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Trehalose Glycopolymers as Excipients for Protein Stabilization

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Abstract

Herein, the synthesis of four different trehalose glycopolymers and investigation of their ability to stabilize proteins to heat and lyophilization stress are described. The disaccharide, α,α -trehalose, was modified with a styrenyl acetal, methacrylate acetal, styrenyl ether, or methacrylate moiety resulting in four different monomers. These monomers were then separately polymerized using free radical polymerization with azobisisobutyronitrile (AIBN) as an initiator to synthesize the glycopolymers. Horseradish peroxidase and glucose oxidase were incubated at 70 and 50 °C, respectively, and β -galactosidase was lyophilized multiple times in the presence of various ratios of the polymers or trehalose. The protein activities were subsequently tested and found to be significantly higher when the polymers were present during the stress compared to no additive and to equivalent amounts of trehalose. Different molecular weights (10 kDa, 20 kDa, and 40 kDa) were tested and all were equivalent in their stabilization ability. However, some subtle differences were observed regarding stabilization ability between the different polymer samples, depending on the stress. Small molecules such as benzyl ether trehalose were not better stabilizers than trehalose, and the trehalose monomer decreased protein activity, suggesting that hydrophobized trehalose was not sufficient and that the polymeric structure was required. In addition, cytotoxicity studies with NIH 3T3 mouse embryonic fibroblast cells, RAW 264.7 murine macrophages, human dermal fibroblasts (HDFs), and human umbilical vein endothelial cells (HUVECs) were conducted with polymer concentrations up to 8 mg/mL. The results showed that all four polymers were non-cytotoxic for all tested concentrations. The results together suggest that trehalose glycopolymers are promising as additives to protect proteins from a variety of stressors.

Keywords

trehalose; glycopolymer; protein; stabilization; heat; lyophilization; excipient; additive; free radical polymerization

Introduction

Proteins are widely used as reagents in laboratories and as therapeutics for many different diseases.^{1,2} However, the instability of proteins during storage and transport increases costs and reduces effectiveness of protein drugs. For example, temperature fluctuations,³ desiccation,⁴ and light⁵ can significantly reduce biological activity of proteins. Proteins endure additional stressors during production and processing. For example, lyophilization,

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Supporting Information. Synthesis of monomers and polymers and their characterization, higher trehalose concentrations, activity assay result of polymers only, CD results, GOX stability study, TGA/DSC data, synthesis of **Benzyl T** and **p(Benzyl PEG)**, and results of additional cell compatibility experiments are available free of charge via the Internet at <http://pubs.acs.org>.

also known as freeze-drying, is the most common method to form protein powders for long-term storage and handling.⁴ However, during the lyophilization process, the protein is exposed to extreme temperature and concentration changes, low pressures, pH changes and desiccation stresses, all of which can induce denaturation or aggregation.^{6–8} Also, even when stored as a powder, proteins denature over time and require the addition of excipients to maintain activity.^{4,9}

Many excipients (additives) have been used to invoke protein stability. The most common excipients include amino acids,^{10,11} osmolytes,^{12–14} sugars,^{15–18} proteins,^{19,20} polymers,^{21–27} and salts,²⁸ which are used to prevent denaturation of pharmaceutical proteins. Amino acids, such as histidine, arginine, and glycine, stabilize proteins through preferential hydration, direct binding, and buffering capacities.^{10,11} Osmolytes are also known protein stabilizers because of their preferential hydration mechanism.¹² Sugars, such as sucrose, lactose and trehalose, are used in both lyophilized and liquid formations to stabilize proteins.⁹ Human serum albumin and gelatins are widely used in protein drugs.⁹ Also, polymers are employed as additives to prevent aggregation and denaturation of proteins. For example, polyethylene glycol or polypropylene glycol added to insulin prevents aggregation of the protein.²⁹ Zwitterionic polymer conjugates also remarkably stabilized proteins²⁴ and nonionic amphipols were effective to solubilize membrane proteins in water and protect them from denaturation.^{25–27} Yet, despite the addition of excipients, many proteins still exhibit instability. Thus, the development of novel, nontoxic additives to stabilize proteins is an important area of research.

In this report, we describe the synthesis and investigation of several new polymers for potential use as excipients. The polymers have pendent trehalose units. Trehalose is a natural non-reducing disaccharide formed by α , α -1,1-linked glucose units, which is generally regarded as safe (GRAS) by the U.S. Food and Drug Administration.³⁰ Interestingly, many reports have shown that trehalose plays a key role in protecting cells and proteins to allow organisms to tolerate anhydrobiosis (desiccation) and cryobiosis (low temperature).³⁰ Currently, trehalose is widely used in the food and cosmetic industries. Also, trehalose is employed as an excipient in protein-based drugs including Advate[®] (Baxter), Avastin[®] (Genentech), Lucentis[®] (Genentech), and Herceptin[®] (Genentech).

Recently, we reported the synthesis of monomer 4,6-*O*-(4-vinylbenzylidene)- α , α -trehalose, which was polymerized using reversible addition-fragmentation chain transfer (RAFT) polymerization.³¹ The resulting polymer had a pyridyl disulfide end-group to conjugate to free cysteines. When the polymer was conjugated to lysozyme, the stability of the protein against heat and lyophilization stress was remarkably increased. We also noted that adding excess of the polymer (not conjugating the polymer) also stabilized the protein, which means the strategy might be applicable to excipients. Herein, we describe the preparation of four different polymers with trehalose side chains. The polymers were added to three different proteins and the proteins were stressed. The activities of the proteins after treatment were evaluated with and without polymer, and to a comparable amount of trehalose. In addition, cytotoxicity of glycopolymers was tested with two mouse and two human cell lines. The results together suggest that the polymers are promising as an additive for protein stabilization. The details of the experiments are discussed below.

Experimental Section

Materials

All the chemicals and proteins were purchased from Sigma-Aldrich and Fisher Scientific and were used without purification unless noted. Trehalose was purchased from The Healthy Essential Management Corporation (Houston, TX) and dried with ethanol and kept under

vacuum before use in synthesis. Trehalose used in stability studies was dried with ethanol, lyophilized, and kept in a closed vial prior to use. Azobisisobutyronitrile (AIBN) was recrystallized from acetone before using. Amplex[®] Red Glucose/Glucose Oxidase Assay kit was purchased from Invitrogen and was used following the manufacturer instructions. 4,6-*O*-(4-Vinylbenzylidene)- α , α -trehalose (**M1**) was synthesized according to a literature procedure.³¹ 3,3'-Diethoxypropyl methacrylate (**2**) was prepared by following a literature procedure.^{32,33}

Analytical techniques

NMR spectra were obtained on Bruker AV 500 and DRX 500 MHz spectrometers. ¹H NMR spectra were acquired with a relaxation delay of 2 s for small molecules and 30 s for polymers. Infrared absorption spectra were recorded using a PerkinElmer FT-IR equipped with an ATR accessory. Gel permeation chromatography (GPC) was conducted on a Shimadzu high performance liquid chromatography (HPLC) system with a refractive index detector RID-10A, one Polymer Laboratories PLgel guard column, and two Polymer Laboratories PLgel 5 μ m mixed D columns. Eluent was DMF with LiBr (0.1 M) at 40 °C (flow rate: 0.60 mL/min). Calibration was performed using near-monodisperse pMMA standards from Polymer Laboratories. HPLC purification of the monomers were conducted on a Shimadzu HPLC system with a refractive index detector RID-10A and one Luna 5 μ m C18(2) column with MeOH and H₂O as an eluent. ESI-MS data were gathered on a Waters LCT premier with ACQUITY LC. Circular Dichroism (CD) spectra were recorded in a Jasco J-715 spectropolarimeter (Jasco, Tokyo, Japan). Differential scanning calorimetry (DSC) was performed on a PerkinElmer Diamond DSC. Approximately 2 mg samples of 1–3 mol % trehalose or **P3** in water were prepared in hermetically sealed aluminum pans. Two heating/cooling scans were performed over a temperature range of –40 °C to 25 °C with a heating/cooling rate of 10 °C/min. Reported values were determined from the second scan. Thermal Gravimetric Analysis (TGA) was performed on a PerkinElmer Diamond TG/DTA using platinum pans. Measurements were performed under 200 mL/min Ar flow over a temperature range of 30 °C to 600 °C at a heating rate of 10 °C/min.

Methods

Representative Free Radical Polymerization—M1 (426 mg, 9.33×10^{-1} mmol) was dissolved in dimethylformamide (DMF, 1.17 mL). AIBN (7.00 mg, 4.26×10^{-2} mmol) was added to the solution. Five cycles of freeze-pump-thaw were undertaken, and the reaction was initiated by immersion in an oil bath at 80 °C. The polymerization was stopped by cooling with liquid nitrogen after 17 h. By ¹H NMR spectroscopy 100% monomer conversion was achieved. The product was purified by dialysis against H₂O for 3 days (MWCO 3,500 g/mol) and dried using a lyophilizer resulting in **P1**. Number-average molecular weight (M_n) of the polymer was 19,500 g/mol and polydispersity index (PDI) was 2.01 by GPC. ¹H NMR (500 MHz in D₆DMSO) δ : 7.11, 6.39, 5.45, 5.23, 4.96, 4.86, 4.41, 4.12, 3.97, 3.78, 3.70, 3.62, 3.48, 3.17, 1.60, 1.24, 0.86. IR: ν = 3384, 2916, 1635, 1380, 1150, 1074, 983 cm⁻¹.

Horseradish Peroxidase Heating Studies—Horseradish peroxidase (HRP, 150 μ g/mL) solution was prepared by adding HRP into pH 7.0, 10 mM sodium phosphate buffer. Stock solutions of 80, 50, 25, and 1 weight equivalent (wt equiv) of **P1**, **P2**, **P3** and **P4** to HRP were prepared. Trehalose was added to be equivalent to the trehalose amount in **P1** for each weight equivalent. 2 μ L aliquots of the HRP solution were mixed with 2 μ L aliquots of each polymer or trehalose solution to give final concentration of 75 μ g/mL of HRP. Samples were incubated at 70 °C for 30 min and a non-heated control sample was stored at 4 °C until the activity assay was performed. 3,3',5,5'-Tetramethylbenzidine (TMB) was used as a substrate and 1 M H₂SO₄ solution as the stop solution. To assess the activity, absorbance at

450 nm was monitored. The assay was repeated in total twelve times (n=12, two separate experiments with six repeats each).

β -Galactosidase Lyophilization Studies— β -Galactosidase (β -Gal, 0.4 mg/mL) solution was prepared by adding β -Gal to pH 7.0 10 mM sodium phosphate buffer. Each 25 μ L aliquot of β -Gal solution was mixed with 75 μ L of H₂O (the control) or 75 μ L of stock solution each containing 10, 5, or 1 wt equiv of **P1**, **P2**, **P3**, **P4**, and trehalose to β -Gal in H₂O to result in a 0.1 mg/mL protein solution. Aliquots of each sample were frozen by immersion in liquid nitrogen before solvent removal via lyophilization. The samples were lyophilized for 12 h for one cycle under 1 mbar. After the first cycle, 100 μ L of H₂O was added and lyophilization was repeated for the next cycles. The control sample was stored at 4 °C until the activity assay was conducted. After the desired number of cycles, the final lyophilized samples were dissolved into 50 μ L of H₂O. 30 μ L of *o*-nitrophenyl- β -D-galactoside (ONPG, 4 mg/mL) was added to the samples. The samples were incubated in dark for 5 min and the reaction stopped with 50 μ L of 1 M Na₂CO₃. Activity was ascertained by reading the absorbance at 405 nm.³⁴ The assay was repeated in total twelve times (n=12, two separate experiments with six repeats each).

Effect of Polymer Molecular Weight on Protein Stabilization—10, 20, and 40 kDa molecular weight **P1** were synthesized. For the HRP stabilization, 25 wt equiv of each **P1** to HRP was added, and the stabilization effect was studied as described before. Also, the same polymers were tested for β -Gal stabilization against lyophilization. β -Gal with 2.5 wt equiv of each **P1** were lyophilized for three cycles and assayed as described above. The assay was repeated in total twelve times (n=12, two separate experiments with six repeats each).

Effect of Trehalose Derivatives and Polymer Analogue on Protein Stabilization

—The stabilization of HRP and β -Gal against heating and lyophilization were tested with the following additives: **P3**, trehalose (**T**), monomer 3 (**M3**), benzyl trehalose (**Benzyl T**), and poly(benzyl ether poly(ethylene glycol) methyl ether) (**p(Benzyl PEG)**). 80 and 10 wt equiv of each of the small molecules and polymers were added to HRP and β -Gal, respectively. The assay was repeated in total twelve times (n=12, two separate experiments with six repeats each).

Cytotoxicity Assay of P1–P4—The cell compatibility of the trehalose glycopolymers to NIH 3T3 mouse embryonic fibroblast cells (NIH 3T3, ATCC), RAW 264.7 murine macrophages (RAW 264.7, from Professor Genhong Cheng), human dermal fibroblasts (HDF, Promocell GmbH), and human umbilical vein endothelial cells (HUVEC, Promocell GmbH) were evaluated using a LIVE/DEAD[®] viability/cytotoxicity assay (Invitrogen). Controls were 20 kDa PEG, trehalose, and buffer only. NIH 3T3 and RAW 264.7 cells were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. HDF cells were cultured in fibroblast growth medium (Promocell) containing 2% fetal calf serum (FCS), 1 ng/mL basic fibroblast growth factor (bFGF), 5 μ g/mL insulin, and 1% penicillin-streptomycin. HUVECs were cultured in endothelial cell growth medium (Promocell) containing 2% FCS (fetal calf serum) with supplements recommended by the supplier. The cells were seeded in 48-well plates (BD Falcon) at a density of 5×10^3 cells per well. After 24 h, culture media were replaced with 200 μ L of the working medium containing known polymer concentrations of 0.1, 0.5, 1, 4, and 8 mg/mL (**P4** up to 1 mg/mL). After incubation for 48 hours, the cells were gently washed twice with pre-warmed D-PBS, and stained with the LIVE/DEAD reagent (2 μ M calcein AM and 4 μ M ethidium homodimer-1). Fluorescent images of each well were captured on an Axiovert 200 microscope with an AxioCam MRm camera and FluoArc mercury lamp. The number of live and dead cells was counted, and percent cell viability was calculated by dividing the number

of live cells by the total number of cells. All experiments were conducted with four repetitions. The results were calculated by using the formula: $100 \times (\text{number of live cells} / \text{total number of cells})$. The data is provided by normalizing each set to the control without any additives.

Statistical Analysis—All the *p*-values were calculated using the independent Student's *t*-test assuming unequal variances.

Results

Synthesis of Trehalose Monomers and Glycopolymers

In order to explore the protein stabilization capability of trehalose glycopolymers, we designed and synthesized four different monomers, **M1** through **M4'** (Scheme 1), and used free radical polymerization to prepare the polymers, **P1–P4**. The position of linkage was either through the 4, 6 (**M1** and **M2**) or 6 (**M3** and **M4'**) hydroxyl of trehalose, the chemistries of linkages were an acetal (**M1** and **M2**), ether (**M3**), or ester (**M4'**), and the polymer backbone was polystyrene (**M1** or **M3**) or polymethacrylate (**M2** or **M4**). We anticipated that this set would allow us to ascertain the effect of polymer backbone or chemistry of linkage on the protein stabilization capability.

4,6-*O*-(4-Vinylbenzylidene)- α , α -trehalose (**M1**)³¹ and 4,6-*O*-(3-propyl methacrylate)- α , α -trehalose (**M2**) were synthesized using styrenyl diethyl acetal and 3,3'-diethoxypropyl methacrylate (**2**) as a starting materials, respectively (Scheme 1).^{31–33} Under acidic conditions with *p*-TsOH and trehalose, **M1** and **M2** were made in 41 and 37% yield. Another monomer, 6-*O*-(4-vinylbenzylidene)- α , α -trehalose (**M3**), was prepared in one step in 20% yield by adding sodium hydroxide and 4-vinyl benzyl chloride to a solution of trehalose. Acetylated trehalose with one of the primary alcohols unprotected was synthesized in three steps by modifying a literature procedure.³⁵ The acetylated trehalose was then reacted with methacryoyl chloride in the presence of triethylamine to give **M4'** in 64% yield. See the ESI for detailed experimental procedures and ¹H and ¹³C NMR spectra for all monomers.

The polymers were all prepared by free radical polymerization utilizing AIBN as an initiator. The polymerization temperature was 80 °C for the styrenyl-based monomers and 65 °C for the methacrylates. The resulting polymers **P1**, **P2**, **P3**, and **P4'** (Scheme 2) were prepared with number average molecular weights (*M_n*) between 14.6 kDa and 23.4 kDa (see ESI for experimental details and for the ¹H NMR spectra of the polymers). **P4'** was then deprotected using a catalytic amount of NaOMe by modifying our previous report;^{36,37} the product precipitated out of solution as the deprotection occurred. **P4** was neutralized with HCl and dialyzed against H₂O resulting in a *M_n* of 10.8 kDa and PDI of 1.52. From ¹H NMR spectroscopy, the peaks corresponding to the methyl protons of the acetate groups at approximately 2 ppm disappeared suggesting successful deprotection (see ESI, Figure S15). Also with IR analysis, the disappearance of the acetyl C=O and C-O stretches, and the appearance of the O-H absorption were observed (see ESI, Figure S16). The molecular weight and polydispersity index for all the polymers are summarized in Table 1.

Heat Burden Studies of Proteins with the Polymers

The activity of HRP after exposure to 70 °C for 30 minutes with and without additives was assayed. HRP is an important heme-containing enzyme, which has been used in biotechnology and bioremediation. However, thermostability of the HRP limits its industrial application.³⁸ The activity of HRP was tested by addition of TMB, which produces a blue by-product in the presence of HRP. HRP samples were prepared without any additive, with

P1–P4, or trehalose. To determine effectiveness of the polymers, 1 wt equiv to 80 wt equiv of each polymer relative to HRP was tested. Weight equivalents were utilized so that the same amount of trehalose was added in each set regardless of which polymer was used, and any inaccuracies or differences in molecular weight of the polymers would not be a factor. Trehalose was added such that the amount was equivalent to the trehalose repeating units in **P1** for each set; in this way, trehalose and the polymer were directly comparable.

Samples that had been heated were tested for remaining HRP activity, and the percent original activities were calculated using the same concentration of HRP without heating and additive (Figure 1). Only 55% of the original activity was observed when HRP itself was heated to 70 °C. Yet, activity was completely retained (100%) for all of the glycopolymers (**P1–P4**) when 50 or 80 wt equiv were added. At 25 wt equiv, differences between the polymers were observed; **P1** stabilized better than no additive (80%) but less than the other polymers that retained HRP activity at 100%. With 1 equiv, all the polymers stabilized the protein slightly better than no additive, while **P3** had a weaker effect compared to the other polymers. The same equivalents of trehalose also stabilized HRP, but the HRP activity was lower compared to the polymers. Increasing the concentration of trehalose up to 4000 wt equiv, improved the stabilization observed (74%, see ESI Figure S17a) but the value was still well below that observed for the polymers. As a control, the polymers alone were tested. **P1–P4** were not substrates for the assay; there was no observed activity in the absence of the enzyme (ESI Figure S18a).

Circular dichroism investigations were undertaken in addition to the activity assays. CD spectra were taken at 70 °C with and without **P2** and also after cooling back to room temperature; the results were compared to pristine HRP. The data showed a similar trend as the activity studies. Loss of some secondary structure was observed for both HRP and HRP with **P2** (ESI Figure S19a). However after cooling, the HRP heated in the presence of **P2** regained its secondary structure to a greater extent. This result suggests that denaturation was reversible and that the polymer may help the protein refold (Figure S19b).

To test an additional enzyme to heat, GOX was also investigated. GOX is also an interesting protein with industrial importance that is currently used in applications ranging from fiber bleaching, food preservatives, to glucose detection kits.³⁹ However, its structural instability is problematic. It was found that 20 wt equiv of trehalose glycopolymers **P1** through **P4** stabilized GOX to 50 °C heat for 30 min better than no additive; however, the effect was not as dramatic because the protein itself was relatively stable at these conditions (see ESI for experimental details and Figure S20). In this case trehalose itself did not stabilize the protein and was statistically the same as no additive at all.

Lyophilization Study

The stabilization of β -galactosidase (β -Gal) was also tested after multiple lyophilization cycles. β -Gal is an important enzyme that catalyzes hydrolysis of β -galactosides including lactose. Furthermore, a recent study shows that it could be used in gene replacement therapy.⁴⁰ However, β -Gal is known to be easily denatured by lyophilization or freeze-thaw cycles.³⁴ The activity of β -Gal was measured by reacting with ONPG, which releases *o*-nitrophenol, a yellow colored product that absorbs light at 405 nm. After 3 cycles of lyophilization, β -Gal had only 47% remaining activity, while 10 wt equiv **P1** through **P4** maintained activity from 85 to 108 %, depending on the polymer (Figure 2). CD confirmed the results (Figure S19c). It has been reported that for GOX adding trehalose results in an increase in affinity of the substrate for the enzyme.³⁹ This was proposed to be due to the sugar enhancing the protein/substrate binding or stabilizing that complex.³⁹ Thus, it is not unprecedented that greater than 100% activity was observed. The result showed that the polymers outperformed trehalose, since the trehalose maintained only 73% of the original

activity maximum. At 5 wt equiv, the polymers maintained activity 80 to 100%, while trehalose was the same as no additive. At 1 wt equiv, the protein was only marginally stabilized by **P1**, **P2** and **P3**.

From this data the styrenyl polymers **P1** and **P3** provided the best stabilization, and were better than trehalose. Unlike the HRP heat burden study, when higher concentrations of trehalose were tested (100, 500, and 1000 wt equiv) β -Gal was completely stabilized (see ESI Figure S17b). However, by using the trehalose polymers, a much smaller amount of trehalose was required for stabilization. To ensure that the polymers themselves did not have activity in the assay giving erroneous results, they were tested (ESI Figure S18c). Again, the polymers were not substrates for the assay, and no activity was observed. Finally, to rule out inaccuracies in the weight equivalent used due to differences in water, TGA of the polymer and trehalose was undertaken (ESI Figure S21). The polymer contained more water (2.4%) than trehalose (1.4%). Interestingly, there was one regime of water loss for the polymer, while for trehalose there was a typically loosely held phase (0.8%) and tightly held water (0.6%). The latter is reported to be due to crystallization of trehalose.⁴¹ Regardless, the TGA data clearly showed that the polymer weight was underestimated in these experiments. Thus water contact was also not a factor, and the polymers did stabilize at lower concentrations based on trehalose content.

Effect of Polymer Molecular Weight on Protein Stabilization

To investigate the effect of polymer molecular weight on protein stabilization, **P1** was synthesized with a M_n of 11.9 (PDI = 2.50) and 40.8 (PDI = 2.80) kDa, and these in addition to the 19.5 kDa **P1** that was used previously were tested. The polymers were added to HRP and β -Gal at 25 wt equiv and 2.5 wt equiv, respectively, prior to heating and lyophilization stresses. The remaining activity after the experiments was same regardless of the M_n or both HRP and β -Gal (Figure 3a and b) showing that the molecular weight had no effect, at least in this regime.

Investigation of Stabilization Properties

During lyophilization, protein denaturation can be observed due to ice formation, a change in concentration of solutes as a result of crystallization (which can lead to osmotic shock or large changes in pH values), or due to cold denaturation.⁴² Trehalose is known to disrupt the hydrogen bonded network of water molecules causing depression of ice formation.⁴³ This leads to a decrease in the enthalpy of melting and crystallization of water and also to a reduction of the temperature of crystallization (T_c).⁴⁴ To evaluate whether or not the polymers have the same properties, DSC was utilized. Trehalose or **P3** (1, 2 and 3 mol % based on trehalose) were added to water. It was found that for both trehalose and the polymer the enthalpy of melting and crystallizations were reduced to a similar extent as was reported in literature (ESI Table S1).⁴⁴ The depression in enthalpy values increased with increasing concentration of trehalose and **P3**. In addition, the T_c was also depressed for both trehalose and the polymer, the relative amount of which depended on the mol %. These results show that the polymer has similar properties in this respect to trehalose and suggest that the trehalose glycopolymers are capable of stabilizing proteins against ice formation induced denaturation or solute crystallization during lyophilization through a mechanism similar to that of trehalose.

The polymers all have hydrophobic backbones attached to the trehalose, meaning that they can be considered nonionic surfactants. To investigate the effect of hydrophobizing trehalose, the stabilization ability of the trehalose monomers and other analogues were determined. For this study, benzyl trehalose (**Benzyl T**) was synthesized, which is a **M3** analogue lacking the vinyl group. Poly(benzyl ether poly(ethylene glycol)) (**p(Benzyl**

PEG)) with a styrenyl backbone and hydrophilic PEG side chains was also prepared in order to understand whether the trehalose component is required (see ESI for the synthesis of **Benzyl T** and **p(Benzyl PEG)**). Interestingly, after heating and lyophilization, **M3** itself decreased HRP activity to 4 % and β -Gal activity to 10 % (Figure 3c and d). This may be due to reaction of the monomer unit with the proteins. In both experiments **Benzyl T** slightly stabilized protein, but less than trehalose itself. In the heat burden study **p(Benzyl PEG)** decreased the activity of HRP compared to having no additive. On the other hand, the stabilization effect of **p(Benzyl PEG)** was similar to trehalose in the case of lyophilization of β -Gal. Neither the **Benzyl T** or **p(Benzyl PEG)** stabilized as well as the polymers, suggesting that the hydrophilic-hydrophobic character in the polymer was not the only factor in stabilization.

P1 and **P2** both contain acetal linkages, which are susceptible for cleavage under slightly acidic conditions. If the acetals did hydrolyze, then polymeric aldehydes would form. Polyaldehydes such as glutaraldehyde are known to stabilize proteins. To determine this, the polymers were subjected to conditions utilized in the activity studies, namely heating to 70 °C for 30 minutes, followed by lyophilization or were incubated for 37 °C for 48 h. The samples were then characterized by ¹H NMR spectroscopy, IR, and the Purpald assay. No aldehyde formation was detected in any case. The hydrophobic backbone of the polymers likely protects the acetal from cleavage.

Examination of Cytotoxicity

To examine the cell compatibility of the trehalose glycopolymers, NIH 3T3, RAW 264.7, HDF, and HUVEC cells were separately cultured in the presence of each polymer. The results were compared to control wells containing media only. The cells were cultured in 48-well plates at a density of 5×10^3 cells per well. After 24 h, the culture media was replaced with the working medium containing each polymer at a concentration of 0.1, 0.5, 1, 4, and 8 mg/mL. After 48 h incubation, the cells were stained with the LIVE/DEAD[®] reagent and fluorescent images were captured for each well. Live and dead cells were counted, and the percent cell viability was calculated by dividing the number of live cells by the total number of cells. All experiments were conducted with four repetitions and averaged. The results for 1–8 mg/mL for **P1–P3** are provided in Figure 4 (see ESI Figure S22 for 0.1–1 mg/mL for **P1–P4**). In each case, the viability of the cells in the presence of the trehalose polymers was the same as no additive, trehalose, and the non-toxic polymer PEG. The cell counts were similar in all cases as well, except for **P2** where fewer cells were observed at the 8 mg/mL concentration. This result is important for applications such as stabilization of cells for transport, where lower cell counts and decreases in proliferation would be a disadvantage. The data confirmed that **P1–P3** trehalose glycopolymers are non-cytotoxic to all the tested four cell lines.

Discussion

Together, the above results show that trehalose glycopolymers are good candidates for use as additives to stabilize proteins. In each of the three proteins studied, the trehalose glycopolymers stabilized the protein significantly better than no additive. In addition, the polymers are non-cytotoxic to at least two mouse and two human cell lines. Trehalose is currently used as one of the excipients in high concentrations (10–100 mg/mL) in protein-based therapeutics, such as Advate[®], Avastin[®], Lucentis[®], and Herceptin[®]. It may be possible to utilize the trehalose glycopolymers as alternative excipients, especially since the polymers stabilized the proteins better than trehalose to each stress. In addition, enzymes such as HRP, GOX, and β -Gal are used in industry and some have potential use in

therapeutic applications. Thus, stabilization of these proteins to stresses which inactivate them is important.

The exact mechanism of how trehalose protects proteins or cells is not known. However, there are three main theories that have been proposed for this osmolyte. These are vitrification,⁴⁵ water replacement,^{46,47} and water entrapment.⁴⁸ DSC results have demonstrated that the polymers are similar to trehalose with respect to suppression of the enthalpy of melting and reduction of the crystallization temperature. However, the polymers stabilized at a lower concentration (based on trehalose to trehalose units) than the sugar itself to lyophilization and were superior all together for heat stabilization. Thus, additional stabilization mechanisms must be in effect. The polymers all have hydrophobic backbones and hydrophilic (trehalose) side chains. Thus, they are nonionic surfactants. Nonionic surfactants are known excipients and are proposed to stabilize proteins by preventing proteins from adsorbing onto hydrophobic surfaces, directly interacting with hydrophobic residues of proteins, and may even act as chaperones for protein unfolding.⁹ To begin to look at the effect of hydrophobic moieties on trehalose, **Benzyl T** (an analogue of the **M3**) and **p(Benzyl PEG)** (which has the polystyrene backbone, but no trehalose) were compared. Neither had the stabilizing ability of the trehalose polymers. The exact mechanisms responsible for the superior stabilization of the trehalose polymers remain to be determined. Yet it is clear that the polymers combine two important classes of excipients, namely the sugars and surfactants.

Different polymers were tested in order to ascertain the importance of attachment site, chemistry, and polymer backbone. **M1**, **M2**, and **M3** were easily prepared in one to two steps without requiring protecting group strategies. Although the yields were low in each case, trehalose is readily available as a food additive and is inexpensive. **M4'** was synthesized by selective protection-deprotection steps. While a longer synthesis, the selective protection-deprotection steps provided reaction selectivity and convenience with regard to purification. The polymers were all polymerized by simple free radical polymerization. **P1–P3** could be utilized directly after synthesis, while **P4** required deprotection. **P1** and **P2** contained six, and **P3** and **P4** had seven hydroxyl groups. All polymers were highly water-soluble. All the polymers were fairly similar in their ability to stabilize HRP, GOX, and β -Gal, especially at high concentrations, although there were slight differences that were observed as lower concentrations were used. There did not appear to be differences with regard to the site of attachment or chemistry of the linkages; the polymer backbone was the dominant effect. For heat treatment of HRP, the polymethacrylate backbone polymers, **P2** and **P4**, stabilized best at lower concentrations. For lyophilization of β -Gal **P1** and **P3**, the styrenyl polymers, were better. With these straightforward syntheses, it should be possible to find a trehalose polymer stabilizer for many different types of proteins and stresses, even at low concentrations of polymer.

Conclusions

In this report, we described the synthesis of four trehalose glycomonomers and their respective homopolymers. The four glycopolymers all stabilized HRP and GOX to heat and β -Gal during lyophilization, compared to no additive, as well as to same amount of trehalose. The cytotoxicity of all prepared glycopolymers were tested with two mouse and two human cell lines. The glycopolymers were non-cytotoxic to all four cell lines. The results show that the trehalose glycopolymers should be further explored as excipients in the pharmaceutical industry to protect protein-based drugs from denaturation, to protect enzymes used industrially as reagents, and in protein research and development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Activity of HRP (75 µg/mL) after 30 min incubation at 70 °C, with no additive, **P1**, **P2**, **P3**, **P4**, or trehalose (**T**). 80, 50, 25, and 1 wt equiv of each polymer were added to the protein. Trehalose was added to be equivalent to the amount of trehalose in **P1** for each set. Data shown as the average of n=12 with standard deviation. *** = $p < 0.001$ and * = $p < 0.05$ relative to no additive. ### = $p < 0.001$ and ## = $p < 0.01$ relative to trehalose.

Figure 2.

Activity of β -Gal (100 μ g/mL) after 3 cycles of lyophilization with no additive, **P1**, **P2**, **P3**, **P4**, and trehalose (**T**) added to the β -Gal. 10, 5, and 1 wt equiv of each polymer were added to the protein. Trehalose was added to be equivalent to the amount of trehalose in **P1** for each set. Data shown as the average of $n=12$ with standard deviation. *** = $p < 0.001$, * = $p < 0.05$ relative to no additive. ### = $p < 0.001$ and ## = $p < 0.01$ relative to trehalose.

Figure 3.

Activity of (a) HRP (75 $\mu\text{g/mL}$) after 30 min heating at 70 $^{\circ}\text{C}$ with 25 wt equiv of 10, 20, 40 kDa P1, (b) β -Gal (100 $\mu\text{g/mL}$) after 3 cycles of lyophilization with 2.5 wt equiv of 10, 20, 40 kDa P1, (c) HRP (75 $\mu\text{g/mL}$) after 30 min heating at 70 $^{\circ}\text{C}$ with 80 wt equiv of trehalose (T), M3, benzyl trehalose (Benzyl T), and poly(benzyl PEG) (p(Benzyl PEG)), and (d) β -Gal (100 $\mu\text{g/mL}$) after 3 cycles of lyophilization with 10 wt equiv of T, M3, Benzyl T, and p(Benzyl PEG).

Figure 4.

Cytotoxicity assay of **P1–P3** and 20 kDa PEG with (a) NIH 3T3, (b) RAW 264.7, (c) HDF, and (d) HUVEC cells, $p > 0.05$ for all polymers relative to the control. The experiments were repeated four times and the results provided as the average with standard deviation relative to the control with no additive.

Scheme 1.
Synthesis of trehalose monomers.

Scheme 2.
Structure of the trehalose polymers.

Table 1

Molecular weight and PDI data of the trehalose polymers.

Polymer	Monomer	Linker to Trehalose	M_n by GPC (kDa)	PDI
P1	M1		19.5	2.01
P2	M2		23.4	2.40
P3	M3		14.6	1.77
P4	M4' (deprotected after polymerization)		10.8	1.52