A brush-polymer/exendin-4 conjugate reduces blood glucose levels for up to five days and eliminates poly(ethylene glycol) antigenicity

Yizhi Qi1, Antonina Simakova2, Nancy J. Ganson3, Xinghai Li1, Kelli M. Luginbuhl1, Imran Ozer1, Wenge Liu1, Michael S. Hershfield3,4, Krzysztof Matyjaszewski2 and Ashutosh Chilkoti1

The delivery of therapeutic peptides and proteins is often challenged by short half-lives and the consequent need for frequent injections that limit efficacy, reduce patient compliance and increase treatment cost. Here, we demonstrate that a single subcutaneous injection of site-specific (C-terminal) conjugates of exendin-4 (exendin)—a therapeutic peptide that is clinically used to treat type 2 diabetes mellitus—and poly(oligo(ethylene glycol) methyl ether methacrylate) (POEGMA) with precisely controlled molecular weights lowered blood glucose for up to 120 h in fed mice. Most notably, we show that an exendin-C-POEGMA conjugate with an average of nine side-chain ethylene glycol (EG) repeats exhibits significantly lower reactivity towards patient-derived anti-poly(ethylene glycol) (anti-PEG) antibodies than two US FDA-approved PEGylated drugs, and that reducing the side-chain length to three EG repeats completely eliminates PEG antigenicity without compromising in vivo efficacy. Our findings establish the site-specific conjugation of POEGMA as a next-generation PEGylation technology for improving the pharmacological performance of traditional PEGylated drugs, whose safety and efficacy are hindered by pre-existing anti-PEG antibodies in patients.

Therapeutic peptides and proteins are an important class of drugs, with more than 100 peptides and proteins approved by the US FDA to treat various diseases and many more in clinical and pre-clinical development1,2. However, the clinical use of all peptides and most proteins (with the exception of antibodies, albumin3 and transferrin4) is challenged by their short plasma half-life, necessitating frequent injections, which can cause an undesirable peak-to-valley fluctuation of the drug concentration in vivo as well as reduce patient compliance and increase treatment cost3. Other limitations of peptide and protein therapeutics may include poor stability, low solubility and immunogenicity6. To address these limitations, various delivery strategies have been developed for the sustained delivery of peptide and protein therapeutics, ranging from particulate systems and depots, to chemical conjugation with long-circulating polymers such as PEG, to recombinant fusion with long-circulating proteins such as albumin or the fragment crystallizable (Fc) domain of antibodies2,8.

PEGylation, or the covalent conjugation of therapeutic peptides and proteins (and more recently nucleotide-based drugs) with the ‘stealth’ polymer PEG, is one of the most widely used approaches to increase the circulation half-life and stability and to reduce the immunogenicity of these therapeutic biomolecules, as evident from the many PEGylated drugs approved by the FDA and the numerous others in development4,9,10. However, after close to four decades of research and over two decades of clinical use, the drawbacks of PEGylation have begun to emerge. Conventional methods for the synthesis of PEGylated conjugates have notable limitations: (i) conjugation involves the reaction between protein-repulsive PEG chains and biomacromolecules, so that even with a large excess of polymer, steric hindrance still results in a low yield of conjugate, typically in the 10–20% range11; (ii) the presence of a large excess of unreacted polymer makes product purification non-trivial; and (iii) conjugation typically involves reacting the chain ends of the polymer with reactive side groups on lysine and cysteine residues, which are often promiscuously distributed on the biomolecule, thus yielding chemically heterogeneous products that can compromise the bioactivity of the drug and greatly complicate regulatory approval12–15.

Furthermore, the immunogenicity of PEG has recently attracted much attention. Anti-PEG antibodies have been induced in patients treated with some PEGylated enzymes. In clinical trials of PEG-uricase16,17 and PEG-asparaginase18, these anti-PEG antibodies have markedly accelerated blood clearance, abrogated clinical efficacy and increased the risk and severity of infusion reactions. Circulating anti-PEG antibodies have also been found in individuals who are naive to PEGylated materials, and are possibly induced by chronic exposure to free PEGs present in commonly used consumer products19,20. High levels of such pre-existing anti-PEG antibodies have recently been linked to serious first-exposure allergic reactions to a PEGylated RNA aptamer, which led to the early termination of a clinical trial21.

To address the synthetic limitations of previous PEG conjugation methodologies, we have previously developed a strategy named sortase-catalysed polymer conjugation22. This strategy exploits the C-terminal native-peptide-ligation mechanism of the enzyme sortase A, derived from Staphylococcus aureus, to attach a polymerization initiator site specifically and stoichiometrically (1:1) at the C-terminus of a peptide or protein to enable grafting of the PEG-like brush polymer POEGMA from the peptide/protein macroinitiator by in situ atom transfer radical polymerization (ATRP) in aqueous buffer23,24. Although POEGMA has been grafted from various model proteins and peptides22,25–29, very few studies have reported POEGMA conjugates of clinically relevant biologic therapeutics for the systematic...
evaluation of the therapeutic potential of this class of conjugates, and conjugation was either carried out post-polymerization\(^{35}\) or was non-specific\(^{31}\). Using our sortase-catalysed polymer conjugation strategy, we grafted POEGMA site-specifically (C-terminal) and stoichiometrically (1:1) from exendin, a peptide drug that is clinically used to treat type 2 diabetes mellitus\(^{35}\). We show that a single subcutaneous injection of exendin-C-POEGMA reduces blood glucose for 120h in fed mice, which is 20 times longer than the reduction resulting from the injection of the unmodified peptide. Most intriguingly, we show that breaking up and appending PEG as short oligomeric side chains of optimized length on the conjugated POEGMA not only retains the long circulation of the POEGMA conjugates but also eliminates their reactivity to patient-derived PEG antibodies. These results demonstrate that the architecture of PEG appended to a biologic drug plays an important role in modulating its antigenicity. This finding is particularly relevant in a clinical context given the growing prevalence of pre-existing anti-PEG antibodies in the general population, which increasingly undermines the safety and efficacy of PEGylated therapeutics. Here, the terms ‘antigenicity’ and ‘immunogenicity’ are not interchangeable. Antigenicity is defined herein as the reactivity of an antigen towards pre-existing antibodies in patients, whereas immunogenicity refers to the intrinsic ability of an antigen to generate antibodies in the body.

**Results**

**Sortase-catalysed C-terminal initiator attachment to exendin.** We exploited the C-terminal native-peptide-ligation mechanism of sortase A to site-specifically attach the ATRP initiator 2-(2-(2-(2-aminoacetamido)acet-amido)acetamido) ethyl)-2-bromo-2-methylpropanamide (AEBMP) to the C-terminus of exendin (Fig. 1). A quaternary fusion protein, abbreviated to exendin-srt-His\(_6\)-ELP, was recombinantly expressed to serve as the sortase-A substrate (Fig. 1a). As explained in an earlier study\(^{32}\), ‘srt’ stands for the native sortase-A recognition sequence LPETG\(^3\), and ‘ELP’ refers to a stimulus-responsive elastin-like polypeptide that was incorporated to enable easy purification of the fusion protein by inverse transition cycling (ITC, Supplementary Fig. 1a), a nonchromatographic protein purification method that we previously developed\(^{34}\). The recognition sequence was deliberately located between the protein and the ELP, so that transpeptidation by sortase A not only attaches the initiator to exendin but also conveniently liberates the purification tag. Sortase A with an N-terminal hexahistidine tag (His\(_6\)-tag) was recombinantly expressed from a plasmid constructed in the earlier study\(^{32}\) and purified by immobilized-metal affinity chromatography (IMAC, Supplementary Fig. 1b). The ATRP initiator AEBMP (Fig. 1) was chemically synthesized with an N-terminal (Gly) motif serving as the nucleophile, as maximum reaction rates for sortase-catalysed C-terminal ligation have been reported with two or more glycines\(^{36}\).

Successful sortase-catalysed initiator attachment (Fig. 1b) resulted in the cleavage of exendin-LPETG-His\(_6\)-ELP into exendin-LPET and G-His\(_6\)-ELP, followed by the attachment of AEBMP to exendin-LPET to generate the macroinitiator product (exendin-C-Br). Sodium-dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the reaction mixture (Fig. 2a) showed >90% conversion to exendin-C-Br as assessed by gel densitometry. Similar to the previous study\(^{32}\), a His\(_6\)-tag was intentionally inserted between srt and ELP on exendin-srt-His\(_6\)-ELP fusion, such that on transpeptidation by His\(_6\)-sortase A, all of the residual reactants, enzyme and side products except the desired product—exendin-C-Br—carried a His\(_6\)-tag. Consequently, elution through an IMAC column yielded pure exendin-C-Br (Fig. 2a) in the eluent while leaving all other unwanted species bound to the resin.

**Synthesis and characterization of exendin-C-POEGMA conjugates.** Next, we conducted in situ activator regenerated by electron transfer (ARGET)-ATRP\(^{36}\) to graft POEGMA from exendin-C-Br (Fig. 1c). An OEGMA monomer with an average mass of ~500 Da or approximately nine side-chain EG repeats (EG9) was used, as shown by liquid chromatography electrospray ionization mass spectrometry (LC/ESI-MS) analysis (Supplementary Fig. 2a). The reaction time was varied to produce EG9 exendin-C-POEGMA conjugates with a range of weight-average molecular mass \(M_n\) values. Size exclusion chromatography (SEC) analysis of exendin-C-Br before polymerization as detected by UV–visible absorbance at 280 nm (Fig. 2b) showed a single peak eluting at 23.7 min. After polymerization, the intensity of the macroinitiator peak greatly diminished and was accompanied by the appearance of peaks at 21.3, 19.5, 17.8, 16.5 and 15.0 min, corresponding to EG9 exendin-C-POEGMA conjugates with increasing \(M_n\) as the reaction time was increased. The results from UV–visible detection were in agreement with those from refractive index (RI) detection (Supplementary Fig. 3a). Integration of the peak areas in the UV–visible chromatograms showed that the average conjugation yield was ~80%. As shown in Table 1, the synthesized conjugates had number-averaged molecular mass \(M_n\) values that ranged from 25.4 to 155.0 kDa, and all conjugates had very narrow dispersities (\(D \leq 1.15\)). The conjugates could be easily and completely purified by a single round of preparative SEC (Supplementary Fig. 3b).

Exendin acts by binding and activating the G protein-coupled glucagon-like peptide (GLP)-1 receptor (GLP-1R), which results in the release of cyclic adenosine monophosphate (cAMP) as a second messenger in a downstream signalling cascade, ultimately leading to the secretion of insulin to regulate blood glucose\(^{37}\). The potency of native exendin and the EG9 exendin-C-POEGMA conjugates were next assessed by quantifying the intracellular cAMP release as a result of GLP-1R activation in baby hamster kidney (BHK) cells that were stably transfected with rat GLP-1R. As shown in Fig. 2c and Table 1, grafting EG9 POEGMA from exendin increases the half-maximum effective concentration \(EC_{50}\) of the peptide in a molecular-weight-dependent manner, which indicates decreased...
The therapeutic efficacy of EG9 exendin-C-POEGMA. The in vivo efficacy of EG9 exendin-C-POEGMA conjugates was assessed in male C57BL/6J mice that were maintained on a 60 kilocalorie (kcal)% fat diet so as to develop a diabetic phenotype. Male C57BL/6J mice were maintained for a week on a 60 kcal% fat diet so as to develop a diabetic phenotype.

The glucose profile of the 155.0 kDa conjugate in particular resembled that of a sustained-release depot, with no peak-to-valley effect to mice measured at various time points post-injection revealed an overall smaller magnitude and duration of the effect dependent on \( M_n \). The area under the overlaid glucose profiles are presented in Supplementary Fig. 6a (overlaid unnormalized glucose measurements in fed mice collectively show that an increase in \( M_n \) delays the onset but prolongs the duration of glucose reduction, and the two higher \( M_n \) conjugates showed an overall smaller magnitude of glucose reduction. This trend is also mirrored by the weight profiles of treated animals (Supplementary Fig. 6c). The two higher \( M_n \) conjugates also showed much more flat and steady glucose profiles. The glucose profile of the 155.0 kDa conjugate in particular resembled that of a sustained-release depot, with no peak-to-valley effect that can cause undesirable side effects.

The in vitro cAMP results and the in vivo \( M_n \)-dependent glucose measurements in fed mice collectively show that an increase in \( M_n \) of the conjugated polymer decreases the potency but increases the circulation duration of the EG9 exendin-C-POEGMA conjugate. We therefore hypothesize that an optimal \( M_n \) of the conjugate exists that can best balance these two opposing effects. The area under

In vivo therapeutic efficacy of EG9 exendin-C-POEGMA. The in vivo efficacy of EG9 exendin-C-POEGMA conjugates was assessed in male C57BL/6J mice that were maintained on a 60 kilocalorie (kcal)% fat diet so as to develop a diabetic phenotype. A dose-dependent study was first performed to determine an adequate dose. A 66.2 kDa EG9 exendin-C-POEGMA conjugate was administered to mice via a single subcutaneous injection of the conjugate at 25, 50 and 85 nmol kg\(^{-1}\) of mouse body weight. Blood glucose levels in fed mice measured at various time points post-injection revealed an overall smaller magnitude increase in the duration of glucose reduction with increasing doses of the conjugate compared with the PBS control (Supplementary Fig. 4a,b and Supplementary Table 1). A similar trend was observed in the mouse body weights, where the mice treated with the highest dose showed considerably more weight loss than those treated with the lower doses (Supplementary Fig. 4c).

Although the weight-lowering benefit of exendin has been well established\(^{6,15}\), overdosing can cause nausea, which can lead to acute weight loss in rodents\(^{14}\). The excessive weight loss seen in mice that received the highest dose suggests the possibility of nausea; therefore all subsequent studies used a dose of 25 nmol kg\(^{-1}\).

To investigate the effect of \( M_n \) on the glucose regulatory effect of EG9 exendin-C-POEGMA conjugates, native exendin and conjugates of four different \( M_n \) values (\( M_n = 25.4, 54.6, 97.2 \) and 155.0 kDa) were tested at a single subcutaneous injection at 25 nmol kg\(^{-1}\) of mouse body weight. While unmodified exendin was only able to lower blood glucose for 6 h relative to the PBS control (Fig. 3a; full glucose profiles are presented in Supplementary Fig. 5), modification with EG9 POEGMA significantly extended the glucose-lowering effect of exendin for up to 12 h, with the onset, magnitude and duration of the effect dependent on \( M_n \) (Fig. 3b–e, Supplementary Table 2). As is evident from the overlaid glucose profiles in Supplementary Fig. 6a (overlaid unnormalized glucose profiles are presented in Supplementary Fig. 6b), an increase in \( M_n \) delays the onset but prolongs the duration of glucose reduction, and the two higher \( M_n \) conjugates showed an overall smaller magnitude of glucose reduction. This trend is also mirrored by the weight profiles of treated animals (Supplementary Fig. 6c). The two higher \( M_n \) conjugates also showed much more flat and steady glucose profiles. The glucose profile of the 155.0 kDa conjugate in particular resembled that of a sustained-release depot, with no peak-to-valley effect that can cause undesirable side effects.

The in vitro cAMP results and the in vivo \( M_n \)-dependent glucose measurements in fed mice collectively show that an increase in \( M_n \) of the conjugated polymer decreases the potency but increases the circulation duration of the EG9 exendin-C-POEGMA conjugate. We therefore hypothesize that an optimal \( M_n \) of the conjugate exists that can best balance these two opposing effects. The area under

![Table 1](https://example.com/table1.png)

**Table 1** | Physical properties and biological activity of exendin and exendin-C-POEGMA conjugates.

| Species | Reaction time (h) | \( M_n \) (Da) | \( M_t \) (Da) | \( \Theta \) (\( M_t \)/\( M_n \)) | \( R_g \) (nm) | \( E_{cAMP} \) (nM) |
|---|---|---|---|---|---|---|
| Exendin | 0.5 | 26,400 | 4,186.6* | 1.00 | 2.2 ± 0.1 | 0.08 ± 0.01 |
| EG9 | 1 | 56,800 | 54,600 | 1.04 | 4.5 ± 0.4 | 0.84 ± 0.09 |
| EG9 | 1.25 | 72,200 | 66,200 | 1.09 | 5.9 ± 0.5 | 2.10 ± 0.08 |
| EG9 | 2 | 100,000 | 97,200 | 1.03 | 6.8 ± 0.7 | 6.67 ± 0.21 |
| EG9 | 3 | 178,000 | 155,000 | 1.15 | 7.6 ± 0.5 | 7.69 ± 0.04 |
| EG3 | 3 | 27,400 | 26,300 | 1.04 | 3.8 ± 0.4 | 3.29 ± 0.27 |
| EG3 | 5.5 | 60,600 | 55,600 | 1.09 | 4.8 ± 0.5 | 4.17 ± 0.13 |
| EG3 | 8 | 82,700 | 71,600 | 1.16 | 5.4 ± 0.6 | 5.11 ± 0.23 |

*Calculated from the amino acid sequence. Default value due to the unmolecular nature of the peptide. \( M_t \) and \( \Theta \) values were determined by SEC-MALS. \( R_g \) was measured by dynamic light scattering. The \( E_{cAMP} \) values of the EG9 and EG3 conjugates were derived from the cAMP response curves in Fig. 2c and Supplementary Fig. 7, respectively. \( R_g \) and \( E_{cAMP} \) values are reported as mean ± SEM, n = 10 for \( R_g \) and n = 3 for \( E_{cAMP} \).
We tested Table 1 - Fig. 3f

ARTICLES

FDA-approved drugs, Adagen (a PEGylated adenosine deaminase din-C-POEGMA conjugate and various controls, including two sorbant assay (ELISA). In a direct ELISA, the 54.6 kDa EG9 exen treated with PEGylated proteins using enzyme-linked immuno to anti-PEG antibodies in plasma samples of patients previously the reactivity of the 54.6 kDa EG9 exendin-C-POEGMA conjugate Antigenicity of EG9 exendin-C-POEGMA conjugates.

post-injection the AUC was reduced by 48% for conjugate-treated cose challenge was reduced by 68% (post-injection, the AUC of blood glucose level over 2 h after glu IPGTT confirmed the prolonged presence of the conjugate in circulation and its significant effect on glycaemic control: at 24 h 54.6 kDa EG9 conjugate or of unmodified exendin at 25 nmol kg (IPGTT) at 24 h and 72 h after a single subcutaneous injection of the obtain further evidence of the efficacy of EG9 exendin-C-POEGMA action. We thus investigated the 54.6 kDa EG9 conjugate further in subsequent experiments.

the curve (AUC) of the glucose profiles in fed mice with respect to a 0% baseline signifies total glucose exposure, which accounts for both the magnitude and the duration of the glucose reduction, and is therefore a manifestation of the combined effect of the two opposing factors. Plotting the AUC of glucose levels as a function of con jugate M₉ yielded a roughly inverted bell-shaped distribution with a minimum at 54.6 kDa (Fig. 3f). This suggests that the 54.6 kDa conjugate is optimal among the tested EG9 conjugates in terms of balancing receptor activation potency and sustained duration of action. We thus investigated the 54.6 kDa EG9 conjugate further in subsequent experiments.

To validate the results from the glucose measurements and to obtain further evidence of the efficacy of EG9 exendin-C-POEGMA conjugates, we performed an intraperitoneal glucose tolerance test (IPGTT) at 24 h and 72 h after a single subcutaneous injection of the 54.6 kDa EG9 conjugate or of unmodified exendin at 25 nmol kg⁻¹. IPGTT confirmed the prolonged presence of the conjugate in circulation and its significant effect on glycemic control: at 24 h post-injection, the AUC of blood glucose level over 2 h after glucose challenge was reduced by 68% (P < 0.0001, Fig. 4a) and at 72 h post-injection the AUC was reduced by 48% for conjugate-treated mice compared with PBS controls (P < 0.01, Fig. 4b). This is in stark contrast to the unmodified exendin group, which was insignificant at both time points (Fig. 4c and d).

Antigenicity of EG9 exendin-C-POEGMA conjugates. We tested the reactivity of the 54.6 kDa EG9 exendin-C-POEGMA conjugate to anti-PEG antibodies in plasma samples of patients previously treated with PEGylated proteins using enzyme-linked immuno sorbant assay (ELISA). In a direct ELISA, the 54.6 kDa EG9 exen din-C-POEGMA conjugate and various controls, including two FDA-approved drugs, Adagen (a PEGylated adenosine deaminase for treating severe combined immunodeficiency disease (SCID)) and Krystexxa (a PEGylated uricase for treating chronic refractory gout), were directly coated on a plate and probed with diluent, an anti-PEG-negative patient plasma sample, or one of two anti-PEG-positive patient plasma samples. As shown in Fig. 5a, although the EG9 exendin-C-POEGMA conjugate did show a small amount of binding to anti-PEG antibodies in the positive plasma samples, the extent of binding was significantly less than that of the two PEGylated-positive controls. This result was confirmed by a competitive ELISA, where Krystexxa was coated on wells, and different amounts of 54.6kDa EG9 exendin-C-POEGMA and controls were added in solution to compete for binding to anti-PEG antibodies in an anti-PEG-positive plasma sample. As can be seen in Fig. 5b, at all tested competing antigen amounts, the antibody binding of 54.6kDa EG9 exendin-C-POEGMA was significantly less than that of the positive control, Adagen.

Exendin-C-POEGMA with shorter side-chain length. The previous results led us to hypothesize that the reduced PEG antigenicity of the EG9 exendin-C-POEGMA conjugate is due to both the branched architecture and the short side-chain length of the conjugated POEGMA. As a minimum length of PEG is presumably needed for antibody recognition and binding, we hypothesized that optimizing the side-chain OEG length may further reduce or possibly eliminate the antigenicity of POEGMA conjugates to anti-PEG antibodies. To test this hypothesis, we synthesized exendin C-POEGMA conjugates using OEGMA monomer with precisely three EG side-chain repeats as seen by LC/ESI-MS (Supplementary Fig. 2b), as evidence in the literature suggests that the antigenic determinant of PEG may be approximately six to seven EG repeats. Three different EG3 exendin-C-POEGMA conjugates with M₉ values of 26.3, 55.6 and 71.6 kDa (Table 1) were synthesized. Assessment of the
conjugate potency by intracellular cAMP ELISA (Supplementary Fig. 7) showed that, similarly to the EG9 conjugates, conjugation of EG3 POEGMA to the C-terminus of exendin increased the $EC_{50}$ (Table 1), indicating a decrease in the receptor activation of the conjugates, although the dependence on $M_c$ was less pronounced.

Antigenicity and efficacy of EG3 exendin-C-POEGMA conjugates. We next tested the reactivity of a 55.6 kDa EG3 exendin-C-POEGMA conjugate to anti-PEG antibodies in patient plasma samples. The 54.6 kDa EG9 conjugate was included as a control to anti-PEG antibodies in patient plasma samples. The 54.6 kDa EG9 and 55.6 kDa EG3 conjugates were administered to fed mice via a single subcutaneous injection at 25 nmol kg$^{-1}$ of mouse body weight. As can be seen from the post-injection glucose profiles in Fig. 6a, unconjugated glucose levels and weight profiles in Supplementary Fig. 8), both conjugates significantly reduced mouse blood glucose for up to 96 h compared with the PBS control. The EG3 conjugates seem to have slightly lower magnitudes of glucose reduction and flatter glucose profiles than their EG9 counterparts.

Pharmacokinetics of exendin-C-POEGMA conjugates. To further confirm the prolonged circulation of exendin-C-POEGMA conjugates and to seek some reasons for the difference between the glucose profiles of EG9 and EG3 conjugates, a pharmacokinetics study was performed with fluorescently labelled exendin, and the 54.6 kDa EG9, 55.6 kDa EG3 and 71.6 kDa EG3 conjugates. Two $M_c$ values of the EG3 conjugate were tested, as the EG3 and EG9 conjugates have different hydrodynamic radii ($R_h$) at the same $M_c$. These $M_c$ values were chosen such that the 54.6 kDa EG9 conjugate ($R_h = 5.4 \pm 0.6$ nm) had an $M_c$ similar to that of the 55.6 kDa EG3 conjugate and an $R_h$ similar to that of the 71.6 kDa EG3 conjugate ($R_h = 5.6 \pm 0.5$ nm). The plasma-concentration–time courses (Fig. 6c, d) were analysed using a non-compartmental fit that characterized the absorption and
elimination phases of the pharmacokinetic profiles to approximate the parameters shown in Table 2.

After subcutaneous injection, unmodified exendin had a very short residence time in circulation, with a short absorption half-life ($t_{1/2a}$ = 0.7 ± 0.1 h) and a short terminal elimination half-life ($t_{1/2el}$ = 1.7 ± 0.2 h). In contrast, the exendin-C-POEGMA conjugates tested increased the absorption time by ~9-fold to 13-fold, with the two EG3 conjugates taking longer than the EG9 conjugate to absorb into circulation. Similarly, the 54.6 kDa EG9 conjugate prolonged the elimination phase of exendin by ~25-fold, whereas the two EG3 conjugates afforded a bigger increase of ~36-fold. These differences in the pharmacokinetics resulted in an ~20-fold increase in the AUC for the conjugates compared with unmodified exendin, indicating that conjugation of POEGMA to the C-terminus of exendin significantly enhanced the cumulative exposure of the peptide in circulation. Although the peak drug concentrations in plasma ($C_{\text{max}}$) of the two EG3 conjugates were lower than that of the EG9 conjugate, consistent with the lower magnitude of glucose reduction seen for the EG3 conjugates in the blood glucose studies in fed mice (Fig. 6a,b), the AUCs of the three tested conjugates were comparable given the longer absorption and elimination half-lives of the EG3 conjugates.

Discussion

Using our previously developed sortase-catalysed polymer conjugation strategy, we synthesized site-specific (C-terminal) and stoichiometric (1:1) exendin-C-POEGMA conjugates with an average overall yield of up to >70%, which compares favourably with the 10–20% overall yield often seen in conventional PEGylation processes. The small fraction of unreacted exendin-C-Br in the in situ ATRP reactions is probably due to hydrolysis or disproportionation at the C-terminus of the macrominitiator, which are common

Figure 5 | Assessment of the reactivity of exendin-C-POEGMA conjugates towards anti-PEG antibodies in patient plasma samples. a, Direct ELISA probing 54.6 kDa EG9 exendin-C-POEGMA conjugate, native exendin, ADA, BSA, Krystexxa and Adagen with diluent (1% BSA in PBS), an anti-PEG negative patient plasma sample, or one of two anti-PEG positive plasma samples. b, Competitive ELISA, where various amounts of exendin, 54.6 kDa EG9 exendin-C-POEMGA, ADA and Adagen were allowed to compete with Krystexxa for binding with anti-PEG antibodies in a positive plasma sample. c, d, Direct (c) and competitive (d) assays as described in a and b performed with a 55.6 kDa EG3 exendin-C-POEGMA conjugate. In all assays, the same unmodified peptide/protein content or similar PEG/OEG content in the case of polymer-modified samples per well were compared. See Methods section for details. Results are plotted as mean ± SEM, n = 3 in a, b; n = 5 in c, d. Data were analysed by two-way ANOVA followed by post hoc Dunnett’s multiple comparison test (**P < 0.01; ****P < 0.0001). OD$_{405}$ optical density at 405 nm.
The stoichiometry of conjugation, and the high degree of $M_w$ tunability and low dispersity, are highly desirable features for polymer conjugates of therapeutic biomolecules, as they translate to a more predictable therapeutic performance. EG9 exendin-C-POEGMA conjugates lowered blood glucose levels in fed mice for up to 20 times longer than unmodified exendin injected at the same dose. This prolonged duration of the therapeutic effect substantially reduces the required administration side reactions of ATRP in aqueous conditions that lead to loss of Br functionality and thus inactivation of the macroinitiator. Additionally, as a controlled radical-polymerization technique, ATRP enabled the synthesis of conjugates with a wide and tunable range of $M_w$ values and very narrow $M_w$ distributions. Together, our synthesis approach gives rise to polymer conjugates of therapeutic biomolecules that are far more homogeneous than PEGylated drugs that are now in clinical use. This full control over the site and stoichiometry of conjugation, and the high degree of $M_w$ tunability and low dispersity, are highly desirable features for polymer conjugates of therapeutic biomolecules, as they translate to a more predictable therapeutic performance.

EG9 exendin-C-POEGMA conjugates lowered blood glucose levels in fed mice for up to 20 times longer than unmodified exendin injected at the same dose. This prolonged duration of the therapeutic effect substantially reduces the required administration

![Graph](https://example.com/graph.png)

**Figure 6** | Assessment of the in vivo efficacy and pharmacokinetics of exendin-C-POEGMA conjugates. **a,b.** Blood glucose levels in fed mice measured before and after a single subcutaneous injection of 55.6 kDa (a) and 71.6 kDa (b) EG3 exendin-C-POEGMA conjugates at 25 nmol kg$^{-1}$ or of PBS at equivalent volume, all administered at $t=0$ h. Blood glucose levels were normalized to the average glucose levels measured 24 h before and immediately before injection. Data were analysed by two-way repeated-measures ANOVA followed by post hoc Dunnett’s multiple comparison test ($n=5$). $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. **c,d.** Exendin (c) and exendin-C-POEGMA conjugates (d) were fluorescently labelled with Alexa Fluor 488 and injected into mice ($n=3$) subcutaneously at 75 nmol kg$^{-1}$ (45 nmol kg$^{-1}$ fluorophore). Blood samples were collected via the tail vein at various time points for fluorescence quantification. Data were analysed using a non-compartmental fit (solid lines) to derive the pharmacokinetic parameters shown in Table 2. Results in all panels are plotted as the mean ± SEM.

**Table 2** | Pharmacokinetic parameters of exendin and the exendin-C-POEGMA conjugates, calculated from data in Fig. 6c,d.

| Exendin/conjugates | $t_{1/2a}$ (h) | $t_{1/2e}$ (h) | $C_{max}$ (nM)* | $t_{max}$ (h)* | AUC (h × nM)$^\dagger$ |
|-------------------|----------------|----------------|----------------|----------------|---------------------|
| Exendin           | 0.7 ± 0.1      | 1.7 ± 0.2      | 371 ± 3.8      | 1.78 ± 0.1     | 217.5 ± 36.5        |
| 54.6 kDa EG9      | 6.2 ± 0.5      | 42.4 ± 2.9     | 56.4 ± 3.9     | 271 ± 0.4      | 4,795.5 ± 440.7     |
| 55.6 kDa EG3      | 7.6 ± 0.7      | 61.2 ± 5.0     | 44.0 ± 2.7     | 28.5 ± 2.3     | 4,775.0 ± 482.9     |
| 71.6 kDa EG3      | 9.0 ± 1.7      | 61.5 ± 3.2     | 37.7 ± 5.0     | 32.4 ± 3.9     | 4,411.2 ± 499.6     |

*Derived from curve fitting. $^\dagger$Calculated from $t=0$ to $\infty$ from curve fitting. Values are reported as mean ± SEM. $t_{max}$: time required to attain $C_{max}$.
frequency, a benefit that is critical to a disease whose treatment outcomes rely heavily on patient compliance. The blood glucose study of the EG9 conjugates showed that an increase in \( M_0 \) was correlated with a slower onset of glucose reduction, and that the two higher \( M_0 \) conjugates provided an overall smaller magnitude of glucose reduction. We speculate that these findings result from, in part, the reduced receptor binding activity and also perhaps a retarded diffusion from the subcutaneous space into systemic circulation, both affected by an increase in conjugate size.

The trends observed in blood glucose levels are also evident in mouse body-weight profiles. A drop in body weight at the beginning of the study is expected for all mice, including for the PBS control group, as the mice are subjected to stress factors including injection and repetitive tail-vein glucose measurements. This stress-induced response is also evident in the sharp spike in their glucose levels at the initial time points. The PBS group in the blood glucose measurement study of the 71.6 kDa EG3 exendin-C-POEGMA conjugate had a more pronounced weight drop than in the other glucose studies. This is probably because mice in these studies were fed a 60 kcal% fat diet, which makes them metabolically less stable, and weight variations are thus more common. The slightly higher weight loss in the PBS group in this particular experiment is consistent with a more sustained initial increase in blood glucose, suggesting that this batch of mice reacted more strongly to the procedural stress.

Two critical—and opposing—features of protein–polymer conjugates that are clearly illustrated by this study are the inverse relationships between conjugate \( M_0 \) and potency versus circulation duration. Up to a threshold value, the \( M_0 \) of the conjugate is expected to be directly proportional to the circulation duration but inversely proportional to the receptor-binding activity of the biomolecule of interest. Plotting the AUC of blood glucose profiles of the tested EG9 exendin-C-POEGMA conjugates as a function of conjugate \( M_0 \), which accounts for both the magnitude and the duration of glucose reduction, gives an inverted bell-shaped distribution with a minimum at 54.6 kDa, suggesting that the 54.6 kDa conjugate is the optimal among the tested conjugates in terms of balancing receptor-activation potency and sustained duration of action. The result also suggests that the renal clearance threshold for these EG9 conjugates is between 25.4 and 54.6 kDa, although the 25.4 kDa conjugate is more potent than the 54.6 kDa conjugate in vitro, its overall glucose-lowering effect is smaller than that of the 54.6 kDa conjugate, and its effect also diminished more quickly, which indicates a much faster renal clearance.

The antigenicity of EG9 exendin-C-POEGMA conjugates, or their reactivity towards pre-existing anti-PEG antibodies in patients, was of particular interest because the serious consequences of PEG immunogenicity have become increasingly apparent. Anti-PEG antibodies have been induced in patients treated with PEGylated drugs and have been shown to correlate with rapid clearance of these drugs\(^{16-18}\). High levels of pre-existing anti-PEG antibodies have also been found in individuals who are naïve to PEGylated agents and are associated with serious and sometimes life-threatening first-exposure allergic reactions\(^{21}\). Therefore, prescreening for polymer conjugates of therapeutic peptides and proteins for antigenicity towards anti-PEG antibodies is crucial.

We found that the 54.6 kDa EG9 exendin-C-POEGMA conjugate showed significantly less reactivity towards anti-PEG antibodies in patient plasmas than Adagen and Krystexxa. More intriguingly, reducing the side-chain length of the conjugated POEGMA to three EG repeats completely eliminated antibody binding. We speculate that the complete elimination of the anti-PEG antigenicity of EG3 POEGMA has two potential molecular explanations. First, three EG repeats may be shorter than the epitope recognized by anti-PEG antibodies, which is consistent with a previous report that the antigenic determinant of PEG may be six to seven repeat units\(^{46}\). Second, distributing PEG as oligomeric side chains may create a 'stacking effect' that hinders antibody access. Given that POEGMA is not present in any current pharmaceutical or consumer product, it is reasonable to speculate that humans would not have any pre-existing antibodies to the polymer. Thus, clinically, the lack of anti-PEG antigenicity of POEGMA conjugates is expected to translate to the complete elimination of serious and sometimes life-threatening first-exposure allergic reactions in patients towards POEGMA conjugates of therapeutics, and should eliminate the accelerated blood clearance of POEGMA–drug conjugates due to pre-existing anti-PEG antibodies in patients. We believe this is a particularly timely and useful finding that will address the pressing problem of increasing levels of pre-existing anti-PEG antibodies in the general population, which is compromising the safety and efficacy of FDA-approved PEGylated drugs and hindering the progress of other PEG conjugates in clinical and pre-clinical development. Given that PEGylation remains the most widely used technology to extend the half-life and improve the bioavailability of biologic drugs clinically, a method to tackle the emerging problem of antigenicity of PEG by modulation of its architecture—from linear to branched—provides a new approach to solve this problem. We note that this study addresses the anti-PEG antigenicity of POEGMA conjugates of biologic drugs but does not make any claims about their intrinsic immunogenicity. Although immunogenicity is also a crucial aspect regarding the safety of this class of conjugates, a thorough investigation would involve extensive in vivo studies performed in multiple species, which is beyond the scope of this study.

Glucose measurement studies confirmed that the elimination of antigenicity did not occur at the cost of in vivo efficacy. While the magnitude of glucose reduction of the EG3 conjugates is slightly lower than that of the EG9 conjugates, the glucose profiles resembled that of a sustained-release depot, without the peak-to-valley effect that is often associated with parenteral drug administration and that can cause unwanted side effects. Pharmacokinetic comparison of the 55.6 kDa and 71.6 kDa EG3 conjugates with the 54.6 kDa EG9 conjugate showed that although the \( C_{\text{max}} \) values of the EG3 conjugates were smaller than those of the EG9 conjugate, their longer absorption and elimination half-lives gave rise to AUCs that are comparable with those of the EG9 conjugate. This finding is consistent with the flatter and steadier glucose profiles of the EG3 conjugates. We speculate that the relatively higher hydrophobicity of EG3 POEGMA may cause its conjugates to remain in the subcutaneous space or lodge in other tissues during their course in circulation for more extended periods of time, thus creating a depot-like effect.

POEGMA was polymerized from the C-terminus of exendin in this study because the N-terminus of the peptide is important for GLP-1R binding and activation\(^{37}\). We note that although we have demonstrated the utility of the sortase-catalysed C-terminal polymer conjugation strategy with exendin in this study, it is a generally applicable platform for improving the pharmacological performance of therapeutic proteins and peptides where the C-terminus is not essential for the activity of the biomolecule. For other biologics where the C-terminal end may be critical to its activity, we have developed a complementary N-terminal approach\(^{38}\). If both the N- and the C-termini of the peptide/protein are critical to its activity, methods developed by us and other investigators can be used for the site-specific incorporation of an ATRP initiator at solvent-accessible sites within the primary amino acid sequence of the peptide or protein drug\(^{44,45}\). These methods collectively offer a general toolbox that should enable site-specific POEGMA conjugation at any desired solvent-accessible site on a peptide or protein.

Collectively, our results establish site-specific POEGMA conjugation as a next-generation PEGylation technology that is highly useful for improving the pharmacological performance of therapeutic biomolecules while providing a solution to the increasing levels of pre-existing anti-PEG antibodies in patients that limit the safety and
efficacy of traditional PEGylated drugs. Although these results are promising, we believe that a deeper understanding of the mechanism of action, and of the efficacy and the safety of exendin-C-POEGMA conjugates—particularly the EG3 variants—is necessary for clinical translation. Future plans include optimizing the $M_n$ of EG3 exendin-C-POEGMA conjugates to maximize efficacy, measuring insulin release, investigating the in vivo biodistribution of the conjugates, and studying the long-term parameters of therapeutic efficacy such as glyated haemoglobin (HbA$_1c$), glycated albumin and pancreatic β-cell proliferation to understand the long-term therapeutic potential of exendin-C-POEGMA conjugates. Furthermore, assessment of the intrinsic immunogenicity of exendin-C-POEGMA conjugates is essential to ensure their long-term safety and efficacy for clinical translation.

**Methods**

**Experimental design.** All in vitro and in vivo experiments include suitable controls; where applicable, a standard negative control and unmodified exendin served as a positive control. The sample sizes for in vivo studies were chosen on the basis of similar studies conducted previously45–47. See the Animal studies section below for details on the animal model used. Mice were randomly grouped before the initiation of each experiment. The investigator was not blinded to group allocation. For the in vivo glucose measurement studies in fed mice, modelled glucose levels were measured until all of the experimental groups no longer showed statistical significance in glucose reduction compared with the PBS control group. All collected data points were included in the data analysis.

**Cloning, expression and purification.** All molecular biology reagents were purchased from New England Biolabs unless otherwise specified. The gene encoding exendin in a pMA-T vector was codon-optimized and synthesized by Life Technologies. The first methionine residue encoding the translational start codon in proteins recombinitely expressed in *E. coli* needs to be cleaved post-translationally for proper function and stability of the protein48. However, the first amino acid of exendin is a histidine, and our past experience and reports in translationally for proper function and stability of the protein in proteins recombinantly expressed in *E. coli*48,49. The gene for sortase A was expressed and purified previously described with minor changes41. Briefly, a reaction mixture consisting of exendin-srt-His6-ELP, His$_6$-sortase A and AEBMP at a 2:1:60 ratio in sortase buffer was incubated at 20 °C for 18h. After the reaction, a reverse His-tag purification was used to isolate the exendin-C-Br macroinitiator by exploiting the fact that it is the only species in the mixture without a His$_6$-tag. Purification was performed on an AKTA Purifier (GE Healthcare) equipped with a photodiode detector set at 280 nm and a HiTrap HP column. Elution through the column with PBS yielded pure exendin-C-Br in the eluent while leaving all other unwanted species bound to the resin. The collected exendin-C-Br was dialysed overnight in PBS (pH 7.4) to remove residual free initiator.

**Macroninitiator characterization.** MALDI-MS was performed on a Voyager-DE Pro mass spectrometer (Life Technologies). Samples at ~25 μg/mL in PBS were dialted 1:10 with 10 mg/mL sinapinic acid in 90:10 water/acetonitrile, with 0.1 v/o trifluoroacetic acid (TFA) as the ionization matrix. The instrument was operated in positive mode with positive ion generation using an N$_2$ laser. Ubiqunon was used as a molecular weight standard to calibrate the instrument.

For LC/MS-MS analysis to confirm site specificity of initiator attachment, 100 μL of ~8 μg/mL exendin-C-Br in PBS was solvent-exchanged into 50 mM ammonium bicarbonate (pH 8.0) on a ZebaSpin desalting column (Thermo Fisher Scientific) followed by trypsin (sequencing grade) digestion overnight at 37 °C for 18h directly in the column. The digestion mixture was collected via centrifugation, dried by vacuum centrifugation and resuspended in 20 μL 2% acetonitrile and 0.1% formic acid in water. 1 μL of the sample was separated on a NanoAcuity ultra-performance liquid chromatography (UPLC, Waters) system equipped with a BEH130 C18 reversed phase column (Waters) using a mobile phase consisting of (A) 0.1% formic acid in water and (B) TFA 0.1% formic acid in acetonitrile. A linear gradient of 5% B to 40% B was performed over 60 min at 400 nm min$^{-1}$, and the separated peptides were ionized by electrospary ionization (ESI) followed by MS analysis on a Synapt G2 HDMS QToF mass spectrometer (Waters). The top four most abundant ions were selected for MS/MS. Mass spectra were processed with Mascot Distiller (Matrix Science) and then submitted to Mascot searches (Matrix Science) against a SwissProt_Ecoli database appended with the custom exendin-C-Br sequence. Search results were imported into Scaffold (v. 4.0, Proteome Software), and scoring thresholds were set to yield a minimum of 99% protein confidence for protein identification. Extracted ion chromatograms were performed in MassLynx (v. 4.1). Experimental isotope distributions of the brominated C-terminal tryptic peptide were compared with a theoretical isotope distribution modelled in Molecular Weight Calculator (v. 6.49, Pacific Northwest National Laboratory; http://nrcr.pnl.gov/software).

**In situ ARGRET-ATRP.** All chemical reagents were purchased from Sigma Aldrich and used as received unless otherwise specified. EG9 OEGMA monomer ($M_n$ 50 Da) or approximately nine cycles chain EG monomer was separated on a Superdex 200 size exclusion column (GE Healthcare) followed by trypsin (sequencing grade) digestion overnight at 37 °C for 18h directly in the column. The digestion mixture was collected via centrifugation, dried by vacuum centrifugation and resuspended in 20 μL 2% acetonitrile and 0.1% formic acid in water. 1 μL of the sample was separated on a NanoAcuity ultra-performance liquid chromatography (UPLC, Waters) system equipped with a BEH130 C18 reversed phase column (Waters) using a mobile phase consisting of (A) 0.1% formic acid in water and (B) TFA 0.1% formic acid in acetonitrile. A linear gradient of 5% B to 40% B was performed over 60 min at 400 nm min$^{-1}$, and the separated peptides were ionized by electrospary ionization (ESI) followed by MS analysis on a Synapt G2 HDMS QToF mass spectrometer (Waters). The top four most abundant ions were selected for MS/MS. Mass spectra were processed with Mascot Distiller (Matrix Science) and then submitted to Mascot searches (Matrix Science) against a SwissProt_Ecoli database appended with the custom exendin-C-Br sequence. Search results were imported into Scaffold (v. 4.0, Proteome Software), and scoring thresholds were set to yield a minimum of 99% protein confidence for protein identification. Extracted ion chromatograms were performed in MassLynx (v. 4.1). Experimental isotope distributions of the brominated C-terminal tryptic peptide were compared with a theoretical isotope distribution modelled in Molecular Weight Calculator (v. 6.49, Pacific Northwest National Laboratory; http://nrcr.pnl.gov/software).

**Characterization of OEGMA monomers.** Monomers diluted 1:20,000 in methanol were separated on an Agilent 1100 LC system equipped with a Zorbax Eclipse Plus C18 column (Agilent) using a mobile phase consisting of (A) 0.3% formic acid in water and (B) 0.3% formic acid in acetonitrile. A linear gradient of 50% B to 95% B was performed over 10 min at 50 °C. Separated samples were ionized by ESI followed by MS analysis on an Agilent MSD ion trap mass spectrometer.

**Physical characterization of exendin-C-POEGMA conjugates.** Analytical SEC was performed on a Shimadzu HPLC system equipped with a UV–visible detector (SPD-10A VP) operating at 280 nm. 50 μL of samples at ~2 mg/mL were separated on a Protein KW-803 column (Shodex) using 0.1M Tris-HCl pH 7.4 as mobile phase at 25 °C with a flow rate of 0.5 ml min$^{-1}$. The conjugation efficiency of in situ ATRP from exendin was calculated by quantifying the AUC of peaks detected at 280 nm.$^{50}$ The sum of the AUCs of the two peaks (corresponding to the unreacted macroninitiator and the conjugate in each chromatogram) was regarded as 100% and the per cent fraction of the...
conjugate peak was calculated as the conjugation efficiency of that particular polymerization reaction.

The fluid line of the analytical HPLC system was connected downstream in series to a DAWN HELEOS II multi-angle light scattering (MALS) detector followed by an Optilab T-REX refractometer (both from Wyatt Technology) for conducting SEC-MALS analysis. The system was calibrated with toluene and normalized with 2.0 mg/ml-bovine serum albumin (BSA, Pierce). Samples were passed through 0.1 μm filters before injection. The refractive index increment (dn/dc) values of the conjugates were determined on an Anton Paar Abbemat 500 refractometer (Anton Paar). Data were analysed in ASTRA (v. 6.0, Wyatt Technology) to compute Mr, Mw and Mda for the conjugates.

Conjugates were purified by a single round of preparative SEC on an AKTA Purifier equipped with a photodiode detector set at 280 nm and a HiLoad 26/600 Superdex 200 PG column using PBS as mobile phase at 4 °C and a flow rate of 2.0 ml/min.

Dynamic light scattering was performed on a DynaPro Plate Reader (Wyatt Technology). Samples were operated at 25 μM and filtered with 0.1 μm filters before analysis. The instrument was operated at a laser wavelength of 831.95 nm, a scattering angle of 90° and at 25 °C. Data were analysed in Dynals mode using Dynamics 6.12.0.3.

General biochemical analysis. The concentrations of the fusion proteins were measured on an ND-1000 Nanodrop spectrophotometer (Thermo Scientific) by UV–visible absorption spectroscopy. The concentrations of exendin and the conjugates for in vitro assays and in vivo studies were assayed using a Bicinchoninic Acid (BCA, Pierce) assay following manufacturer's instructions. SDS-PAGE analysis of sortase A was performed using precast 4–20% Tris-HCl gels (Bio-Rad). SDS-PAGE analysis of all exendin derivatives were performed using precast Tris/Tricine gels (Bio-Rad). Quantification of the sortase reaction conversion was conducted via a gel densitometry analysis using a built-in function in Image Lab (v. 4.0.1, Bio-Rad).

In vitro cAMP ELISA. The activity of native exendin and conjugates was assessed in vitro by quantifying the intracellular cAMP release as a result of a GLP-1 receptor activation in BHK cells that were stably transfected with rat GLP-1R (a gift from Drukker group, University of Toronto, Toronto, Canada)25. Cells were allowed to reach 70–80% confluence in 24-well plates. Before the assay, ~20 μg of peptide or the equivalent of conjugates were treated with 0.5 μg DPP4 (Prosect) overnight to remove the di-alanine leader. On the day of the assay, cells were incubated with 3-isobutyl-1-methylxanthine (IBMX, EMD Millipore) for 1 h to prevent cAMP degradation26, followed by incubation with varying concentrations (0.001–1,000 nM in log-scale increments) of exendin (Genscript) or conjugates for 10 min to trigger GLP-1 receptor activation. 0.1 M HCl was then added to disrupt the cells and release intracellular cAMP. cAMP concentration was measured via a competitive cAMP ELISA according to the manufacturer's protocol (Enzo Life Sciences). Each sample was assayed in triplicate and data were measured on an ND-1000 Nanodrop spectrophotometer (Thermo Scientific) at a scattering angle of 90° and at 25 °C. Data were analysed in Dynals mode using Dynamics 6.12.0.3.

In vivo IPGTT. Mice were randomly divided into groups (n = 5 in Fig. 4a, b, n = 3 in Fig. 4c, d). On day 1, every two groups of mice received a subcutaneous injection of either 54.6 kDa EG9 exendin-C-POEGMA conjugate, exendin as positive control, or PBS at equivalent volume as negative control. Exendin and the conjugate were injected at 25 nmol kg−1. 18 h after injection, one group of mice in each category was fasted by removal of food for 6 h. At the end of the fast period (24 h following injection), mice were given 1.5 g kg−1 glucose (10% w/v solids sterile glucose solution, Sigma) via intraperitoneal injection. Blood glucose levels were monitored by nicking the tail vein and measuring the glucose level in the blood using a glucometer at 0, 20, 40, 60, 90 and 120 min after glucose administration. At 66 h after injection, the remaining groups of mice were subjected to the same protocol and an IPGTT was similarly performed 72 h following injection.

In vivo pharmacokinetics. Exendin, 54.6 kDa EG9, 55.6 kDa EG3 and 71.6 kDa EG3 exendin-C-POEGMA conjugates were fluorescently labelled with Alexa Fluor 488 NHS ester (Thermo Fisher Scientific) via their solvent accessible primary amines on lysine residues and the N-terminus, according to the manufacturer's protocol. Unreacted free fluorophore was removed using a Zeba Spin desalting column (Thermo Fisher Scientific) with the labelled conjugates diluted in PBS at 1 mg/ml.

In vitro anti-P EG ELISA. In the direct ELISA, columns of a 96-well microtitre plate (CoStar) were coated with Krystexxa (Crealta Pharmaceuticals), adenosine deaminase (ADA, Sigma–Tau Pharmaceuticals), Adagen (Sigma–Tau Pharmaceuticals), exendin (Genscript), a 54.6 kDa EG9 exendin-C-POEGMA conjugate, a 55.6 kDa EG3 exendin-C-POEGMA conjugate or BSA (Sigma Aldrich). The antigen solutions for plate coating were prepared in PBS to yield 1 μg of peptide/protein or ~5 μg of PEG/EG in the case of polymer-modified antigens per well on adding 50 μl to each well. The PEG/EG content of the polymer-modified antigens were calculated as follows. Krystexxa contains the tetrameric uricase enzyme (125 kDa total) and the side-chain amino groups on each of its four subunits reacted with 10 kDa PEG N-(00)-nitrophenyl carbonate ester, giving a PEG content of ~78%. Adagen consists of ADA (40.8 kDa) with 11–17 of its side-chain amino groups on solvent-accessible amines functionalized with 5kDa monomethoxy succinyl PEG according to the manufacturer's specifications (Sigma–Tau Pharmaceuticals). For our calculation, we assumed 14 PEG conjugates on one molecule and gave a 60% PEG content. In the case of the exendin-C-POEGMA conjugates, subtracting the poly(methyl methacrylate) backbone (~17% for EG9 POEGMA and ~37% for EG3 POEGMA) gives an OEG content of ~75% for the 54.6 kDa EG9 conjugate and ~58% for the 55.6 kDa EG3 conjugate. After the coated plate was incubated overnight at 4 °C, it was washed with PBS and all wells were blocked with 1% BSA in PBS. One patient plasma sample previously tested negative for PEG antibody and two that were tested positive were diluted 1:400 vol/vol in 1% BSA in PBS. The two positive patient plasma samples were from two different individuals who developed anti-PEG antibodies during a phase II clinical trial of Krystexxa. Following another round of PBS washing, 100 μl of each diluted plasma sample and 1% BSA in PBS were added to replicate wells of each antipeptide. The plate was then incubated at room temperature for 2 h. Wells were then washed with PBS, and 100 μl of alkaline phosphatase-conjugated goat anti-human IgG (Sigma) diluted 1:5250 with 1% BSA in PBS was added to each well. After 1 h incubation at room temperature, wells were washed with PBS followed by Tris-buffered saline. Bound alkaline phosphatase was detected by incubating with p-nitrophényl phosphate (Sigma) in accordance with the directions of the supplier. The phosphatase reaction was stopped by adding 50 μl of 10% NaOH per well and the absorbance at 405 nm was measured on a plate reader (Tecan Infinite M200 Pro, Tecan Austria).

In the competitive ELISA, a microtitre plate was coated with 50 μl of 100 μg ml−1 Krystexxa per well by overnight incubation at 4 °C. Various amounts of ADA, Adagen, a 54.6 kDa EG9 exendin-C-POEGMA conjugate and a 55.6 kDa EG3 exendin-C-POEGMA conjugate were diluted with PBS to yield 0, 0.5, 2, 5, 10 and 20 μg of competing antigen per well on adding 50 μl to each
well. Dilutions of Adagen and the exendin-C-POEGMA conjugates were prepared such that at each competing antigen concentration, similar PEG/PEG contents were compared (as shown in Supplementary Table 3). The diluted competing antigens were mixed with equal volume of a patient plasma sample that tested positive for PEG antibody (diluted 1:200 vol/vol in 1% BSA in PBS) and incubated at 4 °C overnight. The following morning, all wells were washed with PBS then blocked with 1% BSA in PBS. Wells were washed with PBS after blocking, and 100 μl of each concentration of the competing antigen–plasma mixtures was added in replicate wells. After incubation at room temperature for 2 h, alkaline-phosphatase-conjugated IgG was added for colorimetric readout at 405 nm as described above.

Assays in Fig 5a,b were performed with n = 3, while those in Fig 5c,d were performed with n = 5.

Statistical analysis. Data are presented as means ± standard error of the mean (SEM). Blood glucose levels in glucose measurement studies (n = 6) were normalized by the average glucose levels measured 24 h before and immediately before injection. Treatment effects on glucose levels were analysed using two-way repeated-measures analysis of variance (ANOVA), followed by post hoc Dunnett’s multiple comparison test to evaluate individual differences between a treatment and PBS control at each time point. The AUCs of the glucose profiles were compared using one-way ANOVA followed by post hoc Tukey’s multiple comparison test (n = 6). To evaluate the AUC of IPGTT (n = 5), treatment and PBS were compared using an unpaired parametric two-tailed t-test. Both direct and competitive anti-PEG ELISAs (n = 3) were analysed using two-way ANOVA, followed by post hoc Dunnett’s multiple comparison test to evaluate individual differences between exendin-C-POEGMA and the other groups for each plasma sample (direct) or antigen concentration (competitive). A test was considered significant if the P value was <0.05. Statistical analyses were performed using Prism 6 (GraphPad software Inc.).

Data availability. Source data for the figures in this study are available at figshare with the identifier doi: 10.6084/m9.figshare.9796761 (ref. 54). The authors declare that all other data supporting the findings of this study are available within the paper and its supplementary information.

Received 28 April 2016; accepted 3 October 2016; published 28 November 2016

References

1. Leader, B., Baca, J. J. & Golan, D. E. Protein therapeutics: a summary and pharmacological classification. Nat. Rev. Drug Discov. 7, 21–39 (2008).
2. Craik, D. I., Fairlie, D. D., Lisas, S. & Price, D. The future of peptide-based drugs. Chem. Biol. Drug Des. 81, 136–147 (2013).
3. Peters, T. J. Serum albumin. Adv. Protein Chem. 37, 161–245 (1985).
4. Awai, M. & Brown, E. B. Studies of the metabolism of 1-131-labeled human transferrin. J. Lab. Clin. Med. 130, 262–269 (1997).
5. Banga, A. K. Therapeutic Peptides and Proteins: Formulation, Processing, and Delivery Systems (CRC, 2015).
6. Caliceti, P. & Veronese, F. M. Pharmacokinetic and biodistribution properties of poly(ethylene glycol)-protein conjugates. Adv. Drug Deliv. Rev. 55, 1261–1277 (2003).
7. Malik, D. K., Baboota, S., Ahuja, A., Hasan, S. & Ali, J. Recent advances in protein and peptide drug delivery systems. Curr. Drug Deliv. 2, 141–151 (2007).
8. Werle, M. & Berknop-Schurnch, A. Strategies to improve plasma half life time of peptide and protein drugs. Amino Acids 30, 351–367 (2006).
9. Akoncel, S. N. S., Baas, A. S. & Maynard, H. D. FDA-approved poly(ethylene glycol)-protein conjugate drugs. Polym. Chem. 2, 1442–1448 (2011).
10. Nucci, M. L., Shorr, R. G. & Abuchowski, A. The therapeutic value of poly(ethylene glycol)-modified proteins. Adv. Drug Deliv. Rev. 6, 133–151 (1991).
11. Youn, Y. S., Nuin, D. H. & Lee, K. C. High-yield production of biologically active mono-PEGylated salmon calcitonin by site-specific PEGylation. J. Control. Release 117, 371–379 (2007).
12. Gauthier, M. A. & Klok, H. Peptide/protein–polymer conjugates: synthetic strategies and design concepts. Chem. Commun. 2591–2611 (2008).
13. Qi, Y. & Chilkoti, A. Growing polymers from peptides and proteins: a biomedical perspective. Adv. Drug Deliv. Rev. 60, 266–276 (2014).
14. Garay, R. P., El-Gewely, R., Armstrong, J. K., Garratty, G. & Richette, P. Antibodies against polyethylene glycol in healthy subjects and in patients treated with PEG-conjugated agents. Expert Opin. Drug Deliv. 9, 1319–1323 (2012).
15. Ganson, N. J. et al. Pre-existing anti-PEG antibody linked to first-exposure allergic reactions to PEGevacog, a PEGylated RNA aptamer. J. Allergy. Clin. Immunol. 137, 1610–1613 (2016).
16. Qi, Y., Miram, M., Gao, W., McCafferty, D. G. & Chilkoti, A. Sortase-catalyzed initiator attachment enhances high yield growth of a stealth polymer from the C-terminus of a protein. Macromol. Rapid Commun. 34, 1256–1260 (2013).
17. Matyaszewski, K. & Tsarevsky, N. V. Macromolecular engineering by atom transfer radical polymerization. J. Am. Chem. Soc. 136, 6513–6533 (2014).
18. Matyaszewski, K. & Xia, J. Atom transfer radical polymerization. Chem. Rev. 101, 2921–2990 (2001).
19. Sartorelli, D. & Maynard, H. D. Streptavidin as a macroinitiator for polymerization: in situ protein-polymer conjugate formation. J. Am. Chem. Soc. 127, 6508–6509 (2005).
20. Gao, W. et al. In situ growth of a stoichiometric PEG-like conjugate at a protein’s N-terminus with significantly improved pharmacokinetics. Proc. Natl Acad. Sci. USA 107, 16432–16437 (2010).
21. Leader, B., Baca, J. J. & Golan, D. E. Protein therapeutics: a summary and pharmacological classification. Nat. Rev. Drug Discov. 7, 21–39 (2008).
22. Peeler, J. C. et al. Genetic encoded initiator for polymer growth from proteins. J. Am. Chem. Soc. 132, 13575–13577 (2010).
23. Probsteirt, L., Zeller, J., Mathas, J., Zeddam, K. & Steinhoff, M. R. Genome-wide detection and analysis of cell wall-bound proteins with LiPX-TG-like sorting motifs. J. Bacteriol. 187, 4928–4934 (2005).
24. Meyer, D. E. & Chilkoti, A. Purification of recombinant proteins by fusion with thermally-responsive polypeptide. Nat. Biotechnol. 14, 1112–1115 (1999).
25. Morin, H., Bart, S. A., Schink, A. & Pollok, B. A. Sortase-mediated protein ligation: a new method for protein engineering. J. Am. Chem. Soc. 126, 2670–2671 (2004).
26. Jakubowski, W. & Matyaszewski, K. Activators regenerated by electron transfer for atom-transfer radical polymerization of (meth)acrylates and related block copolymers. Angew. Chem. Int. Ed. 45, 4482–4486 (2006).
27. Goke, R. et al. Exendin-4 is a high potency agonist and truncated exendin-(9-39)-amide is an antagonist at the glucagon-like peptide 1 (7-36)-amide receptor of insulin-secreting beta-cells. J. Biol. Chem. 286, 19650–19655 (1993).
28. Winters, M. & Ahren, B. The high-fat diet-fed mouse: a model for type 2 diabetes. Diabetes 53, S215–S219 (2004).
29. Surwit, R. S., Kuhn, C. M., Cochrane, C., McCubbin, J. A. & Feinglos, M. N. Diet-induced type II diabetes in C57BL/6J mice. Diabetes 37, 1163–1167 (1988).
30. Mack, C. M. et al. Antibody action of peripheral exenatide (exendin-4) in rodents: effects on food intake, body weight, metabolic status and side-effect measures. Int. J. Obes. 30, 1332–1340 (2006).
31. Kanosi, S. E., Rupperecht, L. W., Fortin, S. M., De Jonghe, B. C. & Hayes, M. R. The role of nausea in food intake and body weight suppression by peripheral GLP-1 receptor agonists, exendin-4 and liraglutide. Neuropharmacology 62, 1519–1527 (2012).
32. Richter, A. W. & Akerblom, E. Antibodies against polyethylene glycol produced in animals by immunization with monomethoxy polyethylene glycol modified proteins. Int. Arch. Allergy Appl. Immunol. 70, 124–131 (1983).
43. Tsarevsky, N. V., Pintauer, T. & Mattyjaszewski, K. Deactivation efficiency and degree of control over polymerization in ATRP in protic solvents. Macromolecules 37, 9768–9775 (2004).

44. Averick, S. et al. Protein-polymer hybrids: conducting ARGET ATRP from a genetically encoded cleavable ATRP initiator. Eur. Polym. J. 49, 2919–2924 (2013).

45. Bellucci, J. J., Bhattacharyya, J. & Chilkoti, A. A noncanonical function of sortase enables site-specific conjugation of small molecules to lysine residues in proteins. Angew. Chem. Int. Ed. 54, 441–445 (2015).

46. Amiram, M., Lugninbuhl, K. M., Li, X., Feinglos, M. N. & Chilkoti, A. Injectable protease-operated depots of glucagon-like peptide-1 provide extended and tunable glucose control. Proc. Natl Acad. Sci. USA 110, 2792–2797 (2013).

47. Schellenberger, V. et al. A recombinant polypeptide extends the in vivo half-life of peptides and proteins in a tunable manner. Nat. Biotechnol. 27, 1186–1189 (2009).

48. Liao, Y.-D., Jeng, J.-C., Wang, C.-F., Wang, S.-C. & Chang, S.-T. Removal of N-terminal methionine from recombinant proteins by engineered E. coli methionine aminopeptidase. Prot. Sci. 13, 1802–1810 (2004).

49. McDaniel, J. R., Mackay, J. A., Quiroz, F. G. & Chilkoti, A. Recursive directional ligation by plasmid reconstruction allows rapid and seamless cloning of oligomeric genes. Biomacromolecules 11, 944–952 (2010).

50. Ilangoavan, U., Ton-That, H., Schneewind, I. & Clubb, R. T. Structure of sortase, the transpeptidase that anchors proteins to the cell wall of Staphylococcus aureus. Proc. Natl Acad. Sci. USA 98, 6056–6061 (2001).

51. Drucker, D. J. & Nauck, M. A. The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. Proc. Natl Acad. Sci. USA 102, 1802–1807 (2005).

52. Baggio, L. L., Huang, Q. L., Brown, T. J. & Drucker, D. J. A recombinant human glucagon-like peptide (GLP)-1- albumin protein (Albugon) mimics peptidergic activation of GLP-1 receptor-dependent pathways coupled with satiety, gastrointestinal motility, and glucose homeostasis. Diabetes 53, 2492–2500 (2004).

53. Goutelle, S. et al. The Hill equation: a review of its capabilities in pharmacological modelling. Fundam. Clin. Pharmacol. 22, 633–648 (2008).

54. Qi, Y. et al. Dataset for A brush-polymer/exendin-4 conjugate reduces blood glucose levels for up to five days and eliminates poly(ethylene glycol) antigenicity. figshare http://dx.doi.org/10.6084/m9.figshare.3976761 (2016).

55. Zong, Y., Rice, T. W., Ton-That, H., Schneewind, O. & Narayana, S. V. Crystal structures of Staphylococcus aureus sortase A and its substrate complex. J. Biol. Chem. 279, 31383–31389 (2004).

56. Neidigh, J. W., Fesmeyer, R. M., Prickett, K. S. & Andersen, N. H. Exendin-4 and glucagon-like-peptide-1: NMR structural comparisons in the solution and micelle-associated states. Biochemistry 40, 13188–13200 (2001).

Acknowledgements
The authors thank D.M. Gooden at the Duke Small Molecule Synthesis Facility for the synthesis of AE8MP, E.J. Soderblom at the Duke Proteomics Facility for conducting LC/MS-MS, G. Dubay at the Duke Chemistry Mass Spectrometry Facility for LC/ESI-MS support and M.N. Feinglos for discussion of the in vivo results. BHK cells expressing GLP-1R were a gift from the Drucker group (University of Toronto, Canada). This work was supported by the National Institutes of Health (R01-DK092665 to A.C.).

Author contributions
Y.Q. and A.C. conceived and designed the research. Y.Q., A.S., N.J.G., X.L., I.O. and W.L. performed the experiments. A.S. and K.M. provided technical expertise in polymer chemistry. K.M.L. and W.L. contributed to the design of the in vivo studies. N.J.G. and M.S.H. provided materials and technical expertise for the antigenicity studies. Y.Q., N.J.G., K.M.L., M.S.H., K.M. and A.C. analysed and interpreted the results. Y.Q. and A.C. wrote the manuscript and A.S., N.J.G., K.M.L., M.S.H. and K.M. edited the manuscript. All authors discussed the results and commented on the manuscript.

Additional information
Supplementary information is available for this paper.
Reprints and permissions information is available at www.nature.com/reprints.
Correspondence and requests for materials should be addressed to A.C.
How to cite this article: Qi, Y. et al. A brush-polymer/exendin-4 conjugate reduces blood glucose levels for up to five days and eliminates poly(ethylene glycol) antigenicity. Nat. Biomed. Eng. 1, 0002 (2016).

Competing interests
A.C. and Y.Q. have a pending patent on the sortase-catalysed C-terminal polymer conjugation technology (WO 2014194244 A1). M.S.H. is a co-inventor of Pegloticase (Krystexxa) and receives royalties from sales of Pegloticase, along with his employer, Duke University. The results reported in this paper form the basis of US provisional patent applications (62/270,401; 62/329,800; 62/310,534; 62/407,403) filled by A.C., Y.Q., M.S.H. and N.J.G. through the Duke University Office of Licensing & Ventures.