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Trehalose Glycopolymer Enhances Both Solution Stability and Pharmacokinetics of a Therapeutic Protein

Yang Liu^{§,†,‡}, Juneyoung Lee^{§,‡}, Kathryn M. Mansfield[§], Jeong Hoon Ko[§], Sahar Sallam[#], Chrys Wesdemiotis[#], and Heather D. Maynard[§]

[§]Department of Chemistry and Biochemistry and California NanoSystems Institute, University of California, Los Angeles, 607 Charles E. Young Drive, East, Los Angeles, California 90095, United States

[†]Department of Biomedical and Pharmaceutical Sciences, School of Pharmacy, Chapman University, Irvine, California 92618, United States

[#]Department of Chemistry, The University of Akron, 190 East Buchtel Common, Akron, Ohio 44325, United States

Abstract

Biocompatible polymers such as poly(ethylene glycol) (PEG) have been successfully conjugated to therapeutic proteins to enhance their pharmacokinetics. However, many of these polymers, including PEG, only improve the *in vivo* lifetimes and do not protect proteins against inactivation during storage and transportation. Herein, we report a polymer with trehalose side chains (PolyProtek) that is capable of improving both the external stability and the *in vivo* plasma half-life of a therapeutic protein. Insulin was employed as a model biologic, and high performance liquid chromatography and dynamic light scattering confirmed that addition of trehalose glycopolymer as an excipient or covalent conjugation prevented thermal or agitation-induced aggregation of insulin. The insulin-trehalose glycopolymer conjugate also showed significantly prolonged plasma circulation time in mice, similar to the analogous insulin-PEG conjugate. The insulin-trehalose glycopolymer conjugate was active as tested by insulin tolerance tests in mice and retained bioactivity even after exposure to high temperatures. The trehalose glycopolymer was shown to be non-toxic to mice up to at least 1.6 mg/kg dosage. These results together suggest that the trehalose glycopolymer should be further explored as an alternative to PEG for long circulating protein therapeutics.

Graphical Abstract

[‡]Author Contributions

These authors contributed equally. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Conflict of Interest

The authors disclose that there is a patent pending on trehalose glycopolymers.

ASSOCIATED CONTENT

Supporting Information. Experimental details on CTA and RAFT polymer synthesis and characterization, conjugate evaluation by PAGE and MS, higher concentration insulin pharmacokinetic study, and toxicity results.

Keywords

protein; insulin; trehalose glycopolymer; stability; pharmacokinetics; protein-polymer conjugates

INTRODUCTION

Protein drugs have high specificity and potency, and as a result more than 130 proteins or peptides are approved by the FDA.¹ However, proteins also face substantial challenges including short half-lives in the bloodstream, as well as chemical and physical instability upon exposure to environmental stressors leading to short shelf lives.^{2–4} Covalent attachment of poly(ethylene glycol) (PEG) to proteins, or PEGylation, is the most widely used polymer conjugation technique to address the pharmacokinetic challenge of proteins, and ten FDA-approved PEGylated proteins are currently on the market.^{2, 5–7} However, linear PEG does not necessarily improve the stability of proteins to environmental stressors during storage and transport: close to 80% of all protein drugs need to be refrigerated or frozen even in the presence of PEG or other stabilizing excipients.¹ This lowers patient compliance and quality of life and increases costs due to refrigeration during delivery and storage.^{3, 4, 7} More importantly, denaturation of protein drugs as a result of inadequate storage can result in life-threatening events caused by inadequate dosage.

While many PEG alternatives are under development, most have been employed to increase the *in vivo* half-life of protein therapeutics and few have the ability to increase environmental stability.^{7, 8} Protein-polymer conjugates with improved *in vivo* half-lives have been successfully prepared with polymers such as poly-N-(2-hydroxypropyl) methacrylamide (pHPMA),^{9–11} polyoxazolines,^{12–14} and hydroxyethyl starch (HES).^{15, 16} However, conjugating these polymers does not necessarily improve protein stability during storage. There are few recent examples of protein-stabilizing polymers such as zwitterionic carboxybetaine polymer for enzymes,^{8, 17} cationic dendrimer for proline-specific endopeptidase (PEP),¹⁸ and branched or “comb” PEG for oxytocin,^{19, 20} but these have been mainly limited to enzyme or small peptide stabilization. There is still a tremendous need and interest in developing new PEG alternatives that can confer both increased half-life and storage stability for a variety of proteins and stressors.

Herein, we describe a trehalose glycopolymer that we call PolyProtek (Figure 1) to address protein instability during both storage and use. The polymer has the disaccharide trehalose at

the side chains and stabilizes various enzymes and proteins to fluctuations in temperature and lyophilization in solution.^{21–23} We have also employed the trehalose glycopolymer as a resist material and demonstrated that polymer stabilizes proteins to high vacuum and direct electron beam irradiation in solid films.^{24, 25} Trehalose is known to stabilize proteins with a mechanism attributed to vitrification, water replacement, and/or water entrapment.^{26, 27} Yet, the trehalose glycopolymer has outperformed trehalose itself in several studies.^{21, 22, 24} This may be a result of both the osmolyte and nonionic surfactant character of the polymer.^{22, 28} Others have shown that polymers with trehalose side chains can inhibit amyloid formation and form stable nanoparticles for nucleic acid delivery.^{29–31} We hypothesized that trehalose glycopolymers would also stabilize proteins *in vivo* similar to PEG. In the study described here, for the first time we demonstrate that the trehalose glycopolymer can maintain the plasma protein concentration within the therapeutic window over an extended period of time and also stabilize a protein therapeutic at elevated temperature and under mechanical stress. Insulin was chosen as a model protein because of its wide clinical usage and well-established structure and bioactivity assays.³²

RESULTS

The recommended storage for insulin is 2–8 °C because the protein inactivates at a significantly higher rate when it is kept at room temperature.^{33–35} The protein is also prone to aggregation during mechanical agitation associated with transportation.³⁶ Aggregation of insulin exposed to these stressors decreases the activity and poses a risk of diabetic ketoacidosis and other complications for patients.³⁴ Thus, we examined whether or not the trehalose glycopolymer with a polystyrene backbone and acetal-linked trehalose (Figure 1) could stabilize insulin to thermal and mechanical stress. In our initial tests we added the polymer as an excipient. An accelerated thermal stability study of insulin was carried out by comparing results heating insulin with and without the trehalose glycopolymer for 30 minutes. High performance liquid chromatography (HPLC) was employed to evaluate the percent of intact insulin because this technique is established as a way to quantify and distinguish intact insulin from its degradation products caused by aggregation.³³ HPLC analysis of the stressed insulin showed that while insulin significantly degraded after heating or agitation, adding 2 molar equivalents of the trehalose glycopolymer stabilized the protein to a much greater extent (Figure 2a). Though the addition of 2 molar equivalents of PEG during heating extended the initial time to denaturation of insulin, the insulin in the presence of PEG eventually degraded to the same extent as the insulin alone at 30 minutes. Dynamic light scattering (DLS) was employed to study whether or not the trehalose glycopolymer prevented aggregation of insulin. The analysis showed that before stress, the diameter of insulin alone was 4 ± 1 nm and with the trehalose glycopolymer was 7 ± 1 nm. After heating, the diameter increased to 1291 ± 189 nm for the insulin sample, while it remained at 6 ± 2 nm when the trehalose glycopolymer was present (Figure 2b). The mechanical stress stability study was carried out by agitation at 250 rpm and 37 °C for 3 hours. Both the HPLC and DLS analyses showed that the trehalose glycopolymer as an excipient (2 molar equivalent) completely prevented aggregation of the protein (Figure 2c and 2d). Interestingly, the addition of PEG resulted in destabilization of insulin during agitation (Figure 2c). This may be due to the hydrophobic interactions of PEG with exposed

hydrophobic residues of insulin, which has been previously reported for PEG with other proteins.^{37, 38} These results demonstrated that the trehalose glycopolymer effectively prevents insulin aggregation induced by both heat and mechanical stress, which are major mechanisms of insulin degradation.^{39, 40} The data further suggested that the polymer was a good candidate for conjugation to insulin.

A protein-reactive trehalose glycopolymer was thus prepared through reversible addition-fragmentation chain transfer (RAFT) polymerization using either a ketone- or aldehyde-functionalized chain transfer agent ($M_n = 13.8$ kDa and $K = 1.35$ for ketone and $M_n = 9.9$ kDa and $K = 1.10$ for aldehyde end-group polymer) and subsequently conjugated to insulin via reductive amination, which is commonly used to conjugate to amines (Figure 3, Figures S1–S4).^{41, 42} The trithiocarbonate of the RAFT polymer was not reduced under these reductive amination conditions as demonstrated by UV-Vis spectroscopy (Figure S5). However, if required, the trithiocarbonate could be removed using standard protocols.⁴³ Conjugations were confirmed by native gel and western blot (Figures 4c, d and Figure S6–7) and the conjugates were purified via fast protein liquid chromatography (FPLC). Both ketone and aldehyde polymers were able to conjugate to insulin, with the aldehyde polymer providing higher yields in a shorter amount of time (quantitative conversion after 14 hours using 12.5 molar equivalent of the polymer, native polyacrylamide gel electrophoresis (PAGE) in Figure S7 versus S6). Thus, the majority of the studies were conducted with conjugates prepared with the benzaldehyde end-functionalized polymer. PEG aldehyde monomethoxy ether (10 kDa) was also conjugated to insulin as a control (PAGE in Figure S8).

The conjugates were characterized by mass spectrometry. We have observed that the trehalose glycopolymer inhibits evaluation by mass spectrometry and prevents protein ionization even when added to samples; thus a two-step treatment of the sample was necessary for its analysis. First, the trehalose glycopolymer-insulin was treated with formic acid to cleave the polymer chain at the ester leaving a 106 Da linker (corresponding to the 4-hydroxybenzaldehyde moiety) attached to the conjugation site. Second, dithiothreitol (DTT) or *tris*(2-carboxyethyl)phosphine (TCEP) was used to reduce the disulfide bridges of insulin to release chain A (2382 Da) and chain B (3427 Da) plus the mass of the attached linker. The matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrum of the conjugate (Figure S9) shows two species with 106 Da and 212 Da greater mass than insulin, suggesting the presence of mono- and di-substituted conjugates. Note that trace amount of insulin in the conjugate, although not detectable by Coomassie stain and western blot, was visible in the spectrum due to very high ionization efficiency of insulin and non-quantitative nature of polymer cleavage from insulin. Nevertheless, the formic acid treatment was sufficient for characterization of the conjugate. Electrospray ionization mass spectrometry (ESI-MS) of the conjugate confirmed the MALDI results (Figure 4a–4b). Both chain A and chain B exhibited a peak that corresponded to modification with a single polymer. These results suggest that the trehalose glycopolymer was conjugated to the N-terminal glycine of chain A (GlyA1) and to N-terminal phenylalanine or lysine of chain B (PheB1 or LysB29). Since the previously reported reactivity of these amines followed the order GlyA1 > LysB29 >> PheB1,⁴⁴ we expected LysB29 to be the modification site on the chain B. To confirm this, tandem mass spectrometry experiments were performed on the

chain B ion of both native and conjugated insulin (Figure S10). The spectrum of the conjugate exhibited $y_3 + 106$ Da (m/z 451.20) ion, which confirmed that the linker was attached to LysB29 adjacent to the C-terminus.

This analysis indicates that the trehalose glycopolymer was conjugated to GlyA1 and LysB29, consistent with the literature report that these two amine functionalities are shown to be much more reactive towards conjugation than PheB1.⁴⁴ The conjugate was also confirmed by native gel and western blot (Figure 4c and 4d). For the PEG conjugate, MALDI-TOF analysis showed that the PEG conjugate was also mostly mono- and di-substituted (Figure S11a), presumably at GlyA1 and LysB29 as previously reported for PEG conjugates.⁴⁴ This result also agreed with the PAGE result where two overlapping conjugate species are observed (Figure S8). The peaks were separated by roughly 44 m/z , which corresponds to the molecular weight of the PEG repeat unit (Figure S11b).

Once conjugation was confirmed, the thermal and mechanical stability of the insulin-trehalose glycopolymer was evaluated as described above for the added polymer. In both cases, the conjugated polymer stabilized the protein in its monomeric form as shown by both HPLC and DLS. Further, the results of the conjugate closely resembled that of the excipient data for HPLC, demonstrating that conjugated polymer stabilized insulin as well as excess polymer excipient (Figure 2a and 2c). DLS analysis showed that before stress the diameter of the insulin-trehalose glycopolymer conjugate was 6.4 ± 1.2 nm (Figure 2b and 2d). The diameter did not increase after stress like insulin itself (> 1000 nm) and remained low at 4.7 ± 0.7 nm after heating and 6.5 ± 1.3 nm after agitation. Therefore, covalently conjugating two trehalose glycopolymer chains stabilized the monomeric form of insulin as well as adding 2 molar equivalents (10 weight equivalents) of the polymer as an excipient.

Next, the effect of trehalose glycopolymer conjugation on the clearance rate of insulin was studied after tail vein injection into mice. Intravenous administration was used to eliminate subcutaneous absorption as a potential variable. Figure 5 shows the plasma concentration of insulin over time after injection. Both insulin-trehalose glycopolymer and insulin-PEG conjugates exhibited a significantly longer half-life and increased area under the plasma concentration time curve (AUC) compared to unmodified insulin. The native insulin was not detectable at any time point, which is consistent with the extremely short plasma half-life of insulin reported previously.⁴⁵ A much higher concentration (120 Qg/kg versus 16 Qg/kg) was required to observe the insulin (Figure S12). The differences in the plasma levels of insulin-trehalose glycopolymer and insulin-PEG conjugates were statistically insignificant at all time points ($p > 0.1$). It is interesting to note that the PEG in this instance, even though it was the same molecular weight as the trehalose glycopolymer, had a ten-fold larger degree of polymerization (DP = 227 versus 21.9, respectively). The slower clearance observed for both conjugates was likely due to reduced renal filtration from the increased hydrodynamic volume,⁴⁶ as well as increased protease resistance and decreased opsonization as is known for PEGylated proteins,⁴⁷ and this will be verified along with the effect of degree of polymerization of the trehalose glycopolymer in future studies. Together, this data shows that trehalose glycopolymer conjugation is an effective strategy to extend the half-life of exogenously delivered insulin.

Next, the bioactivities of insulin and the insulin-trehalose glycopolymer conjugate were compared in mice. Generally, conjugation of polymers to proteins results in decreased bioactivity of the protein. Previous reports have shown that attachment of even a relatively small 2 kDa PEG decreases the *in vivo* bioactivity of insulin to about 80% of the original activity, and the effect becomes larger when higher molecular weight PEG is conjugated.⁴⁴ In the present work, a loss in activity compared to native insulin was also observed in insulin tolerance tests (ITT) in mice; a five-fold dose of trehalose glycopolymer conjugate (concentration determined by ELISA) was required to achieve equivalent short-term biological activity of native insulin (Figure 6a). A similar result has been reported with insulin conjugated to 20 kDa PEG, which had 17-fold lower binding affinity to the insulin receptor than the native protein.⁴⁸ This effect may be due to steric hindrance to insulin binding to the receptor as well as prolonged blood circulation and delayed transendothelial transport to tissue of action also observed for the basal insulin analogue insulin detemir.⁴⁹ Higher bioactivity may be obtained in the future by site-specific conjugation of the trehalose glycopolymer.⁴⁴ Yet, the results did demonstrate that the conjugate was bioactive and able to reduce blood glucose levels in mice.

The heat stability of the conjugate was further investigated *in vivo* by ITT in mice (Figure 6b). The results confirmed the HPLC and DLS data and showed that the protein insulin retained very low levels of activity after heating (17%). In contrast, insulin heated in the presence of 2 molar (10 weight) equivalents of the trehalose glycopolymer added as an excipient retained 81% of its activity. Importantly, the mice treated with insulin-trehalose glycopolymer conjugate that was heated at 90 °C exhibited full retention of activity relative to the conjugate stored refrigerated, demonstrating that the conjugate is fully active after heating to these conditions.

With these promising results of the stabilization effect of the trehalose glycopolymer both *in vitro* and *in vivo*, we further tested *in vivo* toxicity of the polymer. We have previously investigated the cytotoxicity in four different cell lines and found that the polymer does not decrease cell viability up to 8 mg/mL.²² To evaluate the toxicity *in vivo*, hematological parameters were analyzed to assess if the trehalose glycopolymer, as a nonionic surfactant, would cause hemolysis;⁵⁰ kidney and liver toxicities were also tested since protein-polymer conjugates are known to be mainly cleared through renal and hepatic pathways.⁵¹ A concentration of 1.6 mg/kg was injected via tail vein into mice. This concentration was selected because it is 100-fold higher concentration than was used in the pharmacokinetic studies and 20-fold higher than used in the bioactivity studies. The animals were monitored for signs of stress and weight loss, which were not observed, and after 48 hours the animals were sacrificed for evaluation of the trehalose glycopolymer toxicity.^{52, 53} Liver and kidney enzyme levels for both trehalose glycopolymer and phosphate-buffered saline (PBS) treated groups were both within the normal range (Table S1). In addition, hematological parameters including complete blood count (CBC) were found to be normal and comparable to the control group of PBS alone, and the histology of all the major organs was normal. These promising results suggest that the polymer is biocompatible at least up to 1.6 mg/kg in mice.

DISCUSSION

Our results demonstrate that like PEG, trehalose glycopolymer increases the *in vivo* lifetime of a model therapeutic protein. Yet, the trehalose glycopolymer improves upon PEG by also stabilizing the protein to stressors that typically cause aggregation and reduce the activity of the protein. Enhanced pharmacokinetics is desirable because it translates to fewer doses and better dosing regimens, which improves patient compliance and ultimately the therapeutic efficacy of treatment. Stability to external heat and mechanical stress is additionally helpful for longer term storage of proteins and for better stability during transportation where protein drug solutions can undergo agitation. Thus, this data is the initial work in demonstrating the value of the trehalose glycopolymer (also called PolyProtek) as a potentially superior analog to PEG to enhance both the pharmacokinetics and storage stability of therapeutic proteins. Research along these lines is important to move away from refrigeration of protein therapeutics and to enhance the safety of therapeutics while avoiding the cold chain.

Our study of insulin as a model protein was evaluated only by intravenous injections in mice mainly to test our hypothesis that trehalose glycopolymer could improve the pharmacokinetics of protein drugs. PEG-insulin (Lispro®) has been studied as a once-daily injection for patients with type 1 and type 2 diabetes.^{48, 54, 55} Thus, a major application of a PEGylated analog of insulin would be for subcutaneous injection for use as basal insulin. Absorption of insulin from subcutaneous depots is a nonlinear process, which depends on many factors such as the dissolution and diffusion of insulin or insulin conjugates, local blood flow, and local temperature.^{56, 57} Intravenous administration of conjugates in the current study helps to rule out these confounding factors to accurately test one of the hypotheses, which is that the trehalose glycopolymer improves pharmacokinetics. This was a first test to evaluate whether or not a therapeutic protein could be stabilized *in vivo* with the trehalose glycopolymer. Future studies will entail subcutaneous and muscular injections of the trehalose glycopolymer-insulin conjugate.

We also chose insulin as the model therapeutic protein since insulin instability is clinically relevant and has been reported to cause medical emergencies such as ketoacidosis due to insulin degradation.³⁴ There has been work on insulin stabilization using modified insulin analogs,⁵⁸ small-molecule excipients,^{40, 59, 60} liposomes,⁶¹ and polymeric vehicles,^{62–64} yet due to the large demand of insulin around the world and rapid growth of diabetic population, there is still significant value in the study of additional approaches. Our research shows that adding trehalose glycopolymer as an excipient can prevent heat and mechanical-induced aggregation and inactivation of insulin, and initial studies show that the polymer is non-toxic in mice. These results indicate that the trehalose glycopolymer should also be further investigated as a potential stabilizer of insulin as an excipient.

The current study also lends support for the further study of the trehalose glycopolymer as a versatile polymer for delivery of various therapeutic proteins. With improved pharmacokinetics and storage stability, protein drugs that are in general prone to degradation and elimination pathways could be stored without special precaution and be delivered with extended bioactivity if stabilized. Currently the most frequent strategy to improve protein

stability is through genetic modification of liable amino acid residues. However, this requires *a priori* knowledge of possible degradation pathways. Moreover, modification of even a single amino acid may disrupt the tertiary structure of a protein, making this approach a trial-and-error process that is both time consuming and costly. Trehalose glycopolymer has been shown to be an effective stabilizer of various protein classes including enzymes,^{21–23} growth factors,²⁴ antibodies,^{24, 25} and insulin as a hormone in this work, to external stressors such as heat, lyophilization, electron beam irradiation, and mechanical agitation. Therefore, we expect that trehalose glycopolymer conjugation will be a generalizable and reliable formulation strategy for stabilizing various proteins of clinical value, and investigation of this hypothesis is underway.

CONCLUSION

This report presents research on trehalose glycopolymer (PolyProtek) as a polymer that can enhance the stability and pharmacokinetics of a therapeutic protein. Specifically, we describe the ability of a polymer with pendant trehalose groups to stabilize the model protein insulin to high temperatures and mechanical agitation, and retain its bioactivity by inhibiting protein aggregation. Conjugated to the protein, the polymer increased the concentration of insulin in plasma over time and exhibited bioactivity as shown by the reduction of blood glucose levels even after heating. The *in vivo* toxicity results also suggested that the trehalose glycopolymer is a biocompatible polymer. Together, this research demonstrates that conjugation of the trehalose glycopolymer should be explored as an improvement over PEGylation for protein therapeutics because of its ability to enhance both environmental stability and *in vivo* bioavailability.

EXPERIMENTAL PROCEDURES

Materials

All chemicals were purchased from Sigma-Aldrich and Fisher Scientific and were used without purification unless noted otherwise. Azobisisobutyronitrile (AIBN) was recrystallized from acetone before use. Trehalose was purchased from The Healthy Essential Management Corporation (Houston, TX) and was azeotropically dried with ethanol and kept under vacuum until use. Recombinant human insulin was purchased from Sigma-Aldrich. Human insulin ELISA kit was purchased from Mercodia (Uppsala, Sweden). 2-(Ethyltrithiocarbonate)propionic acid was synthesized according to a literature procedure.⁶⁵ mPEG-propionaldehyde (10.5 kDa by MALDI, $M_n = 1.02$ by GPC) was purchased from Jenkem Technology (Allen, TX). PEG without end group (20 kDa) was purchased from Sigma-Aldrich. Styrenyl acetal trehalose monomer and trehalose glycopolymer (styrenyl acetal trehalose polymer without end-group synthesized by free radical polymerization, $M_n = 29.5$ kDa, $M_w = 2.11$ by GPC) were prepared using previously reported procedure.²²

Preparation of insulin-PEG conjugate

Insulin (0.50 mg, 8.6×10^{-2} Qmol), sodium cyanoborohydride (0.64 mg, 10 Qmol), and 10 kDa mPEG-propionaldehyde (45 mg, 4.5 Qmol, 52 molar eq to insulin) were dissolved in 1 mL of 100 mM sodium acetate buffer, pH 4, in a 1.5 mL protein Lo-Bind[®] tube. The

mixture was incubated at 4 °C for 20 h on a rocker, and the buffer was exchanged to D-PBS, pH 7.4, by centriprep ultrafiltration (MWCO 3 kDa) several times before purification by fast protein liquid chromatography (FPLC). The amount of insulin was assayed by ELISA according to manufacturer's instructions. Briefly, 25 QL of the diluted samples were added to the wells pre-coated with the capture antibody. Buffer containing detection antibody was added (100 QL), and the plate was incubated on a rocker at room temperature (23 °C) for 1 h. The wells were washed six times with 350 QL of the wash buffer. 3,3',5,5'-Tetramethylbenzidine (TMB) solution was added (200 QL), and the plate was incubated at room temperature for 15 min before the addition of 50 QL stop solution. The amount of insulin detected was quantified by absorbance at 450 nm relative to the standards supplied by the manufacturer.

Preparation of insulin-trehalose glycopolymer conjugate

Insulin (0.40 mg, 6.9×10^{-2} Qmol), sodium cyanoborohydride (0.64 mg, 10 Qmol), and ketone end-functionalized trehalose glycopolymer (47.6 mg, 2.75 Qmol, 39 molar eq to insulin) were dissolved in 1 mL of 100 mM sodium acetate buffer, pH 3.5, in a 1.5 mL protein Lo-Bind® tube. The mixture was incubated at 4 °C on a gentle rocker, and the buffer was exchanged to D-PBS, pH 7.4, by centriprep ultrafiltration (MWCO 3 kDa) several times before purification by fast protein liquid chromatography (FPLC). This conjugate was used for pharmacokinetic studies. For benzaldehyde end-functionalized polymer, imine formation is facilitated by conjugation to the aromatic ring and thus the conjugation was conducted at a higher pH. Insulin (1.5 mg, 2.59×10^{-1} Qmol), sodium cyanoborohydride (4 mg, 63.8 Qmol), and benzaldehyde end-functionalized trehalose polymer (31 mg, 3.1 Qmol, 12 molar eq to insulin) were dissolved in 1 mL of 200 mM phosphate buffer, pH 8.0, in a 1.5 mL protein Lo-Bind® tube. The mixture was incubated at 37 °C water bath for 12 h, and the buffer was exchanged to Dulbecco's phosphate-buffered saline (D-PBS), pH 7.4, by centriprep ultrafiltration (MWCO 3 kDa) several times before purification by FPLC. This conjugate was used for bioactivity and stability studies. The amount of insulin was assayed by ELISA as previously described. To determine if the trithiocarbonate could be reduced under these conditions, benzaldehyde end-functionalized trehalose polymer (15.5 mg, 1.5 Qmol) was dissolved in 0.5 mL 200 mM phosphate buffer, pH 8.0 with and without sodium cyanoborohydride (2 mg, 31.9 Qmol) and incubated at 37 °C 12 hours before absorption spectra were obtained.

Stability study

Free insulin, insulin with the trehalose glycopolymer or PEG (20 kDa without end-group, 2 molar equivalents to insulin), and insulin-trehalose glycopolymer conjugate were incubated at 90 °C in PBS at a concentration of 0.5 mg/mL and total volume of 100 QL. At predetermined time interval, each sample was collected and subjected to further analysis. Free insulin, insulin with the trehalose glycopolymer or PEG (20 kDa without end-group, 2 molar equivalents to insulin), and insulin-trehalose glycopolymer conjugate were agitated in PBS at 250 rpm and 37 °C at a concentration of 0.5 mg/mL and total volume of 100 QL in glass vials secured horizontally. At pre-determined time intervals, each sample was collected and subjected to further analysis. For RP-HPLC analysis, each sample was filtered and the concentration of insulin that remained in the sample was determined. Mobile phase

consisted of aqueous phase (Solvent A) and organic phase (Solvent B). Solvent A was 0.1% v/v trifluoroacetic acid (TFA) in deionized distilled water and solvent B was acetonitrile. The solvent gradient used was 30%

Solvent A to 40% Solvent A in 15 min

The insulin was detected with a UV detector at a wavelength of 215 nm. The measurements of the average size of the aqueous suspensions of insulin formulation were carried out on a Malvern Zetasizer Nano-ZS dynamic light-scattering (DLS) analyzer (Malvern Instruments Ltd., Malvern, Worcestershire, UK).

Pharmacokinetics study

Unmodified insulin, insulin-trehalose glycopolymer conjugates, and insulin-PEG were formulated in saline (0.4 Qg/mL). CD1 mice (5–6 wks, female, Charles River Laboratories) were used for the pharmacokinetic studies (n=5 per group). A single dose of either unmodified insulin or insulin conjugates (16 Qg/kg of insulin in 0.4 Qg/mL concentration) was injected through the tail vein and blood samples (30–50 Ql) were taken from the retro-orbital sinus at 3 and 10 min, from the lateral saphenous vein at 20 and 40 min, and by cardiac puncture after euthanasia at 60 min after administration. Blood was collected from each mouse four times and the total amount of blood collection was less than 1.25% of the animal's body weight. Insulin concentrations of the blood plasma samples were determined by ELISA according to the manufacture's protocol as previously described.

Bioactivity of insulin and the insulin-polymer conjugates

Bioactivity was determined by the ITT assay using standard protocols.⁶⁶ CD1 mice (6–8 wks, female, n = 5, Charles River Laboratories) were fasted for 4–6 hours to reduce variability in baseline blood glucose. Pristine insulin, insulin after heating, insulin with addition of trehalose glycopolymer, insulin-trehalose glycopolymer conjugate, and insulin-trehalose glycopolymer conjugate after heating were intravenously injected at appropriate doses. For the stability assay, insulin formulations were injected at the dose of 40 Qg/kg of insulin. To determine the bioactivity of the conjugate, the injection dose was 80 Qg/kg and 16 Qg/kg of insulin was injected as a control. At each prescribed time point, approximately 2 Ql of blood sample was obtained from the tail vein in conscious mice by pricking the tail vein with a needle and sampling the formed blood droplet with a commercially available glucometer to measure the glucose concentration. The percent decrease in blood glucose level was calculated using the following formula:

$$\% \text{ Blood glucose decrease} = \frac{[\text{Glucose}]_{0 \text{ min, heated}} - [\text{Glucose}]_{30 \text{ min, heated}}}{[\text{Glucose}]_{0 \text{ min, not heated}} - [\text{Glucose}]_{30 \text{ min, not heated}}} \times 100$$

Toxicity study

CD1 mice (5–6 wks, female, Charles River Laboratories) were injected subcutaneously with either PBS or trehalose glycopolymer (n=5). The animals received one injection of trehalose glycopolymer at a dose of 1.6 mg/kg. After 48 h, 600 µL of blood was collected by cardiac puncture and the major organs were harvested for histology analysis. Complete blood count

(CBC) was performed with whole blood to determine the hematological compatibility of the polymer. Serum aspartate (AST) and alanine (ALT) aminotransferase activities were determined to assess liver toxicity; serum creatinine (Creat) and blood urea nitrogen (BUN) levels were determined to assess kidney toxicity. A sample from one animal in the control group (PBS) exhibited hemolysis and was discarded from the dataset. Histology sections of heart, lung, liver, spleen, and kidney were assessed by Dr. Gregory Lawson (Division of Laboratory Animal Medicine, UCLA). They were all found to be normal.

Statistical analysis

For assessment of the statistical significance of differences, Student's t-test assuming unequal sample variance was employed. Results were considered significantly different if $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Leader B, Baca QJ, Golan DE. Protein therapeutics: a summary and pharmacological classification. *Nat Rev Drug Discovery*. 2008; 7:21–39. [PubMed: 18097458]
2. Harris JM, Chess RB. Effect of pegylation on pharmaceuticals. *Nat Rev Drug Discovery*. 2003; 2:214–221. [PubMed: 12612647]
3. Frokjaer S, Otzen DE. Protein drug stability: A formulation challenge. *Nat Rev Drug Discovery*. 2005; 4:298–306. [PubMed: 15803194]
4. Manning MC, Chou DK, Murphy BM, Payne RW, Katayama DS. Stability of Protein Pharmaceuticals: An Update. *Pharm Res*. 2010; 27:544–575. [PubMed: 20143256]
5. Alconcel SNS, Baas AS, Maynard HD. FDA-approved poly(ethylene glycol)-protein conjugate drugs. *Polym Chem*. 2011; 2:1442–1448.
6. Pfister D, Morbidelli M. Process for protein PEGylation. *J Controlled Release*. 2014; 180:134–149.
7. Pelegri-O'Day EM, Lin EW, Maynard HD. Therapeutic Protein-Polymer Conjugates: Advancing Beyond PEGylation. *J Am Chem Soc*. 2014; 136:14323–14332. [PubMed: 25216406]
8. Zhang P, Sun F, Tsao C, Liu S, Jain P, Sinclair A, Hung HC, Bai T, Wu K, Jiang S. Zwitterionic gel encapsulation promotes protein stability, enhances pharmacokinetics, and reduces immunogenicity. *Proc Natl Acad Sci U S A*. 2015; 112:12046–12051. [PubMed: 26371311]
9. Tao L, Davis TP. Branched Polymer-Protein Conjugates Made From Mid-Chain-Functional P(HPMA). *Biomacromolecules*. 2009; 10:2847–2851. [PubMed: 19731904]
10. Tao L, Liu J, Xu J, Davis TP. Synthesis and bioactivity of poly(HPMA)-lysozyme conjugates: the use of novel thiazolidine-2-thione coupling chemistry. *Org Biomol Chem*. 2009; 7:3481–3485. [PubMed: 19675903]

11. Tao L, Chen G, Zhao L, Xu J, Huang E, Liu A, Marquis CP, Davis TP. Protein Release from Biodegradable PolyHPMA-Lysozyme Conjugates Resulting in Bioactivity Enhancement. *Chem - Asian J.* 2011; 6:1398–1404. [PubMed: 21416614]
12. Mero A, Fang Z, Pasut G, Veronese FM, Viegas TX. Selective conjugation of poly(2-ethyl 2-oxazoline) to granulocyte colony stimulating factor. *J Controlled Release.* 2012; 159:353–361.
13. Viegas TX, Bentley MD, Harris JM, Fang Z, Yoon K, Dizman B, Weimer R, Mero A, Paust G, Veronese FM. Polyoxazoline: Chemistry, Properties, and Applications in Drug Delivery. *Bioconjugate Chem.* 2011; 22:976–986.
14. Tong J, Yi X, Luxenhofer R, Banks WA, Jordan R, Zimmerman MC, Kabanov AV. Conjugates of Superoxide Dismutase 1 with Amphiphilic Poly(2-oxazoline) Block Copolymers for Enhanced Brain Delivery: Synthesis, Characterization and Evaluation in Vitro and in Vivo. *Mol Pharmaceutics.* 2013; 10:360–377.
15. Liebner R, Meyer M, Hey T, Winter C, Besheer A. Head to Head Comparison of the Formulation and Stability of Concentrated Solutions of HESylated versus PEGylated Anakinra. *J Pharm Sci.* 2015; 104:515–526. [PubMed: 25445200]
16. Liebner R, Mathaes R, Meyer M, Hey T, Winter C, Besheer A. Protein HESylation for half-life extension: Synthesis, characterization and pharmacokinetics of HESylated anakinra. *Eur J Pharm Biopharm.* 2014; 87:378–385. [PubMed: 24681396]
17. Keefe AJ, Jiang S. Poly(zwitterionic)protein conjugates offer increased stability without sacrificing binding affinity or bioactivity. *Nat Chem.* 2012; 4:59–63.
18. Fuhrmann G, Grotzky A, Luki R, Matoori S, Luciani P, Yu H, Zhang B, Walde P, Schlüter AD, Gauthier MA. Sustained gastrointestinal activity of dendronized polymer–enzyme conjugates. *Nat Chem.* 2013; 5:582–589. [PubMed: 23787748]
19. Collins J, Kempe K, Wilson P, Blindaur CA, McIntosh MP, Davis TP, Whittaker MR, Haddleton DM. Stability Enhancing N-Terminal PEGylation of Oxytocin Exploiting Different Polymer Architectures and Conjugation Approaches. *Biomacromolecules.* 2016; 17:2755–2766. [PubMed: 27419537]
20. Collins J, Tanaka J, Wilson P, Kempe K, Davis TP, McIntosh MP, Whittaker MR, Haddleton DM. In Situ Conjugation of Dithiophenol Maleimide Polymers and Oxytocin for Stable Reversible Polymer-Peptide Conjugates. *Bioconjugate Chem.* 2015; 26:633–638.
21. Mancini RJ, Lee J, Maynard HD. Trehalose Glycopolymers for Stabilization of Protein Conjugates to Environmental Stressors. *J Am Chem Soc.* 2012; 134:8474–8479. [PubMed: 22519420]
22. Lee J, Lin EW, Lau UY, Hedrick JL, Bat E, Maynard HD. Trehalose Glycopolymers as Excipients for Protein Stabilization. *Biomacromolecules.* 2013; 14:2561–2569. [PubMed: 23777473]
23. Lee J, Ko JH, Lin EW, Wallace P, Ruch F, Maynard HD. Trehalose hydrogels for stabilization of enzymes to heat. *Polym Chem.* 2015; 6:3443–3448. [PubMed: 26005500]
24. Bat E, Lee J, Lau UY, Maynard HD. Trehalose glycopolymer resists allow direct writing of protein patterns by electron-beam lithography. *Nat Commun.* 2015; 6:6654. [PubMed: 25791943]
25. Lau UY, Saxer SS, Lee J, Bat E, Maynard HD. Direct Write Protein Patterns for Multiplexed Cytokine Detection from Live Cells Using Electron Beam Lithography. *ACS Nano.* 2015; 10:723–729. [PubMed: 26679368]
26. Lins RD, Pereira CS, Hunenberger PH. Trehalose-protein interaction in aqueous solution. *Proteins: Struct, Funt, Bioinf.* 2004; 55:177–186.
27. Sakurai, M. *Water and Biomolecules.* Springer; 2009. Biological functions of trehalose as a substitute for water; p. 219-240.
28. Kale SS, Akamanchi KG. Trehalose monooleate: a potential anti-aggregation agent for stabilization of proteins. *Mol Pharmaceutics.* 2016
29. Wada M, Miyazawa Y, Miura Y. A specific inhibitory effect of multivalent trehalose toward A beta(1–40) aggregation. *Polym Chem.* 2011; 2:1822–1829.
30. Sizovs A, Xue L, Tolstyka ZP, Ingle NP, Wu Y, Cortez M, Reineke TM. Poly (trehalose): sugar-coated nanocomplexes promote stabilization and effective polyplex-mediated siRNA delivery. *J Am Chem Soc.* 2013; 135:15417–15424. [PubMed: 24083547]

31. Tolstyka ZP, Phillips H, Cortez M, Wu Y, Ingle N, Bell JB, Hackett PB, Reineke TM. Trehalose-Based Block Copolyconjugations Promote Polyplex Stabilization for Lyophilization and in Vivo pDNA Delivery. *ACS Biomater Sci Eng*. 2015; 2:43–55. [PubMed: 26807438]
32. Pickup JC. Insulin-Pump Therapy for Type 1 Diabetes Mellitus. *N Engl J Med*. 2012; 366:1616–1624. [PubMed: 22533577]
33. Oliva A, Farina JB, Llabres M. Influence of temperature and shaking on stability of insulin preparations: Degradation kinetics. *Int J Pharm*. 1996; 143:163–170.
34. Pryce R. Diabetic ketoacidosis caused by exposure of insulin pump to heat and sunlight. *Brit Med J*. 2009; 338:a2218. [PubMed: 19124546]
35. Gregory R, Edwards S, Yateman NA. Demonstration of Insulin Transformation Products in Insulin Vials by High-Performance Liquid-Chromatography. *Diabetes Care*. 1991; 14:42–48. [PubMed: 1991434]
36. Sluzky V, Tamada JA, Klibanov AM, Langer R. Kinetics of insulin aggregation in aqueous solutions upon agitation in the presence of hydrophobic surfaces. *Proc Natl Acad Sci U S A*. 1991; 88:9377–9381. [PubMed: 1946348]
37. Lee LLY, Lee JC. Thermal-Stability of Proteins in the Presence of Poly(Ethylene Glycols). *Biochemistry*. 1987; 26:7813–7819. [PubMed: 3427106]
38. Senske M, Tork L, Born B, Havenith M, Herrmann C, Ebbinghaus S. Protein stabilization by macromolecular crowding through enthalpy rather than entropy. *J Am Chem Soc*. 2014; 136:9036–41. [PubMed: 24888734]
39. Bohidar HB. Light scattering and viscosity study of heat aggregation of insulin. *Biopolymers*. 1998; 45:1–8. [PubMed: 9433182]
40. Sluzky V, Klibanov AM, Langer R. Mechanism of insulin aggregation and stabilization in agitated aqueous solutions. *Biotechnol Bioeng*. 1992; 40:895–903. [PubMed: 18601196]
41. Kinstler OB, Brems DN, Lauren SL, Paige AG, Hamburger JB, Treuheit MJ. Characterization and stability of N-terminally PEGylated rhG-CSF. *Pharm Res*. 1996; 13:996–1002. [PubMed: 8842035]
42. Dou H, Zhang M, Zhang Y, Yin C. Synthesis and Purification of Mono3 PEGylated Insulin. *Chem Biol Drug Des*. 2007; 69:132–138. [PubMed: 17381727]
43. Willcock H, O'Reilly RK. End group removal and modification of RAFT polymers. *Polym Chem*. 2010; 1:149–157.
44. Uchio T, Baudys M, Liu F, Song SC, Kim SW. Site-specific insulin conjugates with enhanced stability and extended action profile. *Adv Drug Delivery Rev*. 1999; 35:289–306.
45. Roy FN, Beaulieu S, Boucher L, Bourdeau I, Cohade C. Impact of intravenous insulin on 18F-FDG PET in diabetic cancer patients. *J Nucl Med*. 2009; 50:178–183. [PubMed: 19164226]
46. Caliceti P, Veronese FM. Pharmacokinetic and biodistribution properties of poly(ethylene glycol)–protein conjugates. *Adv Drug Delivery Rev*. 2003; 55:1261–1277.
47. Owens DE 3rd, Peppas NA. Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles. *Int J Pharm*. 2006; 307:93–102. [PubMed: 16303268]
48. Madsbad S. LY2605541--a preferential hepato-specific insulin analogue. *Diabetes*. 2014; 63:390–392. [PubMed: 24464715]
49. Hamilton-Wessler M, Ader M, Dea M, Moore D, Jorgensen PN, Markussen J, Bergman RN. Mechanism of protracted metabolic effects of fatty acid acylated insulin, NN304, in dogs: retention of NN304 by albumin. *Diabetologia*. 1999; 42:1254–1263. [PubMed: 10525669]
50. Zaslavsky BY, Ossipov NN, Krivich VS, Baholdina LP, Rogozhin SV. Action of surface-active substances on biological membranes. II Hemolytic activity of nonionic surfactants. *Biochim Biophys Acta, Biomembr*. 1978; 507:1–7.
51. Webster R, Didier E, Harris P, Siegel N, Stadler J, Tilbury L, Smith D. PEGylated proteins: evaluation of their safety in the absence of definitive metabolism studies. *Drug Metab Dispos*. 2007; 35:9–16. [PubMed: 17020954]
52. Chen Y, Zhang W, Huang Y, Gao F, Sha X, Lou K, Fang X. The therapeutic effect of methotrexate-conjugated Pluronic-based polymeric micelles on the folate receptor-rich tumors treatment. *Int J Nanomed*. 2015; 10:4043–4057.

53. De Jong WH, Borm PJ. Drug delivery and nanoparticles: applications and hazards. *Int J Nanomed*. 2008; 3:133–49.
54. Eli Lilly and Company. A Study of Insulin Peglispro (LY2605541) in Participants With Type 2 Diabetes Mellitus. <<https://clinicaltrials.gov/ct2/show/NCT02106364?term=peglispro&rank=5>>
55. Caparrotta TM, Evans M. PEGylated insulin Lispro, (LY2605541)—a new basal insulin analogue. *Diabetes Obes Metab*. 2014; 16:388–395. [PubMed: 23941279]
56. Brange J, Owens DR, Kang S, Volund A. Monomeric insulins and their experimental and clinical implications. *Diabetes Care*. 1990; 13:923–54. [PubMed: 2226110]
57. Hinds KD, Kim SW. Effects of PEG conjugation on insulin properties. *Adv Drug Delivery Rev*. 2002; 54:505–530.
58. Karas JA, Patil NA, Tailhades J, Sani MA, Scanlon DB, Forbes BE, Gardiner J, Separovic F, Wade JD, Hossain MA. Total Chemical Synthesis of an Intra3A3Chain Cystathionine Human Insulin Analogue with Enhanced Thermal Stability. *Angew Chem Int Ed*. 2016; 55:14743–14747.
59. Hovgaard L, Mack EJ, Kim SW. Insulin stabilization and GI absorption. *J Controlled Release*. 1992; 19:99–108.
60. Hovgaard L, Jacobs H, Mazer NA, Kim SW. Stabilization of insulin by alkylmaltosides. A Spectroscopic evaluation. *Int J Pharm*. 1996; 132:107–113.
61. Park SJ, Choi SG, Davaa E, Park JS. Encapsulation enhancement and stabilization of insulin in cationic liposomes. *Int J Pharm*. 2011; 415:267–272. [PubMed: 21645598]
62. Leobandung W, Ichikawa H, Fukumori Y, Peppas NA. Preparation of stable insulin-loaded nanospheres of poly (ethylene glycol) macromers and N-isopropyl acrylamide. *J Controlled Release*. 2002; 80:357–363.
63. Chalasani KB, Russell-Jones G, Yandrapu SK, Diwan PV, Jain SK. A novel vitamin B 12-nanosphere conjugate carrier system for peroral delivery of insulin. *J Controlled Release*. 2007; 117:421–429.
64. Akiyoshi K, Kobayashi S, Shichibe S, Mix D, Baudys M, Kim SW, Sunamoto J. Self-assembled hydrogel nanoparticle of cholesterol-bearing pullulan as a carrier of protein drugs: complexation and stabilization of insulin. *J Controlled Release*. 1998; 54:313–320.
65. Wood MR, Duncalf DJ, Rannard SP, Perrier S. Selective One-Pot Synthesis of Trithiocarbonates, Xanthates, and Dithiocarbamates for Use in RAFT/MADIX Living Radical Polymerizations. *Org Lett*. 2006; 8:553–556. [PubMed: 16468709]
66. Ayala JE, Samuel VT, Morton GJ, Obici S, Croniger CM, Shulman GI, Wasserman DH, McGuinness OP. Standard operating procedures for describing and performing metabolic tests of glucose homeostasis in mice. *Dis Models & Mech*. 2010; 3:525–534.

Figure 1. Insulin-trehalose glycopolymer conjugate where the polymer improves both the storage stability and *in vivo* plasma half-life (protein structure from the Protein Data Bank 4INS).

Figure 2.

In vitro stabilization of insulin (0.5 mg/mL) by trehalose glycopolymer. (a) HPLC AUC (area under the curve) of insulin peak during heating (90 °C), $n = 3$. (b) Insulin aggregation upon heating (90 °C, 30 min) measured by DLS ($n = 3$, representative image shown). (c) HPLC AUC of insulin peak during agitation (250 rpm, 37 °C), $n = 3$. (d) Insulin aggregation upon agitation (250 rpm, 37 °C, 3 hours) measured by DLS ($n = 3$, representative image shown).

Figure 3. Synthesis of insulin-trehalose glycopolymer conjugate. (a) RAFT polymerization and (b) subsequent conjugation of trehalose glycopolymer to insulin (PDB: 4INS) by reductive amination.

Figure 4.

Characterization of insulin-trehalose glycopolymer conjugate. ESI-MS spectra of (a) chain A and (b) chain B after acid treatment and disulfide reduction each show modification with a single polymer. Native-PAGE after (c) Coomassie staining and (d) western blot show conjugation of aldehyde-functionalized trehalose glycopolymer to insulin (lane 1: insulin, lane 2: trehalose glycopolymer, lane 3: unpurified insulin-trehalose glycopolymer conjugation mixture, lane 4: purified insulin-trehalose glycopolymer, PDB: 4INS).

Figure 5. Pharmacokinetics study of insulin (PDB: 4INS) and polymer conjugates. Blood concentration of insulin in fasted mice after i.v. injection of free insulin, insulin-trehalose glycopolymer, and insulin-PEG conjugates ($n = 5$ each set, 16 Qg/kg of insulin in 0.4 Qg/mL concentration, $p > 0.1$ between conjugates, $p < 0.05$ between insulin and conjugates at all points).

Figure 6.

Bioactivity study of insulin-trehalose glycopolymer conjugate. (a) Blood glucose levels in fasted mice after i.v. injection with unmodified insulin (16 Qg/kg) and insulin-trehalose glycopolymer conjugate (80 Qg/kg) ($n = 5$). (b) Activity of heated insulin, insulin with trehalose glycopolymer excipient (2 molar equivalents), and insulin-trehalose glycopolymer conjugate (90 °C, 30 min) relative to unheated samples during ITT in mice ($n = 4$, * $p < 0.05$ and *** $p < 0.005$).