to a black, round-bottom
752 96-well-plate (Corning). Next, spectrally distinct exendin-, Ex-PEG-, Ex-POEGMA-, OVA-, OVA-PEG-, and OVA-
753 POEGMA-coupled magnetic bead sets were mixed at a concentration of 2500 beads per 50 µl per set. 50µl of the
754 resulting solution was distributed to the wells of a 96-well plate and incubated for 1 hour on an orbital shaker to
755 capture ADAs present in the plasma samples. After incubation, 96-well-plate was placed on a magnetic ring stand
756 (Invitrogen) and incubated for 60 seconds for separation to occur. The supernatant was discarded, and wells were
757 washed with 100 µl of the assay buffer twice. The resulting magnetic bead-ADA complexes were incubated with
758 100µl of 5 µg ml⁻¹ R-Phycoerythrin-conjugated goat anti-mouse IgG (Jackson ImmunoResearch; #115-115-164) for
759 30 minutes to detect IgG subtypes of ADAs. 100µl of 5 µg ml⁻¹ biotin-conjugated goat anti-mouse IgM (Jackson
760 ImmunoResearch; #115-065-075) was used to detect IgM subtypes of ADAs, followed by washes, incubation with
761 100µl of 7.5 µg ml⁻¹ streptavidin-R-phycoerythrin (SAPE; Invitrogen) for 30 minutes, and a final round of washes.
762 Mouse IgG- or IgM-coupled magnetic beads were used as positive controls. They were incubated with 100µl of
763 0.25 µg ml⁻¹ R-Phycoerythrin-conjugated goat anti-mouse IgG or 0.114 µg ml⁻¹ biotin-conjugated goat anti-mouse
764 IgM, followed by 0.171 µg ml⁻¹ SAPE. The negative control was the drug-coupled bead mixture in the assay buffer.

765 Finally, magnetic beads were suspended in 100 μ l of assay buffer, followed by measuring mean fluorescence signal
766 intensity (MFI) using MAGPIX (Luminex) instrument. The average MFI value of each plasma sample and treatment
767 was computed with no exception for outliers. Mouse plasma samples were considered ADA-positive if the average
768 MFI for any given bead type was above the background (see **Supplementary Information**). MFI of each plasma
769 sample for any given bead type was normalized to the positive control.

770 **Histology.** The *db/db* mice used in the long-term efficacy experiment were sacrificed, followed by collecting the
771 organs into 10% neutral-buffered formalin. After fixation in 10% NBF, the tissues were trimmed, paraffin
772 embedded, and sectioned at 5 μ m for light microscopy, followed by H&E staining.

773 **Statistical Analyses.** In all the animal studies, animals were randomized, and group sizes were estimated using
774 open-source G*Power software based on the literature, and adequate power was ensured to detect differences.
775 Data were presented as mean $\pm$ standard error of the mean (SEM) unless otherwise noted differently. Blood
776 glucose data were plotted as a function of time using raw values and normalized values. Normalization reflects
777 the percent change in blood glucose with treatment. It was calculated by dividing raw the fed blood glucose
778 measurement at any given time point by an average value of fed blood glucose levels measured 24 hours ($t=-24h$)
779 and immediately before injection ($t=0h$), as previously described in the literature.¹⁸ Blood glucose and body weight
780 data were analyzed using two-way repeated-measures analysis of variance (ANOVA) followed by *post hoc*
781 Dunnett's multiple comparison test. The AUC for glucose exposure was quantified for each subject using the
782 trapezoid rule and analyzed using two-way ANOVA, followed by *post-hoc* Tukey's multiple comparison test.
783 HbA1c% and immunogenicity data were analyzed using two-way ANOVA, followed by *post-hoc* Tukey's multiple
784 comparison test. A test was considered significant if the *p*-value is < 0.05 (* $p<0.05$; ** $p<0.01$; *** $p<0.001$;
785 **** $p<0.0001$; ns: $p>0.05$). GraphPad Prism 8.0 was used for all statistical analyses.

786 **Acknowledgments.** This work was funded by the National Institutes of Health (R01DK124276-01 to A.C). We thank
787 Dr. Stephen Craig at the Department of Chemistry for allowing us to use his GPC-MALS facilities to analyze
788 POEGMAs and Dr. Peter Silinski at the Duke Chemistry Mass Spectrometry Facility for mass spectrometry analyses

on the monomers and the polymerization initiator. We also thank the Duke University School of Medicine for the use of the Proteomics and Metabolomics Shared Resource, which provided analysis on the stoichiometry of DBCO installment to exendin, and Duke Immune Profiling Core (DIPC) at Duke University Center for AIDS Research (CFAR) for the use of Luminex MAGPIX.

Author Contributions. I.O. and A.C. conceived and designed the research. I.O., A.S., N.Z., and X.L. performed the experiments. J.I.E. imaged the histology slides and interpreted the findings. J.H.C., J.E.C., and D.A.D. and contributed to experimental design. I.O. and A.C. interpreted the results and wrote the manuscript. All authors participated in the discussion of the data and commented on the manuscript.

Competing Interests. I.O. and A.C. have a pending patent on a stimuli-responsive PEG-like polymer-based drug delivery platform (U.S. 17/051,202) and a U.S. provisional patent application on non-immunogenic PEG-like polymer conjugate technology (U.S. 63/169,541).

Data Availability. All data is available in the manuscript or the supplementary materials.

References

- 1 Leader, B., Baca, Q. J. & Golan, D. E. Protein therapeutics: a summary and pharmacological classification. *Nature Reviews Drug Discovery* **7**, 21-39, (2008).
- 2 Fosgerau, K. & Hoffmann, T. Peptide therapeutics: current status and future directions. *Drug Discovery Today* **20**, 122-128, (2015).
- 3 Turner, M. R. & Balu-Iyer, S. V. Challenges and Opportunities for the Subcutaneous Delivery of Therapeutic Proteins. *Journal of Pharmaceutical Sciences* **107**, 1247-1260, (2018).
- 4 Vugmeyster, Y., Xu, X., Theil, F. P., Khawli, L. A. & Leach, M. W. Pharmacokinetics and toxicology of therapeutic proteins: Advances and challenges. *World J Biol Chem* **3**, 73-92, (2012).
- 5 Ekladios, I., Colson, Y. L. & Grinstaff, M. W. Polymer–drug conjugate therapeutics: advances, insights and prospects. *Nature Reviews Drug Discovery* **18**, 273-294, (2019).

812 6 Harris, J. M. & Chess, R. B. Effect of pegylation on pharmaceuticals. *Nature Reviews Drug Discovery* **2**, 214-
813 221, (2003).

814 7 Ganson, N. J., Kelly, S. J., Scarlett, E., Sundry, J. S. & Hershfield, M. S. Control of hyperuricemia in subjects
815 with refractory gout, and induction of antibody against poly(ethylene glycol) (PEG), in a phase I trial of
816 subcutaneous PEGylated urate oxidase. *Arthritis Res Ther* **8**, R12, (2006).

817 8 Hershfield, M. S. *et al.* Induced and pre-existing anti-polyethylene glycol antibody in a trial of every 3-
818 week dosing of pegloticase for refractory gout, including in organ transplant recipients. *Arthritis Res Ther*
819 **16**, R63, (2014).

820 9 Garay, R. P., El-Gewely, R., Armstrong, J. K., Garratty, G. & Richette, P. Antibodies against polyethylene
821 glycol in healthy subjects and in patients treated with PEG-conjugated agents. *Expert Opin Drug Deliv* **9**,
822 1319-1323, (2012).

823 10 Richter, A. W. & Akerblom, E. Polyethylene glycol reactive antibodies in man: titer distribution in allergic
824 patients treated with monomethoxy polyethylene glycol modified allergens or placebo, and in healthy
825 blood donors. *Int Arch Allergy Appl Immunol* **74**, 36-39, (1984).

826 11 Yang, Q. *et al.* Analysis of Pre-existing IgG and IgM Antibodies against Polyethylene Glycol (PEG) in the
827 General Population. *Analytical Chemistry* **88**, 11804-11812, (2016).

828 12 Ganson, N. J. *et al.* Pre-existing anti-polyethylene glycol antibody linked to first-exposure allergic reactions
829 to pegnivacogin, a PEGylated RNA aptamer. *J Allergy Clin Immunol* **137**, 1610-1613 e1617, (2016).

830 13 Armstrong, J. K. *et al.* Antibody against poly(ethylene glycol) adversely affects PEG-asparaginase therapy
831 in acute lymphoblastic leukemia patients. *Cancer* **110**, 103-111, (2007).

832 14 Verhoef, J. J., Carpenter, J. F., Anchordoquy, T. J. & Schellekens, H. Potential induction of anti-PEG
833 antibodies and complement activation toward PEGylated therapeutics. *Drug Discov Today* **19**, 1945-1952,
834 (2014).

835 15 Zhang, P., Sun, F., Liu, S. & Jiang, S. Anti-PEG antibodies in the clinic: Current issues and beyond PEGylation.
836 *Journal of controlled release : official journal of the Controlled Release Society* **244**, 184-193, (2016).

837 16 Fee, C. J. Size comparison between proteins PEGylated with branched and linear poly(ethylene glycol)
838 molecules. *Biotechnology and Bioengineering* **98**, 725-731, (2007).

839 17 Webster, R. *et al.* in *PEGylated Protein Drugs: Basic Science and Clinical Applications* (ed Francesco M.
840 Veronese) 127-146 (Birkhäuser Basel, 2009).

841 18 Qi, Y. *et al.* A brush-polymer/exendin-4 conjugate reduces blood glucose levels for up to five days and
842 eliminates poly(ethylene glycol) antigenicity. *Nature Biomedical Engineering* **1**, 0002, (2016).

843 19 Joh, D. Y. *et al.* Architectural Modification of Conformal PEG-Bottlebrush Coatings Minimizes Anti-PEG
844 Antigenicity While Preserving Stealth Properties. *Advanced Healthcare Materials* **8**, 1801177, (2019).

845 20 Lu, L. *et al.* In vitro and in vivo characterization of a novel long-acting GLP-1 receptor agonist, exendin-4–
846 Fc fusion protein. *RSC Advances* **7**, 54178-54187, (2017).

847 21 Glaesner, W. *et al.* Engineering and characterization of the long-acting glucagon-like peptide-1 analogue
848 LY2189265, an Fc fusion protein. *Diabetes/Metabolism Research and Reviews* **26**, 287-296, (2010).

849 22 Zhang, B., Tang, H. & Wu, P. In Depth Analysis on the Unusual Multistep Aggregation Process of
850 Oligo(ethylene glycol) Methacrylate-Based Polymers in Water. *Macromolecules* **47**, 4728-4737, (2014).

851 23 Sun, S. & Wu, P. On the Thermally Reversible Dynamic Hydration Behavior of Oligo(ethylene glycol)
852 Methacrylate-Based Polymers in Water. *Macromolecules* **46**, 236-246, (2013).

853 24 Badi, N. Non-linear PEG-based thermoresponsive polymer systems. *Progress in Polymer Science* **66**, 54-
854 79, (2017).

855 25 Luginbuhl, K. M. *et al.* One-week glucose control via zero-order release kinetics from an injectable depot
856 of glucagon-like peptide-1 fused to a thermosensitive biopolymer. *Nat Biomed Eng* **1**, (2017).

857 26 Simakova, A., Averick, S. E., Konkolewicz, D. & Matyjaszewski, K. Aqueous ARGET ATRP. *Macromolecules*
858 **45**, 6371-6379, (2012).

859 27 Ramírez-Jiménez, A., Montoya-Villegas, K. A., Licea-Claverie, A. & González-Ayón, M. A. Tunable Thermo-
860 Responsive Copolymers from DEGMA and OEGMA Synthesized by RAFT Polymerization and the Effect of
861 the Concentration and Saline Phosphate Buffer on its Phase Transition. *Polymers (Basel)* **11**, 1657, (2019).

862 28 Lutz, J.-F. Polymerization of oligo(ethylene glycol) (meth)acrylates: Toward new generations of smart
863 biocompatible materials. *Journal of Polymer Science Part A: Polymer Chemistry* **46**, 3459-3470, (2008).

864 29 Ozer, I. & Chilkoti, A. Site-Specific and Stoichiometric Stealth Polymer Conjugates of Therapeutic Peptides
865 and Proteins. *Bioconjugate Chemistry*, (2016).

866 30 Pang, Y., Liu, J., Qi, Y., Li, X. & Chilkoti, A. A Modular Method for the High-Yield Synthesis of Site-Specific
867 Protein-Polymer Therapeutics. *Angew Chem Int Ed Engl* **55**, 10296-10300, (2016).

868 31 J., B. J., Jayanta, B. & Ashutosh, C. A Noncanonical Function of Sortase Enables Site-Specific Conjugation
869 of Small Molecules to Lysine Residues in Proteins. *Angewandte Chemie International Edition* **54**, 441-445,
870 (2015).

871 32 Bebis, K., Jones, M. W., Haddleton, D. M. & Gibson, M. I. Thermoresponsive behaviour of
872 poly[(oligo(ethyleneglycol methacrylate)]s and their protein conjugates: importance of concentration and
873 solvent system. *Polymer Chemistry* **2**, 975-982, (2011).

874 33 Kleinert, M. *et al.* Animal models of obesity and diabetes mellitus. *Nature Reviews Endocrinology* **14**, 140-
875 162, (2018).

876 34 Young, A. A. *et al.* Glucose-lowering and insulin-sensitizing actions of exendin-4: studies in obese diabetic
877 (ob/ob, db/db) mice, diabetic fatty Zucker rats, and diabetic rhesus monkeys (*Macaca mulatta*). *Diabetes*
878 **48**, 1026, (1999).

879 35 Caliceti, P. & Veronese, F. M. Pharmacokinetic and biodistribution properties of poly(ethylene glycol)-
880 protein conjugates. *Advanced Drug Delivery Reviews* **55**, 1261-1277, (2003).

881 36 Yamaoka, T., Tabata, Y. & Ikada, Y. Distribution and tissue uptake of poly(ethylene glycol) with different
882 molecular weights after intravenous administration to mice. *J Pharm Sci* **83**, 601-606, (1994).

883 37 Tang, D. *et al.* C-terminal site-specific PEGylated Exendin-4 analog: A long-acting glucagon like Peptide-1
884 receptor agonist, on glycemic control and beta cell function in diabetic db/db mice. *J Pharmacol Sci* **138**,
885 23-30, (2018).

886 38 Kanoski, S. E., Rupprecht, L. E., Fortin, S. M., De Jonghe, B. C. & Hayes, M. R. The role of nausea in food
887 intake and body weight suppression by peripheral GLP-1 receptor agonists, exendin-4 and liraglutide.
888 *Neuropharmacology* **62**, 1916-1927, (2012).

889 39 Ballav, C. & Gough, S. Bydureon: long-acting exenatide for once-weekly injection. *Prescriber* **23**, 30-33,
890 (2012).

891 40 Gedulin, B. R. *et al.* Pharmacokinetics and pharmacodynamics of exenatide following alternate routes of
892 administration. *International Journal of Pharmaceutics* **356**, 231-238, (2008).

893 41 Richter, W. F., Bhansali, S. G. & Morris, M. E. Mechanistic determinants of biotherapeutics absorption
894 following SC administration. *Aaps j* **14**, 559-570, (2012).

895 42 Yang, Q. & Lai, S. K. Anti-PEG immunity: emergence, characteristics, and unaddressed questions. *Wiley*
896 *Interdiscip Rev Nanomed Nanobiotechnol* **7**, 655-677, (2015).

897 43 Kozma, G. T., Shimizu, T., Ishida, T. & Szebeni, J. Anti-PEG antibodies: Properties, formation, testing and
898 role in adverse immune reactions to PEGylated nano-biopharmaceuticals. *Adv Drug Deliv Rev* **154-155**,
899 163-175, (2020).

900 44 Buse, J. B. *et al.* Liraglutide Treatment Is Associated with a Low Frequency and Magnitude of Antibody
901 Formation with No Apparent Impact on Glycemic Response or Increased Frequency of Adverse Events:
902 Results from the Liraglutide Effect and Action in Diabetes (LEAD) Trials. *The Journal of Clinical*
903 *Endocrinology & Metabolism* **96**, 1695-1702, (2011).

904 45 Ozer, I., Tomak, A., Zareie, H. M., Baran, Y. & Bulmus, V. Effect of Molecular Architecture on Cell
905 Interactions and Stealth Properties of PEG. *Biomacromolecules* **18**, 2699-2710, (2017).

906 46 Richter, A. W. & Akerblom, E. Antibodies against polyethylene glycol produced in animals by immunization
907 with monomethoxy polyethylene glycol modified proteins. *Int Arch Allergy Appl Immunol* **70**, 124-131,
908 (1983).

909 47 Stils, H. F., Jr. Adjuvants and Antibody Production: Dispelling the Myths Associated with Freund's Complete
910 and Other Adjuvants. *ILAR Journal* **46**, 280-293, (2005).

911 48 Verhoef, J. J. F. & Anchordoquy, T. J. Questioning the Use of PEGylation for Drug Delivery. *Drug Deliv Transl*
912 *Res* **3**, 499-503, (2013).

913 49 Fathallah, A. M., Bankert, R. B. & Balu-Iyer, S. V. Immunogenicity of Subcutaneously Administered
914 Therapeutic Proteins—a Mechanistic Perspective. *The AAPS Journal* **15**, 897-900, (2013).

915 50 Ivens, I. A. *et al.* PEGylated Biopharmaceuticals: Current Experience and Considerations for Nonclinical
916 Development. *Toxicol Pathol* **43**, 959-983, (2015).

917 51 Hou, Y., Ernst, S. A., Heidenreich, K. & Williams, J. A. Glucagon-like peptide-1 receptor is present in
918 pancreatic acinar cells and regulates amylase secretion through cAMP. *Am J Physiol Gastrointest Liver*
919 *Physiol* **310**, G26-G33, (2016).

920 52 Wenande, E. & Garvey, L. H. Immediate-type hypersensitivity to polyethylene glycols: a review. *Clinical &*
921 *Experimental Allergy* **46**, 907-922, (2016).

922 53 Sellaturay, P., Nasser, S. & Ewan, P. Polyethylene Glycol-Induced Systemic Allergic Reactions
923 (Anaphylaxis). *J Allergy Clin Immunol Pract*, (2020).

924 54 Garvey, L. H. & Nasser, S. Anaphylaxis to the first COVID-19 vaccine: is polyethylene glycol (PEG) the
925 culprit? *Br J Anaesth*, (2020).

926 55 Badiu, I., Geuna, M., Heffler, E. & Rolla, G. Hypersensitivity reaction to human papillomavirus vaccine due
927 to polysorbate 80. *BMJ Case Rep* **2012**, (2012).

928 56 Bahar, F. G., Ohura, K., Ogihara, T. & Imai, T. Species Difference of Esterase Expression and Hydrolase
929 Activity in Plasma. *Journal of Pharmaceutical Sciences* **101**, 3979-3988, (2012).

930 57 Bader, M. *et al.* Pharmacokinetics and efficacy of PT302, a sustained-release Exenatide formulation, in a
931 murine model of mild traumatic brain injury. *Neurobiol Dis* **124**, 439-453, (2019).

932 58 He, Z. *et al.* Sustained release of exendin-4 from tannic acid/Fe (III) nanoparticles prolongs blood glycemic
933 control in a mouse model of type II diabetes. *J Control Release* **301**, 119-128, (2019).

934 59 Czajkowsky, D. M., Hu, J., Shao, Z. & Pleass, R. J. Fc-fusion proteins: new developments and future
935 perspectives. *EMBO Mol Med* **4**, 1015-1028, (2012).

936 60 Mahmood, I. Application of allometric principles for the prediction of pharmacokinetics in human and
937 veterinary drug development. *Advanced Drug Delivery Reviews* **59**, 1177-1192, (2007).

938 61 Mann, K. V. & Raskin, P. Exenatide extended-release: a once weekly treatment for patients with type 2
939 diabetes. *Diabetes Metab Syndr Obes* **7**, 229-239, (2014).

940 62 Bertsch, T. & McKeirnan, K. ITCA 650. *Clin Diabetes* **36**, 265-267, (2018).

941

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SIOzeretal.pdf](#)
- [nrreportingsummaryOzeretal1.pdf](#)