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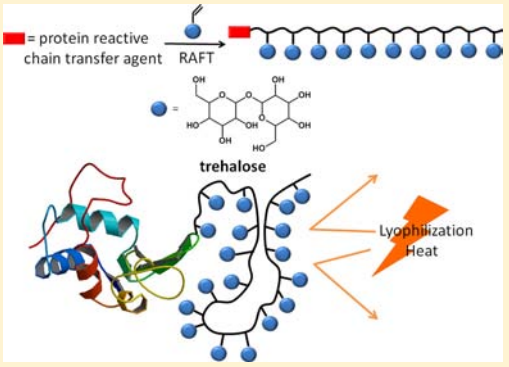
Trehalose Glycopolymers for Stabilization of Protein Conjugates to Environmental Stressors

Rock J. Mancini, Juneyoung Lee, 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 90095-1569, United States

Supporting Information

ABSTRACT: Herein, we report the synthesis of trehalose side chain polymers for stabilization of protein conjugates to environmental stressors. The glycomonomer 4,6-*O*-(4-vinylbenzylidene)-α,α-trehalose was synthesized in 40% yield over two steps without the use of protecting group chemistry. Polymers containing the trehalose pendent groups were prepared via reversible addition−fragmentation chain transfer (RAFT) polymerization using two different thiol-reactive chain transfer agents (CTAs) for subsequent conjugation to proteins through disulfide linkages. The resulting glycopolymers were well-defined, and a range of molecular weights from 4200 to 49 500 Da was obtained. The polymers were conjugated to thiolated hen egg white lysozyme and purified. The glycopolymers when added or covalently attached to protein significantly increased stability toward lyophilization and heat relative to wild-type protein. Up to 100% retention of activity was observed when lysozyme was stressed ten times with lyophilization and 81% activity when the protein was heated at 90 °C for 1 h; this is in contrast to 16% and 18% retention of activity, respectively, for the wild-type protein alone. The glycopolymers were compared to equivalent concentrations of trehalose and poly(ethylene glycol) (PEG) and found to be superior at stabilizing the protein to lyophilization and heat. In addition, the protein−glycopolymer conjugates exhibited significant increases in lyophilization stability when compared to adding the same concentration of unconjugated polymer to the protein.



INTRODUCTION

There is considerable interest in proteins as therapeutics and as biochemical and chemical reagents.[1−3] However, proteins are inherently unstable to thermal fluctuations.[4] Furthermore, most therapeutics are known to degrade upon storage, transport, and use, necessitating regulated temperatures, controlled solvation, and addition of stabilizing excipients.[5−10] Protein activity also diminishes upon exposure to physical or chemical insults such as desiccation,[11] heat,[12] light,[13] and pH change,[14] further complicating application of many biomolecules. As such, attachment of poly(ethylene glycol) (PEG) to proteins has been widely used to increase in vitro and in vivo stability for therapeutic proteins;[7−9] however, PEGylation alone provides limited stability to temperature, desiccation, or storage.

To overcome this problem, lyophilization (freeze-drying) has been widely adopted.[11,15] However, during lyophilization stresses are present on the protein that are tangential to a freeze−thaw process.[16] Therefore, engineering research must be employed to find the optimal buffer formulation for lyophilization of each individual protein type.[17,18] Formation of ice crystals, change in solute concentration, and variation of pH are common stresses present during lyophilization that can contribute to protein denaturation.[19] As a result, high concentrations of excipients are commonly added,[20] particularly for protein therapeutics and biochemical reagents,[21,22] and there is a growing demand for novel stabilizing excipients.[23] Specifically, various carbohydrates have been used to mitigate stressors typically experienced by proteins including thermal fluctuations and lyophilization.[24−26] We report herein polymers based on a natural disaccharide trehalose as potentially new stabilizers for proteins.

In nature, many plants and animals endure complete dehydration stress by accumulating large amounts of trehalose.[27,28] This alpha-linked glucose disaccharide has been known to impart unusual stability to organisms tolerating anhydrobiosis (desiccation) and cryobiosis (low temperature) by protecting cells and proteins.[24,29−33] For instance, Westh and Ramløv reported in 1991 that *Tardigrades*, commonly known as water bears, accumulate trehalose from 0.1% to 2.3% dry weight within 5−7 h under dehydration conditions.[30] In addition, Crowe and co-workers showed *Aphelenchus avenae* (nematodes) can increase the concentration of trehalose to as much as 15% dry body weight in response to desiccation.[31] Other groups have reported that brine shrimp eggs, *Artemia*, survive up to 15 years under anhydrous conditions when trehalose is present.[32] There is much debate over the mechanism of this dramatic stabilization of organisms by

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trehalose, and there are three main hypotheses that are not mutually exclusive: water replacement,[34,35] water entrapment,[36] and vitrification.[37] Proponents of the water replacement hypothesis claim that hydroxy groups in the trehalose molecule interact with proteins via hydrogen bonding and protect them from desiccation. In the water entrapment hypothesis, a layer of water is intimately isolated between the biomolecule and sugars. This surrounding water then protects the protein by maintaining hydration. In the third hypothesis, vitrification, glassy sugar matrices protect protein tertiary and quaternary structure from unfolding. To date, the precise mechanism of how trehalose can stabilize dehydrated organisms has not been proven, and it may be that the mechanism is dependent on the nature of the dehydration method.[20] Most importantly, trehalose acts as a protectant of proteins against environmental stresses. We hypothesized that polymers with pendent trehalose carbohydrates would likewise stabilize proteins to environmental stressors (Figure 1).

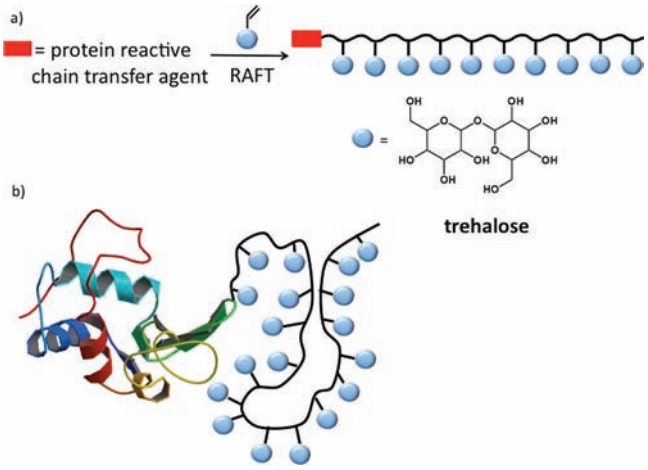

Figure 1. (a) Side chain trehalose polymer with a protein-reactive group at one end. (b) Trehalose polymer conjugates protect proteins from lyophilization and heat.

A unique disaccharide, trehalose, is a food additive that is generally regarded as safe (GRAS).[38] Although endogenous production has not been exhibited by mammals, metabolism occurs in humans through fragmentation about the anomeric center by trehalase enzymes found in the intestinal villi to produce glucose.[39] This stabilization ability combined with the safety of a known metabolic fate has contributed to the proliferation of the use of trehalose as an excipient in protein-based therapeutics, with at least four retail drugs currently having it in their formulation.[27] In this report, trehalose side chain polymers to add to proteins and to conjugate to proteins as stabilization agents were synthesized.

Previously, trehalose-based materials have been produced as cross-linked polymer networks[22] such as polysubstituted trehalose vinylbenzyl ether thermo-set resins.[40] Achieving trehalose linear polymers has been challenging as the anomeric centers are relatively unreactive due to the 1,1-glycosidic linkage.[41] Therefore, typical synthetic routes to produce trehalose-based monomers contain several protecting and deprotecting steps, use of bifunctional monomers targeting the 6,6′ positions, or produce mixtures of regio-isomers that are not well-defined. For example, a simple strategy for synthesizing a trehalose linear polymer was first reported in 1979, but selectivity to form linear polymer versus the branched one was unclear at that time.[42] Polymerization of diamino-type trehalose was explored to overcome the issue of branching, but the overall process was more complicated.[43] Acetalization,[44] enzymes,[45] Diels–Alder reactions,[46] and click chemistry[47,48] have been exploited to synthesize trehalose-based linear polymers, with the latter study being extended to biological systems. However, most incorporate trehalose into the polymer backbone rather than as a side chain. To date, a well-defined trehalose polymer synthesized by controlled radical polymerization has not been reported, and trehalose-based protein–glycopolymer conjugates have yet to be realized. Conjugating a trehalose polymer directly to the protein has the advantage of increasing the effective concentration of polymer relative to protein, thereby enhancing protective effects of the polymer. In addition, for therapeutics, a trehalose-based protein–polymer conjugate could combine the advantages of environmental stabilization due to the sugar with improved pharmacokinetics resulting from the polymer. Herein, we describe the synthesis of a trehalose side chain polymer and stabilization of protein conjugates. Trehalose was attached to a styrene monomer via a 4,6-acetal linkage. Subsequent RAFT polymerization generated well-defined, cysteine-reactive glycopolymers. These were covalently attached to thiolated hen egg white lysozyme, a protein known to lose activity due to heat[49] or lyophilization.[50] The resulting lysozyme–glycopolymer conjugates were exposed to these stresses, and significant increases in retention of activity relative to wild-type protein were observed. Investigation of the stabilization of wild-type lysozyme in the presence of unconjugated polymer is also presented.

RESULTS AND DISCUSSION

Synthesis of a Trehalose-Based Glycomonomer. One typical protection scheme for trehalose involves treatment with dimethoxy toluene to selectively afford acetal protection as 4,6,4′,6′-*O*-dibenzylidene trehalose in high (>90%) yield.[51] Therefore, we envisioned that reaction with 4-vinylbenzaldehyde diethyl acetal (**1**)[52] would exhibit similar regioselectivity to install a polymerizable group. Terephthaldehyde monodiethyl acetal was employed to form **1** via a Wittig reaction in 97% yield. This in turn was used to effect a transacetal reaction with trehalose exclusively at the 4,6 position under acidic conditions affording 4,6-*O*-(4-vinylbenzylidene)-α,α-trehalose in 41% yield (**2**, Scheme 1).

Reactions necessitating anhydrous conditions along with statistical monosubstituion of unmodified trehalose are generally difficult to achieve, partially due to the dramatic decrease in solubility of anhydrous trehalose in organic solvents relative to the dihydrate form.[53] Although a significant amount of bis-functionalized byproduct was formed, production of the desired product **2** occurred in 41% yield after HPLC purification; no other isomers were observed indicating that preference for the 4,6 position was retained for the substituted benzaldehyde diethyl acetal. In addition to ^{1}H NMR, ^{13}C NMR, HMQC, and COSY NMR (see Supporting Information, Figure S1),[54] heteronuclear single quantum coherence total correlation spectroscopy (HSQC-TOCSY) experiments were performed to verify both the selectivity of the reaction as well as the integrity of the product ring systems (see Supporting Information, Figure S2). Furthermore, NOESY NMR was undertaken to demonstrate retention of the unique conformation of the native disaccharide whereby the two glucose subunits are held in a rigid clamshell shape about the α,α-1,1-

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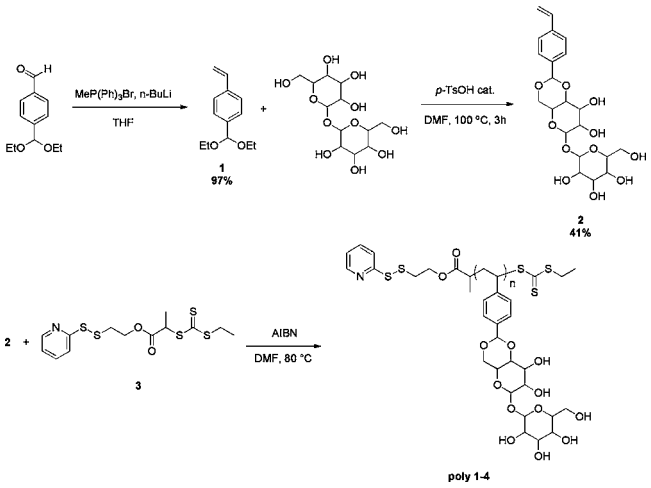
Scheme 1. Synthesis of 4-Vinylbenzaldehyde Diethyl Acetal 1 by Wittig Olefination and Regioselective Reactivity with Trehalose to Yield 4,6-*O*-(4-vinylbenzylidene)-α,α-trehalose 2



glycosidic linkage (see Supporting Information, Figure S3).[55] This result is important as it is thought to be a critical component in generating the unique physiochemical and protective properties of the carbohydrate.[56]

Synthesis of a Thiol Reactive Glycopolymer by RAFT Polymerization. To form polymers to conjugate to proteins and to achieve low polydispersity indices (PDIs) and targeted molecular weights, RAFT polymerization was used. RAFT polymerization readily provides well-defined polymers with protein-reactive end groups.[57] The CTA **3** was prepared with a pyridyl disulfide group because this functionality has been frequently reported as an effective reactive moiety for reaction with free cysteines in proteins.[58,59] Synthesis of CTA **3** was accomplished by esterification of 2-(ethyl sulfanylthiocarbonyl sulfanyl)-propionic acid with a pyridyl disulfide alcohol in 76% yield.

RAFT polymerization of **2** with **3** was performed at 80 °C (Scheme 1). The ratio used for polymerization was [CTA]:[monomer]:[AIBN] = 1:29:0.2, with a concentration of 0.8 M monomer. After 6 h, the polymerization was stopped to obtain 77% conversion. The polymer was dialyzed against aqueous sodium bicarbonate with a MWCO 2000 g/mol. The molecular weight of the polymer was analyzed by ^{1}H NMR spectroscopy and was 9600 Da by comparing the integration of the end-group pyridine peaks to the aromatic ring from styrene (see Supporting Information, Figure S4). The PDI by GPC was 1.07, demonstrating that a well-defined polymer was formed. The kinetic study of the trehalose polymer demonstrated that PDIs were well below 1.1 throughout the polymerization (see Supporting Information, Graph S1). The [CTA]:[monomer]:[AIBN] ratios were then altered to obtain other molecular weights ranging from 4200 to 19 000 g/mol (Table 1, Poly 1−4) with narrow PDIs obtained in all cases.

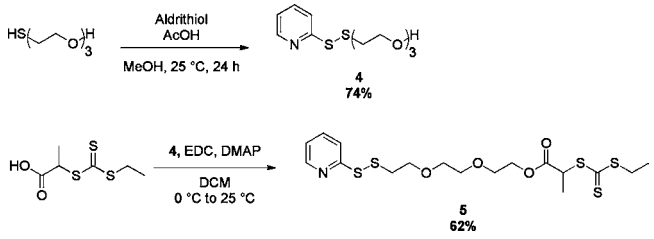
In addition, a new CTA **5** was devised to contain a hydrophilic triethylene glycol spacer between the trithiocarbonate and pyridyl disulfide end-group (Scheme 2). Tri(ethylene glycol) (TEG) was modified by tosylation followed by refluxing with thiourea.[60] Upon addition of base, the resulting 1-mercapto-tri(ethylene glycol) was treated with Aldrithiol to obtain activated disulfide **4**. Subsequent carbodiimide-mediated coupling to the acid moiety on 2-

Table 1. RAFT Polymerization of 2 with 3 or 5[a]

entry	CTA	CTA:M:AIBN	target M_n	conv. (%)	M_n NMR	PDI
Poly 1	3	1:21:0.2	7000	72	4200	1.05
Poly 2	3	1:29:0.2	9600	77	9400	1.07
Poly 3	3	1:35:0.2	13800	84	14700	1.11
Poly 4	3	1:50:0.2	20000	85	19000	1.14
Poly 5	5	1:20:0.4	9000	94	8000	1.10
Poly 6	5	1:60:0.2	11400	40	15400	1.13
Poly 7	5	1:76:0.2	25800	73	24500	1.20
Poly 8	5	1:120:0.4	44800	81	49500	1.47

[a]Polymerization was conducted in DMF at 80 °C with 0.8 M monomer concentration for all trials.

Scheme 2. Activation of the Known Starting Material 1-Mercaptotriethylene Glycol with Aldrithiol to Yield Activated Disulfide 4 before Subsequent Carbodiimide Coupling to Produce the Thiol Reactive Chain Transfer Agent 5



(ethyl sulfanylthiocarbonyl sulfanyl)-propionic acid afforded the thiol reactive chain transfer agent **5** in 62% yield. RAFT polymerization of **2** in the presence of **5** was shown to proceed for a range of molecular weights (Table 1, Poly 5−8) with slight deviation from narrow polydispersities obtained for the highest molecular weight attempted (Poly 8). After purification by dialysis (1 kDa MWCO, H_2O, 3 days), these thiol reactive glycopolymers were characterized by NMR spectroscopy to confirm end-group retention and complete removal of monomer (see Supporting Information, Figure S7).

Conjugation of Glycopolymer to Thiopropionyl Hen Egg White Lysozyme. Trehalose polymers were then conjugated to thiolated lysozyme. Thiols were added to hen egg white lysozyme by treatment with *N*-succinimidyl-S-acetylthiopropionate (SATP) in a procedure known to covalently attach thioacetate functionality to proteins via amide bonds.[61] After deprotection with the hydroxyl amine and removal of excess SATP, free thiols were quantified on the thiolated lysozyme (LyzSH) by Ellman's assay resulting in a thiol:protein ratio of 1:1.4 (71% thiol). The LyzSH was then conjugated to various trehalose polymers (Poly 2, 5−8, Scheme 3). After incubation with trehalose polymers, conjugates were seen by SDS-PAGE (see Supporting Information, Figures S6 and S11). The conjugation yield was lower for Poly 2 as compared to Poly 5−8. One possible explanation was that the TEG linker moves the thiol reactive end group away from the bulky and hydrated trehalose side chain of the polymer improving access to the protein surface.

Lysozyme−glycopolymer conjugates (Lyz−Poly 5−8) were purified by fast protein liquid chromatography (FPLC) with subsequent runs of LyzSH and glycopolymer alone used to determine which fractions would be pure of starting materials (see Supporting Information, Figure S10). These fractions were collected and utilized in subsequent studies. Additionally, SDS-

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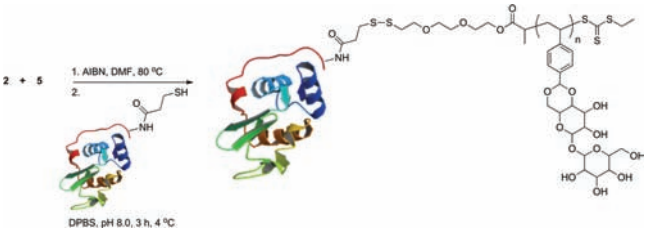
Scheme 3. RAFT Polymerization of 2 with CTA 5 to Produce the Thiol Reactive Glycopolymers Poly 5 through Poly 8[a]



[a]The glycopolymers were then conjugated to thiolated lysozyme.

PAGE was used to confirm isolation of the conjugate as demonstrated by the absence of a lysozyme band under nonreducing conditions. Reappearance of the band under reducing conditions indicated that the polymer was conjugated to the lysozyme through a reducible disulfide bond (see Supporting Information, Figure S12). Activities of the purified Lyz–Poly conjugate fractions were confirmed by observing active lysis of the FITC labeled Gram-positive bacteria *Micrococcus luteus* in the EnzChek lysozyme activity assay.

Lysozyme–Glycopolymer Conjugate Resistance to Environmental Stress. The ability of the polymers to stabilize lysozyme to lyophilization was first verified, and the results were compared to trehalose at the same concentrations. Poly 5–8 at 100 equivalents to protein were added (not conjugated) to wild-type Lyz containing no free thiols. Samples with trehalose added at equivalent concentrations relative to the trehalose in the various polymers were also tested. The samples were exposed to 10 lyophilization cycles, and the protein activity was determined. Wild-type protein retained 16% activity after this treatment, while with a 100-fold excess of polymer, full retention (100%) of activity was observed (Figure 2). The same effect was seen regardless of the molecular weight

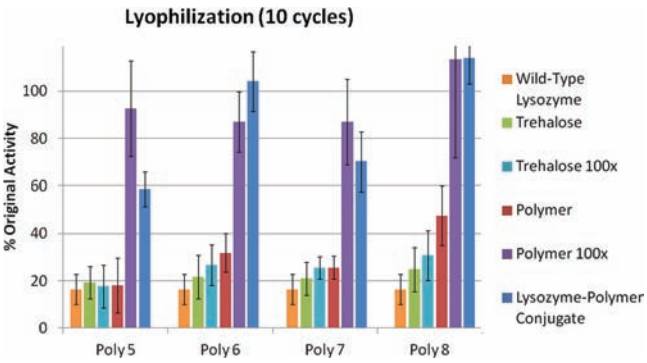


Figure 2. Activity of the lysozyme–glycopolymer conjugate, wild-type lysozyme with glycopolymer (1 or 100 equiv relative to lysozyme), or wild-type lysozyme with trehalose (1 or 100 equiv relative to polymer monomer units) as excipients exposed to 10 cycles of lyophilization. Data shown are repeated 6 times with $p < 0.01$ for all polymer 100× and conjugate samples relative to wild-type.

tested (8000 to 49 500 Da). Trehalose at equivalent concentrations to that in the 100-fold polymers only stabilized the lysozyme between 18 and 31%. The results confirm that the polymers are able to protect the protein during lyophilization. Furthermore, the data show that the polymer is significantly more effective than trehalose at the same concentration relative to monomer units present in the polymer. This indicates that stabilization effects of trehalose are enhanced by using a polymer where the entropic barrier to having several carbohydrate moieties organized around the protein has already been included in the excipient. Trehalose itself has been proposed to stabilize proteins to desiccation via water replacement, water entrapment, and vitrification. The mechanisms by which the trehalose polymers stabilize the protein are not yet understood, and investigative studies are underway. Regardless of the mechanism, the results suggest that trehalose polymers may be useful as a replacement for the disaccharide in formulations of unmodified biomolecules, particularly in instances where the additional materials properties imparted by the polymer would be advantageous.

To investigate the ability of the polymer to stabilize when conjugated to the protein, Lyz–Poly 5–8 were exposed to the same 10 lyophilization cycles. In this case, the conjugates exhibited between 59 and 100% retention of original activity compared to 16% retention by wild-type lysozyme (Figure 2). The smallest molecular weight Lyz–Poly 5 had the lowest activity, while the largest polymer conjugate Lyz–Poly 8 had full retention of activity. However, the activities of the medium-sized polymers did not directly correlate to molecular weight. It is possible that this is due to differing numbers of polymers attached to the protein in the fractions analyzed; the number of polymers attached in the collected fractions could not be accurately estimated by SDS-PAGE due to the low concentrations involved. Importantly though, the conjugates were all significantly more stable compared to samples containing a similar concentration of unattached polymer added (1 equiv to protein); addition of polymer resulted in only 18–47% retention of lysozyme activity. These results show that conjugating the polymer to the protein is advantageous with regard to environmental stability. Studies to determine the in vitro and in vivo stability of protein conjugates prepared from these polymers are underway to evaluate pharmacokinetic properties and potential use of the polymer in therapeutic conjugates.

Trehalose is also known to stabilize biomolecules to increases in heat.[24,62,63] Therefore, an identical array of lysozyme samples were also stressed by exposure to a heat burden of 90 °C for 1 h (Figure 3). Although trehalose was found to confer marginal stabilization at the highest concentration tested (31% activity retention for 100 equiv comparable to Poly 8), the trehalose

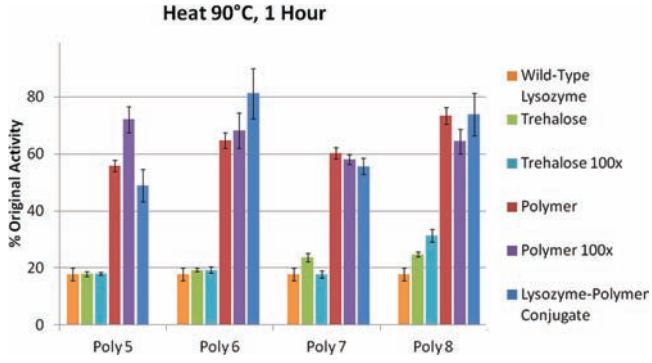


Figure 3. Activity of the lysozyme–glycopolymer conjugate, wild-type lysozyme with glycopolymer (1 or 100 equiv relative to lysozyme), or wild-type lysozyme with trehalose (1 or 100 equiv relative to polymer monomer units) as excipients exposed to a heat burden of 90 °C for 1 h. Data shown are repeated 6 times with $p < 0.001$ for all polymer and conjugate samples relative to the wild-type.

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polymer effects superior protection to heat, both added and as a conjugate. Up to 81% retention of activity was observed under these rigorous conditions. In this particular study, the protective effect was not concentration dependent as adding 1 equiv and 100 equiv or conjugating the polymer gave similar retention of activity for all molecular weights tested. However, in other studies, which were longer in duration and at a different concentration of protein, some concentration dependence was observed (data not shown). Overall, the results demonstrate that the glycopolymers stabilize lysozyme to high temperatures. Furthermore the data suggest that the polymers should be investigated further as an excipient and conjugate to mollify the rigorous storage requirements that are typical for proteins, particularly during transport where large fluctuations in temperature may be observed.

Additional initial studies were conducted to compare trehalose glycopolymers to the commonly used excipient poly(ethylene glycol) (PEG). As such, PEG (M_n = 2−20 kDa) was combined with wild-type lysozyme at 1 or 100 equiv and stressed by lyophilization or heat in an identical fashion as before. The lyoprotective effect of PEG (see Supporting Information, Figures S13 and S14) was found to depend on weight. Glycopolymer at 1 equiv performed similarly to 1 equiv of PEG, and 100 equiv of glycopolymer was a more effective lyoprotectant than PEG depending on the molecular weight. Thermal stability of PEG relative to the glycopolymers was also investigated by exposing the relevant samples to a heat burden of 90 °C for 1 h as before. Although PEG mitigates the thermal stress to a minimal extent, the trehalose glycopolymer outperforms PEG based on DP or molecular weight for all samples tested (see Supporting Information, Figures S13 and S14). The data collectively indicate that trehalose side chain polymers are highly effective and superior to PEG as stabilizers of a representative protein, lysozyme, to heat stress, and similar or better stabilizers to lyophilization stress.

■ CONCLUSION

A monomer based on trehalose has been synthesized through installation of a vinylbenzylidene moiety to produce 4,6-*O*-(4-vinylbenzylidene)-α,α-trehalose. Thiol-reactive CTAs were utilized to effect RAFT polymerization of the glycomonomer resulting in well-defined polymers with pendent trehalose functionalities and thiol-reactive end groups. Glycopolymers of different molecular weights were conjugated to hen egg white lysozyme modified to contain a free thiol. It was found that a longer spacer length between the reactive end group and polymer chain provided better conjugation yields. The resulting purified protein−glycopolymer conjugates exhibited active cell lysis. When unconjugated polymers were added to wild-type lysozyme, significant increases in resistance to lyophilization and heat stresses were observed relative to wild-type protein, protein stabilized by trehalose alone, or protein with added PEG. When the polymer was conjugated to the protein, the observed lyophilization stability was significantly enhanced. The results suggest that the polymer could be potentially useful in protein formulations.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details, NMRs, GPCs, UV−vis, kinetic data, FPLC, and SDS-PAGE results for glycopolymer−protein conjugates, and additional activity studies. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

maynard@chem.ucla.edu

Notes

The authors declare no competing financial interest.

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