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

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

Biologics are potent, highly specific, and well-tolerated and have become an important class of drugs.\textsuperscript{1} Despite their promise, most of them have a short half-life due to rapid renal elimination and in vivo degradation.\textsuperscript{2} Their short in vivo half-life hence necessitates frequent injections, resulting in a peak-and-valley profile in drug concentration that is pharmacologically suboptimal. It also incurs a high treatment cost and suboptimal patient compliance because of the need for frequent drug administration and the side effects associate with a peak-and-valley drug concentration profile.\textsuperscript{3,4} One of the most common approaches to overcome these challenges is the covalent attachment of biologics to polyethylene glycol (PEG), a technology that is colloquially termed PEGylation.\textsuperscript{5} PEGylated drug conjugates have a much longer plasma half-life than the native drug due to their larger size leading to slower renal clearance, reduced opsonization that reduces clearance by the reticuloendothelial system (RES), and improved solubility.\textsuperscript{6} Unfortunately, PEGylation has several significant limitations, some of which have come to the forefront recently. First, PEG was initially believed to be non-immunogenic. However, it is now well accepted that PEGylated therapeutics induce PEG antibodies upon treatment.\textsuperscript{7,8} Pre-existing PEG antibodies have also been reported in up to 70\% of the population who are naïve to PEGylated therapeutics, possibly due to chronic exposure to PEG in consumer products and because PEG is used as an excipient in many drug formulations.\textsuperscript{9-11} Both induced and pre-existing PEG antibodies have caused a severe allergic reaction and accelerated clearance in some patients, reducing the drugs’ clinical efficacy.\textsuperscript{7,8,12,13} These issues have collectively led to the early termination of several clinical trials of PEGylated drug candidates and the withdrawal of several PEGylated therapeutics from the market.\textsuperscript{12,14,15} Second, attempts to improve the pharmacokinetics (PK) of PEG have focused on synthesizing branched and star-shaped PEGs. However, these architectures have a modest effect on PK\textsuperscript{16} and have antigenic and immunogenic profiles similar to linear PEG.\textsuperscript{12} Third, PEG forms vacuoles in major organs due to its non-biodegradable structure and clearance by the RES.\textsuperscript{17} Clearly, optimization of PEG is now at an asymptote, and new architectures that radically depart from linear PEG are needed to address these limitations.
Motivated by these needs, we have developed a “next-gen” PEG-like polymer, poly[oligo(ethylene glycol) methyl ether methacrylate] (POEGMA). POEGMA is an amphiphilic, hyperbranched polymer that breaks up the long ethylene glycol sequences in PEG and presents them as much shorter oligomeric ethylene glycol (OEG) side-chains along a hydrophobic backbone. It has been previously shown that POEGMA with three EG unit long side-chains improves the PK of exendin-4, a peptide drug used in the clinic to treat type 2 diabetes (T2D) and subsequently referred to as exendin.\textsuperscript{18} The POEGMA conjugate of exendin (Ex-POEGMA) also showed no \textit{in vitro} binding to human-derived PEG antibodies, presumably because the short OEG side-chains in POEGMA lack the anti-PEG epitope.\textsuperscript{18,19} Although these results were encouraging, the soluble Ex-POEGMA conjugate reported previously only showed a modest improvement in the PK of the drug. This study also did not compare the PK and efficacy of POEGMA conjugates with PEG conjugates, nor did it report the intrinsic immunogenicity of POEGMA that could occur upon repeated administration of POEGMA-drug conjugates, which are critical for its clinical translation.

This paper has two goals. The first goal was to devise a strategy to further enhance the half-life of exendin beyond the four days of therapeutic action of a soluble POEGMA conjugate in mice\textsuperscript{18} and do so without compromising its lack of reactivity to pre-existing PEG antibodies. Improving the PK of POEGMA beyond the duration exhibited by soluble POEGMA conjugates is important because recent long-acting delivery technologies such as Fc fusions have been shown to work for 4-6 days in mice\textsuperscript{20,21} and 7 days in humans\textsuperscript{21} so that a next-generation PEGylation technology must match—at a minimum— the performance of Fc conjugates. To address this goal, we report on the design and optimization of a gel-like injectable depot of an Ex-POEGMA conjugate that achieves sustained release from the depot into the bloodstream while retaining its lack of reactivity towards PEG antibodies. We identify an optimal Ex-POEGMA depot that maximizes blood glucose control in hyperglycemic mice and investigate its PK and efficacy benefits. The second goal was to investigate the intrinsic immunogenicity and toxicity of a POEGMA-drug conjugate and compare it to PEG. We report the remarkable result that Ex-POEGMA 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. 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) using activator-regenerated by electron transfer atom transfer radical polymerization (ARGET-ATRP). 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. We also fortuitously found that the phase transition temperature (Tt) of POEGMA was sensitive to the OEG side chain length, with shorter side-chains having a lower Tt due to the decreased hydrogen bonding. The Tt of a homopolymer POEGMA with 3EG-long side chains (EG3) was too high to form an s.c. depot, while the Tt 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 Tt 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 Tt 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
Resonance (NMR) spectroscopy (Supplementary Fig. 7-8). We also synthesized an EG3 POEGMA homopolymer as a non-depot forming —soluble— control. The monomer composition of POEGMA was defined as the percentage of EG2 (or EG3) monomer content in the total polymer. We used the nomenclature of EGX%w, where X is the length of the EG chain (2 or 3), and the subscript % is the percentage of the monomer in the total polymer. All synthesized polymers were relatively monodisperse, as measured by gel permeation chromatography-multi-angle light scattering (GPC-MALS), with a polydispersity (D) of <1.2 (Fig. 1a; Supplementary Fig. 9). Their degree of polymerization (DP) ranged from 100 to 400, translating to a weight-averaged molecular weight (M_w) range of 20-80 kDa. Each polymer’s monomer composition, M_w, number-averaged molecular weight (M_n), D, DP, hydrodynamic size (R_h), and T_t are shown in Supplementary Fig. 7-10 and summarized in Supplementary Table 1.

All polymers showed sharp and thermally reversible phase behavior, as seen by the sharp increase in optical density as the temperature was increased and by the fact that their optical density decreased back to baseline as the temperature was decreased below the T_t (Fig. 1b). Their phase behavior also showed no thermal hysteresis, as seen by the overlapping turbidity curves for the heating and cooling cycle. The EG3_100% POEGMA had a T_t of ~48ºC at 25 µM, consistent with a previous report, confirming that it cannot form an s.c. depot. The T_t of the POEGMA copolymers was a function of the EG2 content, as the T_t decreased with the increasing molar ratio of the more hydrophobic EG2 monomer. All copolymers phase transitioned between 25 and 34ºC, suggesting that all copolymers were potential depot-forming constructs. The T_t of POEGMA also showed an inverse concentration dependence. The T_t increased with POEGMA dilution (Fig. 1c), suggesting that sustained release of a POEGMA conjugate from the s.c. depot should be possible in vivo, in response to continuous dilution at the 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—and M_w (Fig. 1d). These data clearly showed that the key molecular variables that control the phase behavior of 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). To circumvent this issue, we site-specifically conjugated POEGMA to exendin at its C-terminus using bio-orthogonal click chemistry. We chose the C-terminus as the
POEGMA conjugation site, as the N-terminus of exendin is critical to its function. To do so, we first covalently conjugated a bio-orthogonal triglycine dibenzocyclooctyne (DBCO) group to the C-terminus of exendin via sortase A-mediated native peptide ligation, yielding exendin-DBCO. To ensure that the conjugation is both bioorthogonal—to avoid side-reactions of POEGMA with any internal residue in exendin—and site-specific, we chose to synthesize azide-terminated POEGMA copolymers. The azide moiety readily reacts with the DBCO group and does not react with any other chemical groups in exendin, yielding a site-specific conjugate with a 1:1 stoichiometry ([Supplementary Fig. 11]).

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

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

Table 1. Characterization of exendin variants. EG2% was calculated using NMR spectroscopy. Mn, Mw, and Đ values were determined by size exclusion chromatography multi-angle light scattering (SEC-MALS). Rg was measured by dynamic light scattering (DLS) (n=10). DP was calculated by dividing polymer Mw by the monomer Mw. The EC50 of exendin variants was determined from the dose-response curves in Fig. 2c, Fig. 2i, and Fig. 3c (n=10). Data are reported as mean ± standard error of the mean (SEM). Tc was measured at 500 µM. *Calculated
from the amino acid sequence. †Default value due to the monodisperse nature of the peptide. The indices in column 1 of the table — *T<sub>t</sub>, *M<sub>w</sub>, and polymer type are explained in the text.

In the first phase of optimization, to identify an Ex-POEGMA conjugate with an optimal T<sub>t</sub>, we first conjugated exendin to POEGMAs that have a varied monomer composition and hence T<sub>t</sub>, but all of which had a near-constant *M<sub>w</sub> of ~57 kDa (Table 1; index *T<sub>t</sub>). We chose an *M<sub>w</sub> of ~57 kDa, as it was previously shown to be the *M<sub>w</sub> that maximized the PK of a soluble Ex-POEGMA conjugate (Table 1; Supplementary Fig. 12). All conjugates had a low polydispersity and had a similar R<sub>h</sub> (Table 1; Supplementary Fig. 13). In addition, they showed reversible phase behavior (Fig. 2a), and the T<sub>t</sub> of all conjugates were between 28-32ºC, allowing the conjugates to remain in solution at room temperature and transition into an insoluble coacervate when injected s.c. at an injection concentration of 500 µM (see circled data in Fig. 2b). The T<sub>t</sub> of the conjugates also showed an inverse concentration-dependence (Fig. 2b), indicating that they should release from the depot into the blood in response to dilution at the depot’s margins. The conjugates with a greater EG2 content had a lower T<sub>t</sub>, as the EG2 unit is more hydrophobic than the EG3 unit. (Table 1; Supplementary Fig. 14). Finally, the conjugates were tested for their ability to activate exendin’s endogenous receptor, termed glucagon-like peptide 1 receptor (GLP1R), in an in 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 (EC₅₀) 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 EC₅₀ 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 Mₚ but varied Tₜ 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 Tₜ. Circled data shows Tₜ 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 Tₜ but varied Mₚ as
temperature increased, demonstrating phase behavior at 25µM and (H) at varying concentrations to demonstrate inverse concentration-dependence of T_c. (I) In vitro activity of the conjugates (n=6). (J) Blood glucose normalized to t=0, (K) AUC of blood glucose, and (L) 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. Data were analyzed by two-way repeated-measures ANOVA followed by post hoc Dunnett’s multiple comparison test. *The last time point that blood glucose for treatment is significantly lower than that of PBS treated mice. The AUC for glucose exposure was analyzed using two-way ANOVA, followed by post-hoc Tukey’s multiple comparison test. #The conjugate with the lowest AUC for glucose exposure. Data showed the mean ± SEM and were considered statistically significant when p<0.05.

We next examined the fed blood glucose control provided by the Ex-POEGMA conjugates by administering them s.c. into 11-week-old male diet-induced obese (DIO) C57BL/6J mice. The equivalent dose of exendin and equivalent volume of PBS were used as positive and negative controls, respectively. Mice treated with exendin and the conjugates had lower blood glucose levels (Fig. 2d; Supplementary Fig. 15) and body weight (Fig. 2f) than the PBS-treated group. Exendin provided a shorter temporal duration of control than the conjugates, likely due to its short half-life. The most hydrophobic conjugate tested—Ex-POEGMA_28.4—provided only modest control, possibly because it released only a small amount of drug from the depot. The Ex-POEGMA_29.9 conjugate outperformed others by providing six days of glucose control and had the lowest area under the curve (AUC) for glucose exposure (Fig. 2e), which accounts for both magnitude and duration of blood glucose reduction. This result was consistent with the literature, where the optimal T_C of an injectable elastin-like polypeptide (ELP) drug depot was also reported to be ~30°C.

Having identified an Ex-POEGMA conjugate with an optimal T_C, we next identified an optimal M_w. Our strategy was to synthesize Ex-POEGMA conjugates with the optimal T_C of ~30°C but with varying M_w (Table 1; Index M_w)—18.9 kDa, 54.3 kDa, 99.3 kDa, and 171.4 kDa (Supplementary Fig. 16). As the T_C is a function of the M_w, we carried out an iterative set of polymer syntheses to create POEGMA copolymers that have the similar T_C —close to ~30 °C across a range of concentrations— but varied M_w by titrating the ratio of EG2 to EG3 in the monomer feed of the synthesis, leading to the set of polymers shown in Table 1 and listed under Index: M_w. The polymers with a lower M_w had a greater mole fraction of the more hydrophobic EG2 monomer in the POEGMA copolymers
and vice versa (Supplementary Fig. 17). These polymers were then conjugated to exendin. The resulting Ex-POEGMA conjugates had varied $R_h$ (Supplementary Fig. 18) consistent with their different $M_w$, but all showed thermally reversible phase behavior (Fig. 2g). However, the $T_t$ of the conjugates had a slightly different inverse concentration dependence, as seen by the different slopes of the log(concentration) versus $T_t$ plots (Fig. 2h). Importantly, these plots intersected at 500 µM and ~30°C (see circled data in Fig. 2h), a temperature that fortuitously corresponds to the previously identified optimal $T_t$. This concentration of 500 µM was hence chosen as the injection concentration for all in vivo studies to identify the optimal $M_w$ with constant $T_t$ of ~30°C. No significant correlation was observed between in vitro activity of the conjugates as seen by their EC$_{50}$ and the $M_w$ of conjugates (Fig. 2i).

The conjugates were next administrated into 11-week-old male DIO mice ($n=6$), with PBS as a negative control. Mice treated with the conjugates had lower blood glucose (Fig. 2j; Supplementary Fig. 19) and body weight (Fig. 2l) than the PBS-treated group. The conjugate with the lowest $M_w$ tested—Ex-POEGMA$_{18.9}$—controlled blood glucose only one day and had a significantly higher glucose exposure as seen by the AUC (Fig. 2k) than all other treatment groups. This data suggests that Ex-POEGMA$_{18.9kDa}$ was cleared from the circulation much faster than the other conjugates because of its significantly smaller size ($R_h$ ~2.6 vs. ~4-5 nm for the other conjugates) that is lower than the renal excretion threshold, defined as the size of serum albumin (~3.6 nm).$^{18,25}$ This data is consistent with the literature, where PEG and ELP conjugates show a prolonged half-life at an $M_w$ threshold of 30 kDa$^{35,36}$ and 35.8 kDa$^{25}$, respectively. In addition, the higher $M_w$ Ex-POEGMA conjugates differed in their duration of glucose control. Ex-POEGMA$_{99.4}$ and Ex-POEGMA$_{171.4}$ showed modest glucose control for three days, while Ex-POEGMA$_{54.3}$ outperformed the rest with six days of glucose control and had the lowest AUC for glucose exposure. This conjugate —Ex-POEGMA$_{54.3}$— was defined as the $T_t$- and $M_w$- optimized conjugate and is subsequently referred to as Ex-POEGMA$_{opt}$ in this paper. Finally, the optimal injection dose of this conjugate was determined to be 1000 nmol kg$^{-1}$ in a dose-escalation study, which completed the optimization process of the conjugate (Supplementary Fig. 20).
**Short-term efficacy.** We next investigated the short-term efficacy of Ex-POEGMA<sub>opt</sub> compared to its soluble POEGMA and PEG counterparts. We synthesized the soluble counterpart of Ex-POEGMA<sub>opt</sub>—termed Ex-POEGMA<sub>sol</sub>—using POEGMA consisting of only EG3 monomers at the near-identical $M_w$ of $\sim$57 kDa (Supplementary Fig. 21). In preliminary studies, we found that linear PEG has a much larger hydrodynamic size than hyperbranched POEGMA of the same $M_w$, resulting in conjugates with a much larger $R_h$ at the same $M_w$. Because this difference could complicate side-by-side efficacy comparison of the conjugates by affecting their kidney clearance rates, we synthesized both $M_w$-matched and $R_h$-matched exendin-PEG conjugates—termed Ex-PEG<sub>Mw</sub> and Ex-PEG<sub>Rh</sub> (Table 1; Supplementary Fig. 22-23). Ex-POEGMA<sub>opt</sub> reversibly phase transitioned below body temperature (Fig. 3a), allowing it to remain as a solution in a syringe at room temperature but to transition into an insoluble coacervate phase when injected s.c., as tested at a concentration of 500 µM (see circled data in Fig. 3b). In contrast, Ex-POEGMA<sub>sol</sub> phase-transitioned well above body temperature at all concentrations, indicating that it cannot form a depot and would remain soluble when injected into the s.c. space. Neither Ex-PEG<sub>Mw</sub> nor Ex-PEG<sub>Rh</sub> showed phase behavior (Fig. 3a). The conjugates showed no difference in their EC<sub>50</sub> in activating the GLP1R in an *in vitro*, cell-based assay (Fig. 3c).

The conjugates were next administered s.c. into 11-week-old male DIO mice ($n=6$) at the equivalent, optimal dose, with PBS included as a control. Mice treated with the conjugates had lower fed blood glucose levels than the control (Fig. 3d; Supplementary Fig. 24). Ex-PEG<sub>Rh</sub> and Ex-PEG<sub>Mw</sub> controlled fed blood glucose for three and four days, respectively (Fig. 3d), consistent with the literature. The one-day longer glucose control provided by Ex-PEG<sub>Mw</sub> could be attributed to its larger size, and hence slower clearance. In accord with the literature, Ex-POEGMA<sub>sol</sub> also provided four days of blood glucose control. In contrast, Ex-POEGMA<sub>opt</sub> outperformed its soluble POEGMA counterpart and the two PEGylated exendin controls by providing six days of fed blood glucose control and had the lowest glucose exposure of all conjugates (Fig. 3e), showing the enhanced efficacy provided by sustained release of the conjugate from an injectable s.c. depot. All soluble conjugates resulted in a much sharper and more significant weight loss than Ex-POEGMA<sub>opt</sub> (Fig. 3f), possibly because a sudden increase in drug
concentration of soluble conjugates can induce nausea and trigger transient weight loss, which is consistent with common side effects of exendin.\textsuperscript{38}

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

To confirm these results, we next tested if Ex-POEGMA\textsubscript{opt} also provided glycemic regulation by performing an intraperitoneal (i.p.) glucose tolerance test (IPGTT) in $db/db$ mice on days 1, 3, and 5 post-injection of the treatments. Exendin-treated mice displayed similar glucose intolerance compared to the PBS-treated mice, indicated by hyperglycemia, presumably because it cleared from circulation before IPGTTs were performed due to its short half-life (Fig. 3j-l).\textsuperscript{34,40} There was a clear trend in glucose exposure on days 1 and 3 (Supplementary Fig. 26), with Ex-POEGMA\textsubscript{opt} regulating blood glucose more efficiently than Bydureon. Ex-PEG\textsubscript{Rh} had a progressive loss of glycemic lowering by Day 3 due to its faster clearance. Notably, Ex-POEGMA\textsubscript{opt} showed superior glycemic regulation to Ex-PEG\textsubscript{Rh} throughout the study by providing the lowest glucose exposure among the treatments. This difference was also observed when the experiment was repeated using DIO mice (Supplementary Fig. 27).

However, the magnitude of the difference was not as great, possibly due to the milder display of T2D allowing lower circulating Ex-PEG\textsubscript{Rh} concentrations to show therapeutic effect. The test time did not affect glucose
exposure in the Ex-POEGMA<sub>opt</sub> treatment group (p>0.99) (Fig. 3l), indicating that Ex-POEGMA<sub>opt</sub> maintained its superior glycemic regulation ability throughout the study.

Figure 3. Ex-POEGMA<sub>opt</sub> outperforms its soluble POEGMA and PEG counterparts and a clinical sustained-release exendin formulation, Bydureon, in fed blood glucose and glycemic control. The optical density of treatments (A) as temperature increased and decreased, demonstrating reversible phase behavior for Ex-POEGMA<sub>opt</sub> and Ex-POEGMA<sub>sol</sub> at 25µM and (B) at varying concentrations to demonstrate inverse concentration-dependence of T<sub>t</sub>. Circled data show T<sub>t</sub> 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, (F) and percent weight change relative to weight at t=0 after treating
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 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 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 blood glucose was quantified. Data were analyzed by two-way repeated-measures ANOVA, followed by post hoc Dunnett’s multiple comparison test. *The last time point that blood glucose for treatment is significantly lower than that of PBS treated mice. The AUC for glucose exposure was analyzed using two-way ANOVA, followed by post-hoc Tukey’s multiple comparison test. #The conjugate with the lowest AUC for glucose exposure. Data showed the mean ± SEM and were considered statistically significant when p<0.05.

Pharmacokinetics. We next investigated the PK of Ex-POEGMA_{opt} and the PEGylated controls to better understand the differences in the short-term efficacy profiles of the treatments. We fluorescently labeled exendin, Ex-POEGMA_{opt}, Ex-PEG_{Rh}, and Ex-PEG_{Mw} (Supplementary Fig. 28), followed by s.c. administration into naive DIO mice (n=4). The PK parameters were determined from the drug’s plasma concentration (Fig. 4a-b) and are shown in Table 2. All conjugates had much slower absorption kinetics than exendin, suggesting that the conjugates were primarily taken up from the s.c. space into the blood through the lymphatic system due to their large size. Ex-POEGMA_{opt} had the slowest absorption (t_{1/2 absorption}=9.0 ± 1.8h) among the treatments, possibly due to its amphiphilic structure and sustained release. Exendin had a fast elimination half-life (t_{1/2 elimination}=2.1 ± 0.5h), defined as the time needed to reach half-maximal blood concentration, consistent with its small size (Fig. 4a).

The conjugates had much longer elimination half-lives due to their slower renal clearance (Fig. 4b). Ex-PEG_{Rh} (t_{1/2 elimination}=23.1 ± 6.4h) and Ex-PEG_{Mw} (t_{1/2 elimination}=34.9 ± 9.1h) prolonged exendin’s circulation by ~11- and ~17-fold, respectively. The 55.6 kDa soluble Ex-POEGMA conjugate with a matching M_w as the optimal depot-forming POEGMA conjugate had a t_{1/2 elimination} of 61.2 ± 5.0h. In contrast, Ex-POEGMA_{opt} outperformed all conjugates by prolonging exendin’s circulation by ~46-fold with a t_{1/2 elimination} of 97.3 ± 5.6h, and it provided the highest drug exposure (AUC) among the treatments. These results clearly demonstrate the PK benefits of sustained release from a depot. This study also allowed us to infer that the minimal effective concentration for Ex-POEGMA_{opt} is 5.1 nM, which corresponds to the concentration of Ex-POEGMA_{opt} after 6 days post-injection.
Figure 4. Pharmacokinetics of Ex-POEGMA\textsubscript{opt}. Fluorescently labeled (A) exendin and (B) Ex-PEG\textsubscript{MW}, Ex-PEG\textsubscript{Rh}, and Ex-POEGMA\textsubscript{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\textsubscript{Rh}, and (E) Ex-POEGMA\textsubscript{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 ± SEM.

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

Table 2. Pharmacokinetic parameters of Ex-POEGMA<sub>opt</sub>. Pharmacokinetic parameters were calculated from the data given in Figure 4. Data were fitted to a one-phase exponential decay curve and analyzed via non-compartmental pharmacokinetic analysis using GraphPad Prism 8 software. Absorption phase pharmacokinetics of Ex-PEG<sub>Rh</sub> in immunized mice were not calculated (N/C) due to its low goodness-of-fit values. Data showed the mean ± SEM.

| Treatment       | t<sub>1/2</sub> absorption (h<sup>-1</sup>) | t<sub>1/2</sub> elimination (h<sup>-1</sup>) | t<sub>max</sub> (h)* | C<sub>max</sub> (nM)* | AUC (h x nM) |
|-----------------|------------------------------------------|------------------------------------------|---------------------|----------------------|--------------|
| Naïve Mice      |                                          |                                          |                     |                      |              |
| Exendin         | 0.6 ± 0.1                                 | 2.1 ± 0.5                                | 1.6 ± 0.2           | 31.3 ± 15            | 188 ± 18     |
| Ex-PEG<sub>Rh</sub> | 6.6 ± 0.5                                | 23.1 ± 6.4                               | 16.6 ± 2.2          | 34.6 ± 2.4           | 1585 ± 112   |
| Ex-PEG<sub>Mw</sub> | 6.6 ± 1.7                                | 34.9 ± 9.1                               | 19.5 ± 4.9          | 35.3 ± 4.5           | 2206 ± 84    |
| Ex-POEGMA<sub>opt</sub> | 9.0 ± 1.8                                | 97.3 ± 5.6                               | 32.1 ± 5.8          | 35.8 ± 2.6           | 3664 ± 167   |
| Immunized Mice  |                                          |                                          |                     |                      |              |
| Exendin         | 0.8 ± 0.1                                 | 1.9 ± 0.5                                | 1.7 ± 0.3           | 36.9 ± 6.8           | 159 ± 18     |
| Ex-PEG<sub>Rh</sub> | N/C                                      | 11.4 ± 5.4                               | N/C                 | N/C                  | 424 ± 35     |
| Ex-POEGMA<sub>opt</sub> | 12.1 ± 2.0                               | 105.7 ± 12                               | 42.6 ± 6.4          | 30.3 ± 4.0           | 3664 ± 167   |
Figure 5. Long-term efficacy of Ex-POEGMA<sub>opt</sub>. (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<sub>Rh</sub>, Ex-POEGMA<sub>opt</sub>, Bydureon, or PBS for eight weeks. (C) Percent glycated hemoglobin (Hb1Ac%). Data represent the mean ± 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. In humans, IgG responses are also observed in some individuals, though the mechanism of class switching is not well understood. 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<sub>opt</sub>). We administrated sterile and endotoxin-free Ex-POEGMA<sub>opt</sub>, Ex-PEG<sub>Mw</sub>, 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
conjugation chemistry than Ex-PEG\textsubscript{Mw} and Ex-POEGMA\textsubscript{opt} (urethane linkage in OVA-based conjugates vs. strained alkyne-azide linkage in exendin-based conjugates) (Supplementary Figure 31; Supplementary Table 2). Each drug-conjugated bead set had a different fluorescent barcode, allowing us to measure the signal detected by each of them when multiplexed. Mouse IgG- or IgM- coupled beads in diluent were used as positive controls, while the multiplexed beads in diluent served as a negative control. The immunoassay’s optimization and validation are summarized in the Supplementary Information, and supplementary data are shown in Supplementary Fig. 31-32 and Supplementary Tables 2-8.

Ex-POEGMA\textsubscript{opt}, Ex-PEG\textsubscript{Mw}, and exendin treatments induced a transient IgM response against exendin (Fig. 6b-c). No anti-exendin IgG response was detected (Supplementary Fig. 33). This data is consistent with the literature\textsuperscript{44}, where only mild induction of anti-exendin antibodies was reported in clinical trials. In contrast, Ex-PEG\textsubscript{Mw} induced a significant and persistent anti-PEG IgM response (Fig. 6b-c), consistent with the literature.\textsuperscript{7,8,42} PEG specificity was indicated by the significant signal measured with both Ex-PEG and OVA-PEG beads. Anti-PEG antibodies were strictly IgM, and no class switching to IgG was observed (Supplementary Fig. 33). PEG-specific IgM response increased with the number of Ex-PEG\textsubscript{Mw} injections (Supplementary Fig. 34). Notably, the induced PEG antibodies did not bind POEGMA conjugated bead sets, indicating that POEGMA eliminates PEG antigenicity in agreement with the literature.\textsuperscript{18,19} Strikingly, Ex-POEGMA\textsubscript{opt} did not induce anti-POEGMA antibodies, indicating that it is non-immunogenic.

We further tested the immunogenicity of POEGMA and compared it to PEG using highly immunogenic OVA as its conjugation partner. Briefly, OVA, OVA-PEG, OVA-POEGMA, and PBS (negative control) were repeatedly injected s.c. into naïve C57BL6/J mice (n=10) in accordance with the timeline in Fig. 6a. The Luminex immunoassay was used to quantify the ADAs induced by the treatments with minor changes from the previous experiment. Briefly, OVA, OVA-PEG, and OVA-POEGMA coupled beads were used to quantify ADA responses to the full immunizing material, while Ex-PEG and Ex-POEGMA coupled beads were employed as irrelevant protein conjugates to measure responses to the polymer domains only. The exendin-coupled bead was used as a cross-
reactivity control. Free, PEG-conjugated, and POEGMA-conjugated OVA induced significant anti-OVA IgG responses by Day 44 (Supplementary Fig. 35a-b). The OVA-PEG conjugate induced lower titers of anti-OVA IgG than unmodified OVA (Supplementary Fig. 35b). This result was attributed to the steric hindrance imparted by the PEG, allowing the conjugate to expose fewer OVA epitopes to the immune system. The differences in anti-OVA antibody titers were not as drastic when POEGMA was used. We speculate that this is because POEGMA provides less steric hindrance than PEG to its conjugation partners due to its more compact architecture. PEG was highly immunogenic, as seen by the high titers of PEG-specific ADAs detected in OVA-PEG-immunized mice plasma with Ex-PEG beads (Supplementary Fig. 35c-d). Similar to when exendin was the conjugation partner, PEG-specific ADAs were restricted to the IgM class (Supplementary Fig. 35c-d), possibly due to the lack of T-cell help for class switching, as reported in the literature. The PEG-specific IgM titer was ~60-fold higher with OVA-PEG than Ex-PEG, possibly due to the higher immunogenicity of OVA. In contrast, only a mild anti-POEGMA IgM response was detected after the first drug injection. The anti-POEGMA response was not detectable at later time points (Supplementary Fig. 36d) and did not mature into an IgG response (Supplementary Fig. 36h), further supporting our conclusions that POEGMA appears to be significantly less immunogenic than PEG.
Figure 6. **Ex-POEGMA\textsubscript{opt} does not induce anti-POEGMA antibodies.** (A) Timeline of PBS, exendin, Ex-PEG\textsubscript{Mw}, or Ex-POEGMA\textsubscript{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\textsubscript{Mw}, or Ex-POEGMA\textsubscript{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 ± 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.\textsuperscript{46}
Freund’s adjuvants (FA) significantly enhance the titer and affinity of the immune response induced towards co-administered immunogens by extending their presentation, stimulating the innate immune system, and facilitating T-cell help, commonly allowing immunogens to reveal their most immunogenic state.\(^{47}\) We hypothesized that POEGMA should at most induce a weak IgM immune response. We tested this hypothesis by administering PBS (- control), OVA, OVA-PEG, and OVA-POEGMA s.c. into naïve C57BL/6J mice (n=10) in FA emulsions. Blood was collected by following the timeline in Fig. 6a, processed into plasma, and tested by a Luminex immunoassay. Strikingly, a POEGMA-specific IgM response was observed transiently and disappeared after the first injection (Supplementary Fig. 37-38). In contrast, CFA/IFA emulsions magnified the anti-OVA and anti-PEG responses by ~2-fold. Notably, no change in the specificity and subtype of the immune response developed towards the treatments was detected (Supplementary Fig. 37). In sum, this data confirmed the immunogenicity benefits of POEGMA over PEG even delivered with a potent adjuvant.

**Effect of ADAs on long-term efficacy.** When immunogenicity and long-term efficacy data were evaluated together, they suggested that the inferior blood glucose control with the increasing number of injections and poor HbA1c% control by Ex-PEG\(_{Rh}\) could result from anti-PEG antibody interference. To test this hypothesis, we next measured the ADA levels in the plasma of long-term treated mice. To do so, plasma was collected on day 66 after 8 weekly treatments and assayed for ADAs by the Luminex immunoassay. Both exendin and Bydureon induced a mild anti-exendin IgM response. Notably, Ex-POEGMA\(_{opt}\) did not induce anti-POEGMA antibodies and resulted in a much weaker anti-exendin IgM response, possibly due to the non-immunogenic nature of POEGMA and the shielding of exendin’s immunogenic epitopes by POEGMA (Supplementary Fig. 39a). Notably, anti-exendin IgM titers were higher in Ex-PEG\(_{Rh}\) treated mice plasma, and an anti-exendin IgG response was detected (Supplementary Fig. 39b). Ex-PEG\(_{Rh}\) also induced both PEG-specific IgM and IgG responses. Given the absence of exendin- and PEG-specific IgGs in the prior immunogenicity experiment, the observation of IgGs towards both 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.\textsuperscript{42}

We next investigated if the ADAs could neutralize the treatments. We analyzed long-term treated mice plasma for neutralizing antibodies (NAb) using the cell-based assay developed to test the GLP1R-binding by exendin variants with minor modifications.\textsuperscript{44} Assay design and optimization are summarized in the \textit{Supplementary Information} and given in \textit{Supplementary Table 9} and \textit{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 (\textit{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\textsubscript{Mw} by incubating the conjugate with the plasma samples of the mice immunized with FA emulsion of OVA-PEG. Ex-PEG\textsubscript{Mw} showed no change in activity (\textit{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\textsubscript{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.\textsuperscript{42,48} We also hypothesized that Ex-POEGMA\textsubscript{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\textsubscript{Rh}, and Ex-POEGMA\textsubscript{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 (\textit{Fig. 4c; Table 2}), indicating that anti-exendin antibodies were not PK-altering, consistent with the literature.\textsuperscript{44} Importantly, Ex-PEG\textsubscript{Rh} showed significantly different PK profiles in naïve and immunized mice (\textit{Fig. 4d; Table 2}). Its absorption was significantly lower in the immunized mice, suggesting local lymph nodes' involvement in its elimination.\textsuperscript{49} Ex-PEG\textsubscript{Rh}’s elimination half-life was \textasciitilde2-fold shorter in the immunized mouse (t\textsubscript{1/2 elimination}=11.4 \pm 5.4 h vs. 23.1 \pm 6.4 h), indicating the PK-altering nature of anti-PEG antibodies. Together, the \textasciitilde4-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<sub>Rh</sub> treatment. Notably, Ex-POEGMA<sub>opt</sub> 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<sub>opt</sub> had any histopathological effects and compared it to Ex-PEG<sub>Rh</sub>, which induces significant vacuolization in major organs, and with Bydureon, exendin, and PBS (- control).<sup>50</sup> We collected kidney, pancreas, spleen, and thyroid tissues from the <i>db/db</i> mice (<i>n</i>=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<sub>Rh</sub> 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<sub>Rh</sub>-treated mice (Supplementary Fig. 43d), possibly because of GLP1R targeting by exendin<sup>51</sup> and vacuole formation by the conjugated PEG. No changes were noted within the splenic or thyroid tissues treated with Ex-PEG<sub>Rh</sub> (Supplementary Fig. 44). Importantly, Ex-POEGMA<sub>opt</sub> 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 ~30°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 ~30°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 ~30°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 ~30°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. 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. 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
positive population, which is now rampant because of chronic exposure to PEG through excipients in drugs and
consumer products. These findings are highly topical because up to 70% of the human population has pre-existing
antibodies to PEG,\textsuperscript{11} and billions of individuals are in the process of being immunized with a PEG-containing
liposome formulation of the Pfizer and Moderna vaccines for COVID-19. There is also a growing consensus that
PEG is responsible for the immediate-type hypersensitivity reactions\textsuperscript{52,53} seen with the Moderna and Pfizer COVID-
19\textsuperscript{54} and other vaccines.\textsuperscript{55} Fourth, another favorable feature of POEGMA is that it does not exhibit the PEG-induced
vacuolization problem, presumably due to its partially biodegradable and non-immunogenic structure. We
speculate that cleavage of the OEG side chains from the polymethacrylate backbone through the ester bonds by
esterase enzymes in the blood\textsuperscript{56} reduces overall hydration, thereby conjugate size, allowing POEGMA to clear by
the kidneys. Finally, injectable and depot-forming POEGMA technology allows chromatography-free purification
of conjugated drugs, eliminating a significant time-, labor-, and cost-intensive step in manufacturing PEGylated
drugs.

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

The POEGMA depot technology has several advantages over other drug delivery technologies. First,
POEGMA combines sustained-release and extended circulation in one molecule. Most exendin formulations, such
as Bydureon, release the unmodified exendin peptide into the bloodstream that, after release, circulates only for
a few hours, while the POEGMA depot releases exendin as a POEGMA conjugate that has \textasciitilde46-fold longer half-life
in the circulation than the unmodified peptide. The molecular design of the POEGMA conjugate also eliminates
the variable drug loading seen with PLGA\textsuperscript{57} and nanoparticle-based formulations\textsuperscript{58} as exendin is covalently conjugated with POEGMA at a known—equimolar—stoichiometry. In recent years, the Fc fusion technology—wherein a peptide or protein drug is presented on an IgG Fc scaffold—has become increasingly popular because it provides a long plasma half-life due to the Fc component of the fusion\textsuperscript{59} and because its manufacturing leverages the established expertise of the bioprocessing industry in overexpression of antibody drugs in engineered mammalian cell lines. However, the Fc fusion technology is not a panacea for all peptide and protein drugs that have a short circulation time, as not all drugs can be expressed as Fc fusions because even subtle changes in the Fc structure can dramatically alter the potency and function of the fused drug.\textsuperscript{59} In contrast to Fc fusions restricted to genetically encodable peptides and proteins, the injectable POEGMA drug depot technology applies to all biologics—peptide, protein, and oligonucleotide drugs that have suboptimal PK. The fact that the injectable POEGMA depot technology is competitive with Fc fusion can be inferred by comparing the results presented herein exendin with a preclinical study on Trulicity—a once-weekly FDA-approved Fc fusion of an exendin analog GLP-1. Trulicity required twice-weekly injections in \textit{db/db} mice to adequately control blood glucose, whereas a single injection of Ex-POEGMA\textsubscript{opt} provided six days of blood glucose control in the same animal model. Allometric scaling suggests that Ex-POEGMA\textsubscript{opt} can be administered twice-monthly to T2D patients,\textsuperscript{60} necessitating less frequent injections and thereby promoting better patient adherence, currently a major problem in diabetes management. Third, a POEGMA depot releases a steady amount of drug into circulation to achieve a desired therapeutic effect. In contrast, PLGA\textsuperscript{57} and nanoparticle-based sustained-release exendin formulations\textsuperscript{58} have a burst release profile, characterized by a peak-and-valley PK.\textsuperscript{61} Fourth, Ex-POEGMA conjugate eliminates the need for surgical implantation, replacement, and removal seen with an exendin pump —TCA 650 (Intarcia),\textsuperscript{62} thereby patient discomfort. Finally, POEGMA shields exendin’s immunogenic epitopes, while free exendin-releasing technologies do not have a mechanism for reduced immunogenicity, resulting in higher ADA titers and potentially altered drug efficacy. Our results supported this assertion, where no anti-exendin ADAs were detected after long-term treatment with Ex-POEGMA\textsubscript{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 (CuBr₂) in ultrapure water with 18.2 MΩhm 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 µl 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 $D$ of POEGMA were assessed by GPC-MALS. POEGMA was solubilized in tetrahydrofuran (THF) at 2 mg ml⁻¹, followed by filtration through a 0.22 µm Teflon syringe filter. 50 µl of the solution was separated on an Agilent PLgel mixed-C column (105 Å, 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 $D$.

**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
in the optical density with temperature indicates the occurrence of the phase transition. The \( T_t \) was defined as the
temperature at the inflection point of the optical density versus the temperature curve and is calculated as the
maximum of the first derivative using GraphPad Prism 8.0 software. The reversibility of the phase behavior was
investigated by monitoring the optical density as the temperature was gradually decreased from a temperature
above the \( T_t \) back to 15ºC.

**Protein expression and purification.** Exendin was recombinantly expressed in *E. coli* from a synthetic gene in a
pMA-T expression vector as an ELP fusion protein with a sortase-A recognition site (LPETG) and a polyhistidine
tag, yielding exendin-LPETG-His\(_6\)-ELP (ESE). The ELP tag enables rapid non-chromatographic purification of ESE,
while the LPETG peptide acts as the sortase ligation site for the ATRP initiator. Both ESE and His\(_6\)-Sortase A were
available from a previous study and expressed and purified to >95% purity, as previously described.\(^{18}\) The purity
of all proteins was visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 4-
20% precast Tris-HCl gels (Bio-Rad), followed by staining with Simply Blue Safe Stain (Thermo Scientific) and gel
densitometry analysis using Image Lab software (Bio-Rad). The ESE concentration was measured using a
Bicinchoninic Acid (BCA) assay (Pierce) according to the manufacturer’s instructions. The His\(_6\)-Sortase A
concentration was measured by UV–visible spectroscopy on an ND-1000 Nanodrop spectrophotometer (Thermo
Scientific) and the known extinction coefficient of the protein (14440 M\(^{-1}\) cm\(^{-1}\)).

**Synthesis, purification, and characterization of exendin-DBCO.** A bio-orthogonal DBCO group was installed on
the C-terminus of exendin by sortase A-mediated native peptide ligation, yielding exendin-DBCO. Briefly, ESE (100
\( \mu \)M) and His\(_6\)-Sortase A (50 \( \mu \)M) were reacted in the presence of triglycine-DBCO (Gly\(_3\)-DBCO) (5 mM; Click
Chemistry Tools) in ligation buffer (50 mM Tris, 150 mM NaCl, and 10 mM CaCl\(_2\); pH 7.5) at room temperature for
16 hours. Exendin-DBCO was purified from the reaction mixture by reverse immobilized metal affinity
chromatography using an AKTA Purifier (GE Healthcare) equipped with a photodiode array operating at 220 and
280 nm and a HisTrap HP (GE Healthcare) column. Exendin-DBCO was collected in the flow-through as it was the
only species that did not carry an oligohistidine tag, and hence did not bind to the resin. Exendin-DBCO was
concentrated by ultrafiltration using Centricon 70 (Millipore Sigma) filters with a 3,000 Da molecular weight cut-off (MWCO), followed by dialysis into cold water and lyophilization. Stoichiometric (1:1) attachment of DBCO to exendin was confirmed by Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight mass spectroscopy (MALDI-TOF-MS).

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

**Physical characterization of exendin conjugates.** The conjugates were characterized in terms of their $M_n$, $M_w$, and $D$ by SEC-MALS using an Agilent 1260 analytical HPLC equipped with a UV detector operating at 280 nm (Agilent), a DAWN HELEOS II MALS detector (Wyatt Technology), and an Optilab T-rEX refractive index detector (Wyatt Technology). The DAWN HELEOS II MALS detector was annually calibrated in toluene and normalized with 2 mg ml$^{-1}$ bovine serum albumin (Pierce) before each analysis. The exendin conjugates were dissolved in 10 mM phosphate buffer at pH 7.4, followed by filtration through a 100 nm syringe filter (Whatman). 50 µl of the 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 $D$ 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, Prospec 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
exendin variants. The effective half-maximal dose (EC_{50}) of each exendin variant was determined by fitting the dose-response curve to a four-parameter logistic, nonlinear regression model using GraphPad Prism 8 software.

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

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

**Fed blood glucose measurements.** In the short-term efficacy experiments, the fed blood glucose was measured after a single s.c. injection of the treatments. On the day of injection, the tail was sterilized with alcohol pads (BD). The first drop of blood collected from a tiny incision on the tail vein was wiped off. The second drop of blood was used to measure fed blood glucose using a hand-held glucometer (AlphaTrack, Abbott). The treatments were solubilized in PBS and kept on ice before injection to prevent phase transition, followed by s.c. administration into mice. Bydureon was prepared for injection according to the manufacturer's instructions. Fed blood glucose levels were measured 24 h and immediately before injection, at 1-, 4-, and 8-h post-injection, and every 24 h after that...
until no significant effect of treatments on fed blood glucose was observed. Body weight was tracked daily. In the long-term efficacy experiment, fed blood glucose and body weight were tracked every three days.

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

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

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

The fluorophore-labeled treatments were administered into DIO C57BL/6J mice via a single s.c. injection at 1000 nmol kg⁻¹ (45 nmol kg⁻¹ fluorophore). Ten µl of blood was collected from a tiny incision on the tail vein into tubes containing 90 µl of 1,000 U ml⁻¹ heparin (Sigma) at 5-min, 1-, 2-, 4-, 8-, 24-, 48-, 72-, 96-, 120-, 144- and 168-h. Blood samples were centrifugated at 1600 g at 4°C for 15 minutes for plasma. Fluorophore concentration in
plasma samples was detected by a Victor plate reader (Perkin Elmer) at 485 nm (excitation) and 535 nm (emission). PK parameters were derived by plotting the drug’s plasma concentration as a function of time and fitting it to a non-compartmental PK model for the absorption and elimination phases using GraphPad Prism software. The absorption phase described the time between injection at 0-h and $t_{max}$, where the drug concentration was maximum. The elimination phase described the time after $t_{max}$. $t_{max}$ was calculated from equation:

$$t_{max} = \frac{2.303}{\log \left( \frac{k_a}{k_e} \right)}$$

where $k_a$ and $k_e$ were the apparent absorption and elimination rate constants.

The rate constants were determined from the linear regression slope of the log (drug concentration) versus time graph using equation $k = -2.303 \times \text{slope}$. Half-lives of treatments in each phase were determined from the equation:

$$t_{1/2} = \frac{0.693}{k}$$

The maximum drug concentration ($C_{max}$) was calculated at $t_{max}$. Minimal effective conjugate concentration was calculated by triangulating the concentration values based on the duration of blood glucose control determined in short-term efficacy experiments in DIO mice.

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

**Immunogenicity.** We tested the immunogenicity of POEGMA and compared it to that of PEG in three sets of immunogenicity experiments. In the first set, sterile and endotoxin-free exendin, Ex-PEG$_{Mw}$, and Ex-POEGMA$_{opt}$ were s.c. administrated into 7-week-old C57BL/6J mice ($n=10$) at an equivalent dose (1000 nmol kg$^{-1}$) and concentration (500 µM). In the second set of experiments, mice were injected with OVA (Invivogen), OVA-PEG$_{10K}$, 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 emulsified in an equal volume of sterile CFA/IFA (Invivogen) and then administrated into 7-week-old C57BL/6J 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 injections. An equal injection volume of PBS (or PBS emulsified in CFA or IFA) was used as a negative control in all experimental sets. All drugs were administrated into mice every 17 days three times (Day 0, 17, and 34). The
Injection site was the loose skin over the shoulders and was kept the same across the injections. Blood samples were collected seven days before the first injection (Day -7) and ten days after each injection (Day 10, 27, and 44), followed by plasma isolation via centrifugation at 4°C at 1600 g for 15 minutes, aliquoting on ice, and storage at -80°C until analysis.

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

In a typical LMI assay, 50µl of the diluted plasma samples (n=3) were transferred to a black, round-bottom 96-well-plate (Corning). Next, spectrally distinct exendin-, Ex-PEG-, Ex-POEGMA-, OVA-, OVA-PEG-, and OVA-POEGMA-coupled magnetic bead sets were mixed at a concentration of 2500 beads per 50 µl per set. 50µl of the resulting solution was distributed to the wells of a 96-well plate and incubated for 1 hour on an orbital shaker to capture ADAs present in the plasma samples. After incubation, 96-well-plate was placed on a magnetic ring stand (Invitrogen) and incubated for 60 seconds for separation to occur. The supernatant was discarded, and wells were washed with 100 µl of the assay buffer twice. The resulting magnetic bead-ADA complexes were incubated with 100µl of 5 µg ml⁻¹ R-Phycoerythrin-conjugated goat anti-mouse IgG (Jackson Immunoresearch; #115-115-164) for 30 minutes to detect IgG subtypes of ADAs. 100µl of 5 µg ml⁻¹ biotin-conjugated goat anti-mouse IgM (Jackson Immunoresearch; #115-065-075) was used to detect IgM subtypes of ADAs, followed by washes, incubation with 100µl of 7.5 µg ml⁻¹ streptavidin-R-phycocerythrin (SAPE; Invitrogen) for 30 minutes, and a final round of washes. Mouse IgG- or IgM-coupled magnetic beads were used as positive controls. They were incubated with 100µl of 0.25 µg ml⁻¹ R-Phycoerythrin-conjugated goat anti-mouse IgG or 0.114 µg ml⁻¹ biotin-conjugated goat anti-mouse IgM, followed by 0.171 µg ml⁻¹ SAPE. The negative control was the drug-coupled bead mixture in the assay buffer.
Finally, magnetic beads were suspended in 100 µl of assay buffer, followed by measuring mean fluorescence signal intensity (MFI) using MAGPIX (Luminex) instrument. The average MFI value of each plasma sample and treatment was computed with no exception for outliers. Mouse plasma samples were considered ADA-positive if the average MFI for any given bead type was above the background (see Supplementary Information). MFI of each plasma sample for any given bead type was normalized to the positive control.

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

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

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**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.

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