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Substituted Polyesters by Thiol–Ene Modification: Rapid Diversification for Therapeutic Protein Stabilization

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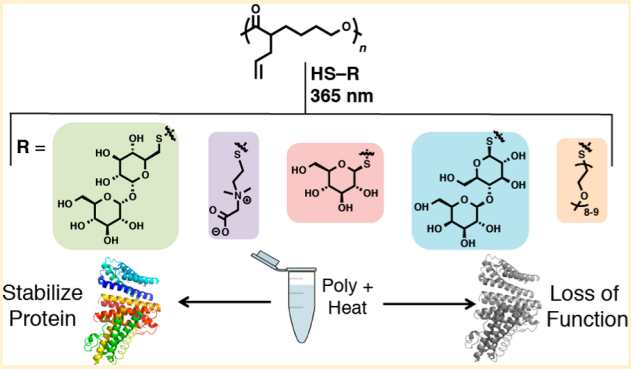
Supporting Information

ABSTRACT: Many proteins, especially those used as therapeutics, are unstable to storage and shipping temperatures, leading to increased costs in research and industry. Therefore, the design and synthesis of novel stabilizers is an important area of investigation. Herein we report new degradable polymers that stabilize proteins to environmental stressors such as refrigeration and elevated temperature. Specifically, polycaprolactones with different pendant groups were synthesized and surveyed for their ability to stabilize an important therapeutic protein to storage and shipping conditions. Ring-opening polymerization (ROP) of an allyl-substituted caprolactone monomer was carried out using the organocatalyst 1,5,7-triazabicyclo[4.4.0]-dec-5-ene (TBD) to yield a well-defined, alkene-substituted degradable polymer, which was used as a common backbone to control for the degree of polymerization. Relevant side chains such as trehalose, lactose, glucose, carboxybetaine, and oligo(ethylene glycol) were installed via postpolymerization thiol–ene reactions. These degradable polymers were then employed as excipients for the stabilization of the therapeutic protein granulocyte colony-stimulating factor (G-CSF) against storage at 4 °C and shipping temperatures of 60 °C. The best stabilization was observed using the trehalose- and zwitterion-substituted polyesters. Both the trehalose- and carboxybetaine-substituted pCL were further investigated with regard to molecular weight dependence, and it was found that the molecular weight was minimally important for stabilization to refrigeration, but critical for G-CSF stabilization at elevated temperatures. Both high performing zwitterionic and trehalose polyesters were also degraded, and the polymers and degradation products were shown to be noncytotoxic. This work provides potential biocompatible polymers for stabilization of the important therapeutic G-CSF, as well as a general platform for the future discovery of new polymeric protein stabilizers.



INTRODUCTION

Due to their substrate specificity and biological function, proteins have unique and essential roles in various industries. For example, proteins are used as reagents for improving chemical transformations, as cosmetic additives, as supplements for improving nutrition of animal feed, and as biological therapeutics. However, the stabilization of certain proteins during storage and transport, especially those used as therapeutics, can be critical to maintain structure and activity. Conditions such as UV exposure,[1] heat,[2] lyophilization,[3] and excessive agitation[4] can lead to protein unfolding, aggregation, or loss of biological activity. Measures to prevent this loss of activity, such as the maintenance of a refrigeration chain for delicate protein therapeutics, increase costs and may still result in inactivated protein.

As a result, a number of compounds are used as excipients or additives to maintain protein activity.[5] For instance, osmolytes and carbohydrates such as trehalose, sorbitol, and sucrose have been shown to maintain protein activity through preferential hydration or protein interactions.[6] Arginine, histidine, and other amino acids have also been shown to stabilize proteins through binding interactions, buffering, or hydration mechanisms.[7−9] Moreover, proteins such as human serum albumin (HSA) have been used as bulking agents or to prevent protein adsorption.[5] Furthermore, surfactants such as polysorbate (Tween) or modified polysaccharides such as hydroxyethyl starch (HES) have been employed to prevent protein unfolding and aggregation.[5,10,11] Excipients have also been used in nonbiological therapeutics. For instance, the recently approved hyperkalemia drug patiromer includes sorbitol in its formulation to improve stability.[12] However, therapeutics still suffer from activity loss despite the presence of these excipients, prompting further development of improved materials.

Synthetic polymers comprise another promising class of excipients used to stabilize proteins against environmental stressors. Polymers such as anionic polyacrylate, poly(glutamic acid), carboxylated polyamidosaccharides, and block copoly-

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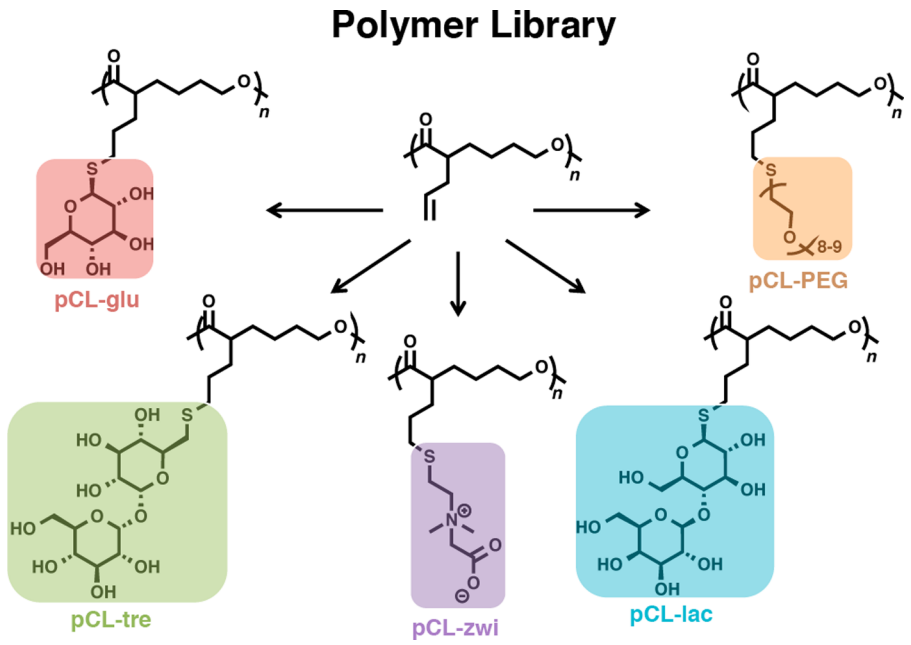

Figure 1. Illustrative scheme of pCL backbone and modification with thiols using thiol−ene chemistry to produce a small library of degradable polymers.

mers of poly(ethylene glycol) (PEG) and poly(histadine) have been shown to stabilize a variety of proteins to stressors such as heat, aggregation, and lyophilization.[13−18] Other charged polymers such as poly(ethylenimine) or heparin mimicking polymers can stabilize a variety of enzymes or growth factors using electrostatic interactions.[19−22] Zwitterions have also been shown to have significant stabilizing ability due to their hydration and protein repulsion properties.[23] Additionally, thermoresponsive copolymers have been used for refolding denatured proteins.[24] We have previously developed styrene- and methacrylate-based polymers with trehalose side chains and shown that these polymers protect lysozyme, horseradish peroxidase (HRP), and glucose oxidase (GOX) against elevated temperatures both as excipients and as protein−polymer conjugates.[25,26] And others have investigated the use of trehalose in polyacrylamide polymers to inhibit amyloid protein aggregation and in polycationic nanoparticles for delivery of siRNA.[27,28]

Though synthetic polymers show promise in stabilization of proteins, most are nondegradable and thus will not be cleared from biological systems or will persist in the environment. For instance, PEG is the most widely used biocompatible polymer, but has been shown to induce the formation of antibodies in 32−46% of patients during a clinical trial because of its persistence in vivo.[29,30] Additionally, vacuolation in rats has been reported upon injection with high molecular weight (40 kDa) PEG.[31] Small-molecule excipients that have been widely used for therapeutic formulation present other disadvantages. For instance, sorbitol is widely used and effectively maintains protein activity, yet has been shown to result in GI tract complications such as bleeding, ulcers, and necrosis.[32] Other high-performing excipients include the nonionic surfactants Tween 20 and Tween 80, which effectively prevent protein aggregation but have been shown to undergo auto-oxidation, resulting in the formation of damaging peroxides.[33] Therefore, the development of novel, degradable, and functional polymers has been a subject of recent interest, especially for biological applications.[34,35] Degradable polymers might alleviate immunogenic responses, while also enabling the use of higher molecular weight polymers, which typically cannot be employed due to difficulty in clearance. In addition, enzymes are widely employed in applications such as in detergents or animal feed, where the protein stabilizers should be biodegradable to avoid unwanted environmental buildup. Therefore, there is significant need for well-controlled, homogeneous, and degradable synthetic materials for biological and environmental concerns.

Herein we report the synthesis of degradable stabilizing polyesters using ring-opening polymerization (ROP). The polymers were prepared by first synthesizing alkene-functionalized polycaprolactones, followed by the installation of desired side chains using high-yielding thiol−ene reactions. A variety of materials were easily synthesized by varying mercaptan identity and the resulting materials protected granulocyte colony-stimulating factor (G-CSF) against loss of biological activity when added as excipients. We expect that these polymers can function as protein stabilizers in a variety of fields due to their combination of biodegradability and stabilization abilities.

RESULTS

The nature of the degradable polymer backbone was an important consideration in the design of a modular system for protein stabilization. We have previously observed that trehalose polymers with hydrophobic backbones have demonstrated good protein stabilization,[26] and hypothesized that the nonionic surfactant character of these materials was an important contributor to their desirable properties.[5] Therefore, the FDA-approved polymer poly(caprolactone) (pCL) was selected because of its hydrophobic and biodegradable nature. Previous examples have introduced functional side chains onto pCL using a variety of postpolymerization click chemistries to avoid chemical incompatibilities with ROP conditions and also to minimize steric interference during polymerization. For instance, aminooxy-functionalized PEG chains have been added to ketone-modified pCL through oxime click chemistry,

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resulting in graft copolymers.[36] Alkyne- and alkene-functionalized valero- and caprolactone monomers have been synthesized and polymerized to yield polyesters with reactive handles for later installation of PEG and peptide side chains.[37,38] We chose to synthesize our polyester backbone with reactive alkene side chains and use it as a common precursor to introduce stabilizing functionalities via postpolymerization thiol−ene reactions. Thiol−ene is a particularly attractive type of "click" modification because it combines efficiency, a metal-free nature, and a tolerance of both water and oxygen.[39,40] Using this type of chemistry allows for the ready introduction of different functional and potentially stabilizing moieties onto the polymer side chains by using a variety of mercaptans (Figure 1). Additionally, with this postpolymerization approach, the backbone length would be the same between the different classes to rule out differences in stabilizing ability due to changes in degree of polymerization.

Synthesis of a Library of Functionalized Polyesters. The desired alkene-functionalized caprolactone monomer was synthesized by adding allyl bromide to CL in the presence of lithium diisopropylamide following a literature procedure[37] and polymerized using ROP. A degree of polymerization (DP) of 40 was targeted because it would result in functionalized polymers with molecular weights between 20.9 and 12.8 kDa. The organic catalyst 1,5,7-triazabicyclo[4.4.0]dec-5-ene (TBD) was used due to its fast polymerization kinetics at room temperature and narrow dispersity ($Đ$) for the ROP of functional lactones.[41−44] The initiator 3-methyl-1-butanol was employed because its distinctive ^{1}H NMR peaks allowed for good characterization.[45] Using a monomer concentration of 2 M, high conversion and good control over molecular weight were achieved, with $Đ$ = 1.08, a degree of polymerization (DP) of 36, and a number-average molecular weight of 5600 Da by ^{1}H NMR and 5400 by gel permeation chromatography (pCL-allyl$_{40}$, Table 1). Initially, the polymer was purified by dialysis in dichloromethane/methanol. However, polymers purified using this method had unidentified impurities, which resulted in significant loss of protein activity in later experiments (data not shown). Purification by silica gel column chromatography successfully removed the impurities, and subsequent polymers were therefore purified using this method.

Table 1. Molecular Weights and GPC Data for the Library of Polyesters (DP40)

	polymer	Mn (^{1}H NMR)	Mn (GPC)	Đ
starting polymer	pCL-allyl$_{40}$	5600	5400	1.08
protected	pCL-trehalose-OAc$_{40}$	34 700	28 400	1.06
	pCL-glucose-OAc$_{40}$	20 300	22 300	1.06
	pCL-lactose-OAc$_{40}$	29 900	28 900	1.07
deprotected	pCL-trehalose-OH$_{40}$	18 500	12 700	1.17
	pCL-glucose$_{40}$	11 300	18 300	1.09
	pCL-lactose$_{40}$	18 000	16 900	1.17
	PCL-PEG$_{40}$	20 400	23 600	1.07
	pCL-zwitterion$_{40}$	12 500	5100[a]	1.19[a]

[a]GPC run in buffer/MeCN with PEG standards.

The allylated polymer was then used in radical thiol−ene reactions to install the desired pendant stabilizing groups (Scheme 1). The photoinitiator 2,2-dimethoxyphenylacetophenone (DMPA) was used because of its demonstrated high efficiency in photoinitiated thiol−ene reactions.[46] A series of easily accessible thiols (**A**−**D**) were selected containing sugars or oligo(PEG) that as small molecules are known stabilizing excipients.[5] Thiolated trehalose was synthesized in five steps and 53% overall yield from trehalose using trityl and acetate protecting groups (Scheme S1). Briefly, monohydroxyl trehalose heptaacetate was synthesized as previously described.[26] A tosylate ester was installed and displaced using potassium thioacetate. Selective cleavage of the thioester using hydrazine acetate then led to thiolated trehalose **A**. Thiolated lactose **C**[35,47] and thiolated mPEG **D**[48] were synthesized as previously described.

Use of acetate-protected saccharide mercaptans was found to be important for good miscibility between the pCL backbone and the thiol, giving clean conversion to the acetylated glycopolymers. In all cases, three equivalents of thiol per alkene were used to ensure complete reaction of the alkene side-chains. After polymer modification, removal of the acetate esters using potassium carbonate in methanol/chloroform[26] or hydrazine hydrate[49−52] led to the desired glycopolymers without hydrolysis of the polyester backbone. Complete modification was confirmed by disappearance of the alkene peaks in the ^{1}H NMR (representative data in Figure 2a, full data provided in the Supporting Information (SI)) as well as clean shifts in GPC molecular weight (representative data in Figure 2b, full data provided in the SI).

The carboxybetaine zwitterionic pCL polymer was synthesized taking inspiration from a literature procedure for a nondegradable polymer (Scheme 2).[53] 2-(Dimethylamino)-ethanethiol hydrochloride was added to the pCL-allyl$_{40}$ backbone polymer using photoinitiated thiol−ene conditions and subsequently treated with sodium bicarbonate to neutralize the hydrochloride salt. Exposure to *t*-butyl bromoacetate quaternized the amine and hydrolysis of the *t*-butyl ester with trifluoroacetic acid led to the formation of the zwitterion. No acidic backbone scission was observed by GPC or ^{1}H NMR analysis.

All polymers were characterized by GPC and ^{1}H NMR to determine molecular weight and dispersity (Table 1). During the deprotection, there was unlikely to be chain scission, as the library of substituted pCL all gave narrow molecular weight distributions between 1.19 and 1.07 and the peak shapes were generally well-defined and symmetrical (Figures S26, S35, S36). Using poly(methyl methacrylate) standards, the M_N by GPC for the DMF-soluble polymers varied from 12.7 to 23.6 kDa. The zwitterionic material was not DMF-soluble and was instead analyzed using PEG standards, making direct molecular weight comparison difficult. However, because a postpolymerization approach was used to synthesize these materials, the same backbone was used to construct all polymers in the study. Therefore, while the molecular weight varied due to side chain identity, the DPs of all polymers (i.e., backbone length) compared were identical.

Assessment of Stabilizing Ability. Next the ability of the polymer to protect protein activity against environmental stressors was assessed. The therapeutic protein granulocyte colony-stimulating factor (G-CSF) was selected to compare excipient efficacy due to its clinical importance. G-CSF is FDA-approved as filgrastim (Neupogen), and lenograstim and is used therapeutically to increase the neutrophil granulocyte count during chemotherapy.[54] G-CSF is highly unstable at

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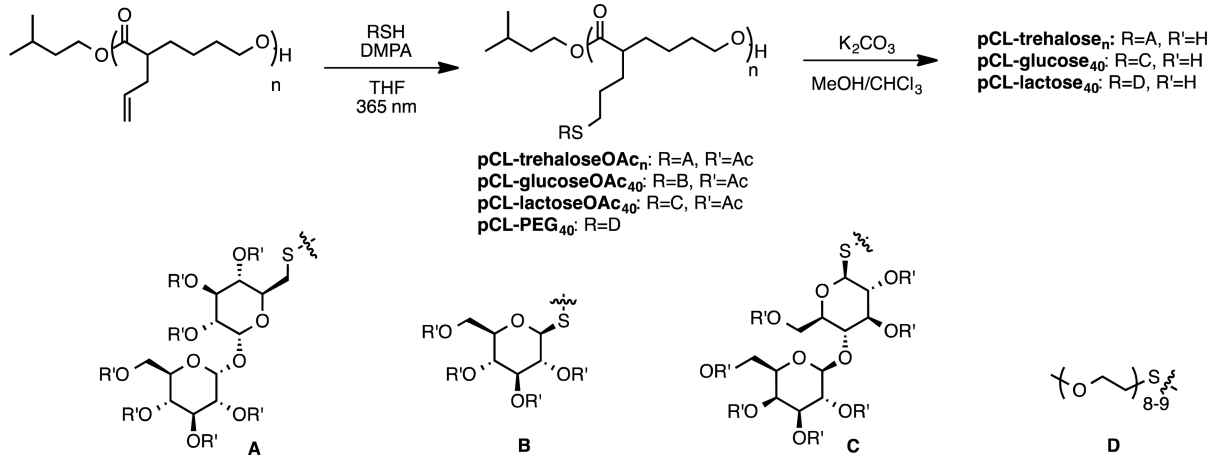
Scheme 1. Synthetic Scheme of Thiol−Ene Modification of pCL-Allyl Polymers with Acetyl-Trehalose, Acetyl-Glucose, Acetyl-Lactose, and PEG Thiols, Followed by Deprotection of the Acetylated Sugars



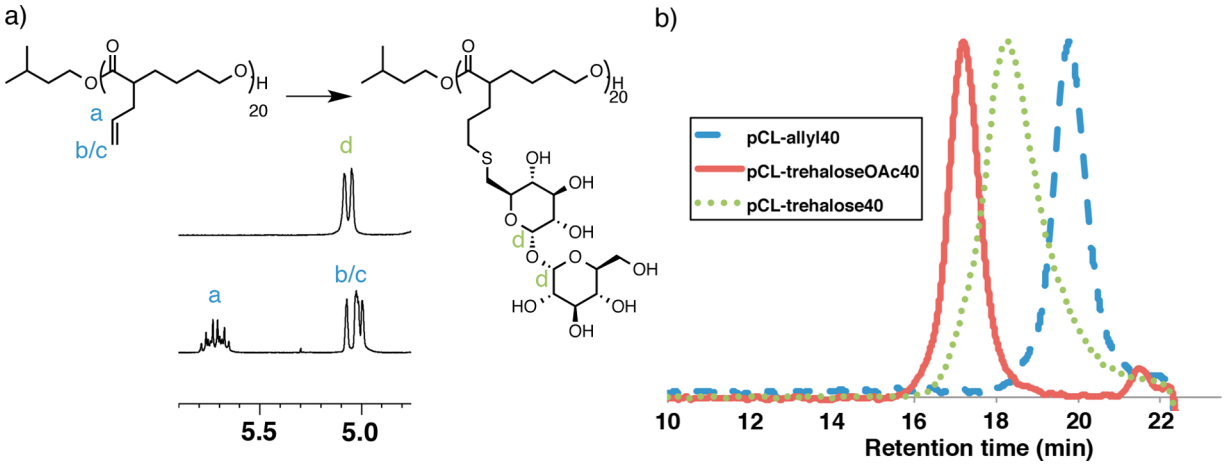
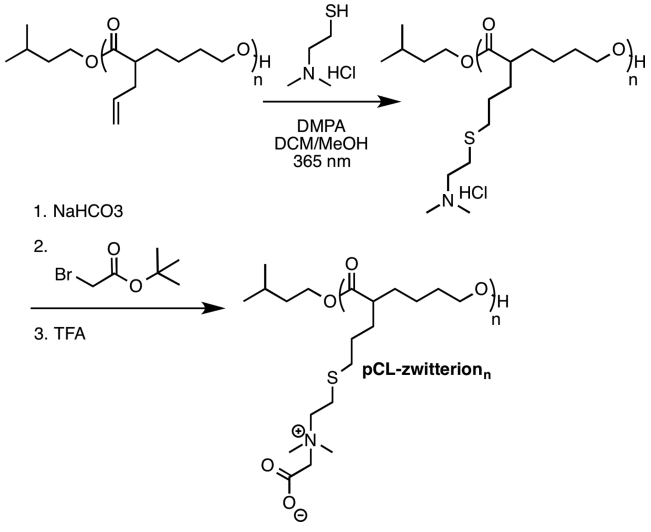


Figure 2. Characterization of trehalose modification of pCL using thiol−ene chemistries. (a) The ^{1}H NMR traces before and after modification showing a disappearance of the alkene resonance peaks at 5.0 and 5.7 ppm and the appearance of resonance peaks corresponding to trehalose anomeric protons. (b) GPC characterization of pCL-allyl$_{40}$ before and after modification showing a shift toward a higher molecular weight species. After deprotection of the trehalose, a shift toward a lower molecular weight species was observed showing complete modification and deprotection of the polymer.

Scheme 2. Synthetic Scheme for the Synthesis of Zwitterionic Polymer pCL-Zwitterion$_n$



physiological pH and is therefore stored at pH 4.0; still at this pH the protein readily degrades upon storage or subjection to heat.[55] The side chain identity was varied to determine the relative stabilizing ability of the functional groups. To investigate storage at refrigeration temperatures, pCL polymers were added to G-CSF at 100 wt equiv to protein and the protein was stored for 90 min at 4 °C at 1 μg/mL and pH 4.0. Protein activity was determined by measuring cell proliferation in murine myeloid leukemia NFS-60 cells, which is enhanced in the presence of G-CSF, and compared to the proliferation of freshly diluted protein.[56]

After stressing at 4 °C, G-CSF with no additive only exhibited 133 ± 6% cell proliferation, a drastic reduction compared to fresh G-CSF (Figure 3a). Addition of the pCL-glucose and pCL-lactose polymers was not statistically different than no additive, indicating that, for this protein, the polymers were not effective stabilizers. Interestingly, when the pCL-PEG polymer was added to the G-CSF solution, significantly lower cell proliferation was observed. PEG has been shown to associate with hydrophobic moieties on the protein surface due to its amphiphilic nature[57] and has been previously observed to lower protein thermal stability.[58] A similar mechanism may be a

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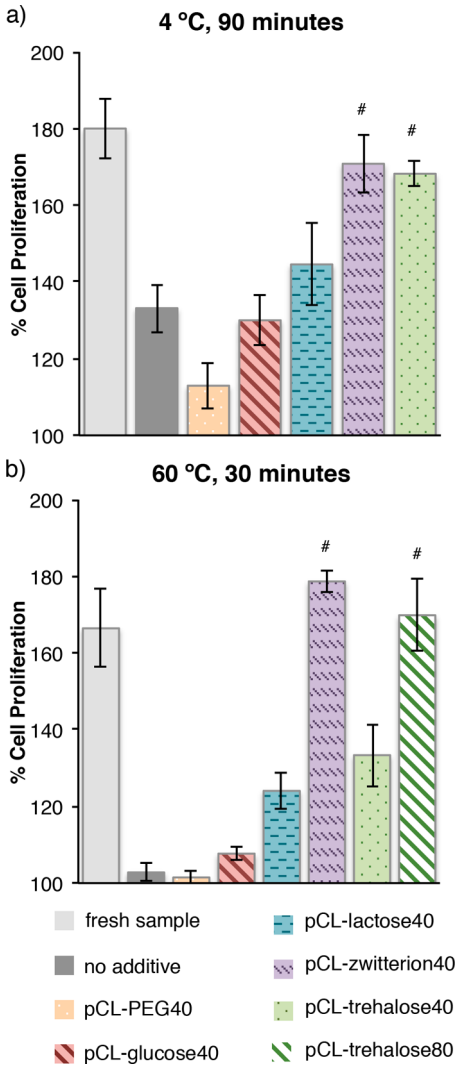

Figure 3. Effect of side chain identity on stabilization of G-CSF at pH 4.0 to (a) storage conditions at 4 °C for 90 min and (b) thermal stress at 60 °C for 30 min. # = no statistical difference from the fresh sample (p > 0.01), Student's t test. Data shown as the average of six experimental repeats and six well repeats with standard deviation.

factor for the destabilizing effect of this pCL-PEG polymer. Both the zwitterionic and trehalose side chains significantly outperformed the other polymers, stimulating 171 ± 7% and 168 ± 3% cell proliferation, respectively. Both stabilizing polymers were not statistically different than the fresh sample, indicating that both are equally effective at preventing G-CSF activity loss under these conditions. G-CSF was also stressed at 60 °C for 30 min; this is representative of the maximum temperature inside truck and shipping containers during transport.[59] As expected, G-CSF lost more than 95% of the native activity after heating; addition of the pCL-PEG polymer was not statistically different than no additive (Figure 3b). The pCL-glucose and the pCL-lactose polymers were moderately stabilizing. Addition of the trehalose (133 ± 8%) and zwitterionic (179 ± 3%) side chain polymers resulted in the highest cell proliferation, and the pCL-zwitterion$_{80}$ was not statistically different than the fresh sample. We observed that the zwitterionic polymers retained greater activity than the trehalose side chain polymers to heat. Since one hypothesis of why trehalose provides stabilization is due to clustering of the sugar around flexible polar residues on the protein surface,[60,61] we included a larger trehalose CL polymer (preparation vide infra) in the heat study. In this case, a 40 kDa pCL-trehalose$_{80}$ polymer was statistically the same as the zwitterionic polymer, showing that the larger trehalose pCL stabilizes as well as the zwitterionic polymer and suggesting a molecular weight dependence of the trehalose polymer stabilization ability.

Testing of Different Molecular Weights and Comparison to Common Excipients. To further test this potential molecular weight dependence, various CL trehalose polymer sizes were synthesized. Using previously optimized ROP conditions, well-defined pCL-allyl polymers were synthesized with DP between 10 and 80 and $Đ$ < 1.25 (Table 2). These

Table 2. Molecular Weights and GPC Data for the Library of pCL-Trehalose and pCL-Zwitterion Polymers with Variable DP

	polymer	Mn (^{1}H NMR)	Mn (GPC)	Đ
starting polymers	pCL-allyl$_{10}$	1600	ND[a]	ND[a]
	pCL-allyl$_{20}$	3600	2400	1.21
	pCL-allyl$_{40}$[b]	5600	5400	1.08
	pCL-allyl$_{80}$	12 400	12 200	1.08
trehalose-OAc	pCLtrehalose-OAc$_{10}$	10 600	9600	1.07
	pCL-trehalose-OAc$_{20}$	20 200	15 400	1.06
	pCL-trehalose-OAc$_{40}$[b]	34 700	28 400	1.06
	pCL-trehalose-OAc$_{80}$	67 000	53 100	1.06
trehalose-OH	pCL-trehalose-OH$_{10}$	6200	5600	1.09
	pCL-trehalose-OH$_{20}$	14 400	8100	1.15
	pCL-trehalose-OH$_{40}$[b]	18 500	12 700	1.17
	pCL-trehalose-OH$_{80}$	41 000	17 000	1.39
zwitterions	pCL-zwitterion$_{10}$	3200	1700[c]	1.17[c]
	pCL-zwitterion$_{20}$	6400	3000[c]	1.12[c]
	pCL-zwitterion$_{40}$[b]	12 400	5100[c]	1.19[c]
	pCL-zwitterion$_{80}$	25 400	8900[c]	1.19[c]

[a]Too small for GPC analysis. [b]Same entry as in Table 1. [c]GPC run in buffer/MeCN with PEG standards.

DPs were selected so that, after modification with thiolated trehalose, the molecular weight of the pCL-trehalose polymers would be between 5 and 40 kDa, assuming quantitative conversion. For the smallest pCL-allyl polymer, matrix assisted laser desorption ionization (MALDI) was used to confirm the molecular weight (Figure S13). Modification was again carried out using photoinitiated thiol−ene chemistry, yielding a series of trehalose-modified pCL polymers. This series demonstrated increased dispersity ($Đ$) with increasing molecular weight. At high molecular weights (Table 2, pCL-trehalose$_{80}$) $Đ$ was increased to 1.39 and the GPC molecular weight was correspondingly lower than that predicted by ^{1}H NMR. The peak shape was also asymmetrical and extended toward the low molecular weight side (Figure S37a). To confirm that this peak broadening was not due to hydrolysis of the backbone esters, the molecular weight of pCL-trehalose$_{80}$ was also measured on an aqueous size exclusion chromatography (SEC) system (Figure S37b). In aqueous solution, no asymmetry was

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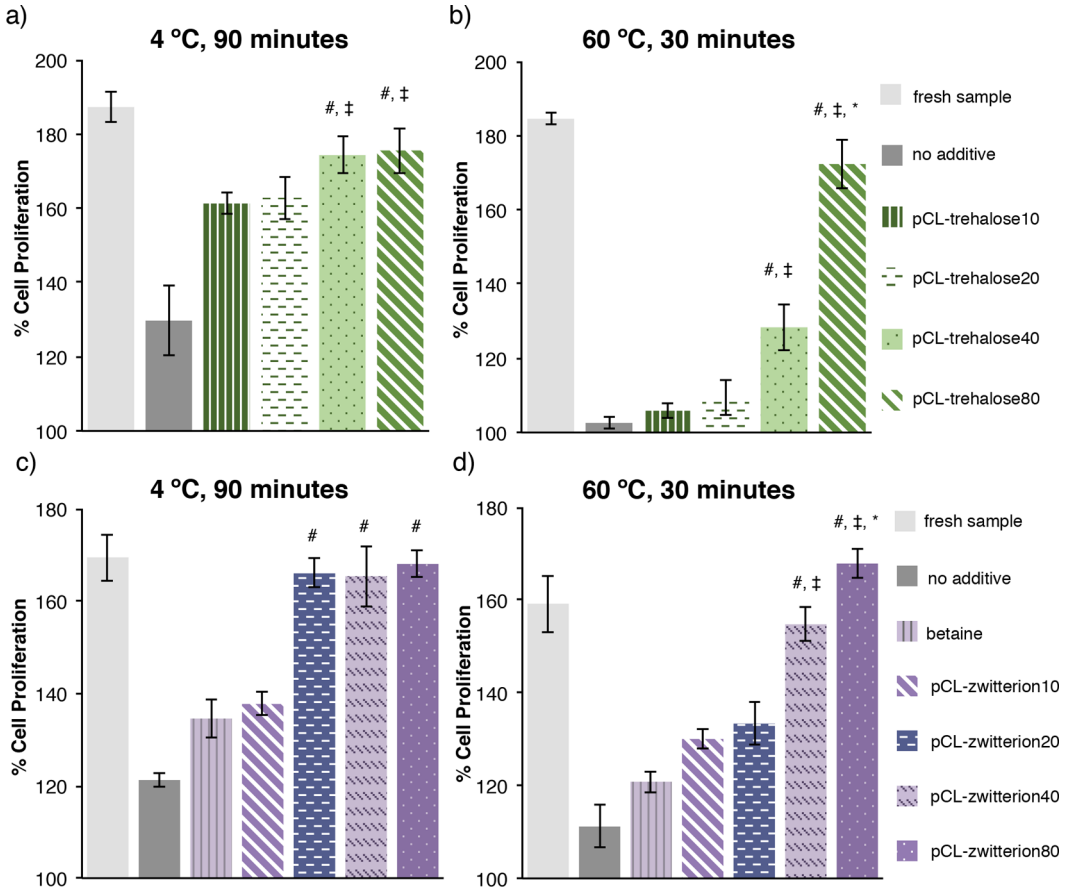

Figure 4. (A) Effect of pCL-trehalose molecular weight on G-CSF stabilization at pH 4.0 to storage at 4 °C for 90 min. (B) G-CSF stabilization to thermal stress at 60 °C for 30 min. Data shown as the average of six experimental repeats and six well repeats with standard deviation. (C) Effect of pCL-zwitterion molecular weight on G-CSF stabilization at pH 4.0 to storage at 4 °C for 90 min. (D) G-CSF stabilization to thermal stress at 60 °C for 30 min. Data shown as the average of four experimental repeats and six well repeats with standard deviation. All polymers exhibited statistically significant stabilization ($p < 0.05$) relative to no stabilizing additive. A dependence on molecular weight was also observed in that greater molecular weight polymers showed greater stabilization (# = $p < 0.01$ relative to DP10 polymers, ‡ = $p < 0.01$ relative to DP20 polymers, * = $p < 0.01$ relative to DP40 polymers, Student's t test).

observed and the calculated dispersity was lower (1.26). The dragging observed at high molecular weights was therefore hypothesized to be a result of interactions with the stationary phase of the GPC column. Similarly, a series of pCL-zwitterion polymers were synthesized using photoinitiated thiol−ene chemistry on the pCL-allyl backbones. Analysis by GPC showed that they were well-defined and demonstrated clear shifts in molecular weight with increasing pCL-allyl DP (Table 2).

Both sets of polymeric backbones were then subjected to the same stability tests using 100 wt equiv of polymer. First the trehalose polymers were tested and a very slight dependence of protein activity on molecular weight was observed upon storage at 4 °C (Figure 4a). Larger polymers offered improved stabilization compared to smaller polymers, but there was no significant difference between the stabilizing effects of DP40 and DP80 polymers, or between the DP10 and DP20 polymers. The series of pCL-trehalose polymers were also used as stabilizers against 60 °C heating (Figure 4b). In this case, a drastic molecular weight dependence was observed, with the pCL-trehalose80 polymer exhibiting the highest cell proliferation. It should be noted that despite the increase in molecular weight, the concentration of stabilizing units in solution remained constant at 69 weight equivalents of trehalose or 190 μM, indicating that the observed changes in stabilizing ability were solely due to the molecular weights of the polymers.

Similar experiments were carried out using the zwitterionic backbone. Upon exposure to the milder 4 °C stressor, only a moderate dependence on molecular weight was observed (Figure 4c). While the pCL-zwitterion$_{10}$ polymer sample exhibited reduced cell proliferation, there was no statistical difference between the DP20, DP40, and DP80 polymers. They were statistically the same as the fresh sample, indicating the presence of a molecular weight threshold for complete stabilization ability. However, when the protein was heated to 60 °C for 30 min, separation between the polymer additives was observed (Figure 4d). At this temperature, the performance of the DP20, DP40, and DP80 polymers was significantly different from each other, and only the two largest polymers retained comparable activity to the pristine sample.

Additional experiments were carried out to better understand the observed dependence on molecular weight. To determine if shorter polymers could demonstrate improved stabilization at higher weight equivalents, we stressed G-CSF at 60 °C for 30 min and added pCL-trehalose$_{40}$ and pCL-zwitterion$_{40}$, varying the amount of polymer in solution between 1 and 500 wt equiv (Figure S38). We were curious to determine if the DP40

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polymers would match the stabilizing performance of higher molecular weight DP80 polymers when more weight equivalents were used. Instead, there was a distinct plateau, and for both polymers only 100 wt equiv were required to see the best stabilization, without further improvement at the higher concentrations tested. This is strong evidence that the number of repeat units on the polymer chain has a distinct effect on the polymer's stabilizing ability. Trehalose has been previously shown to demonstrate a clustering effect in computational studies, self-organizing near polar residues on proteins.[61] The molecular weight trends observed support a multivalency effect in these materials, where increased equivalents offer inferior protection compared to a preorganized or pregrouped set of stabilizing units. This sort of molecular weight effect has been previously reported in other systems.[62]

The stabilizing abilities of the pCL polymers to protect G-CSF from 60 °C thermal stress were additionally compared to commonly used small-molecule excipients: sucrose, trehalose, betaine, sorbitol, and Tween 80 (Figure 5). These compounds

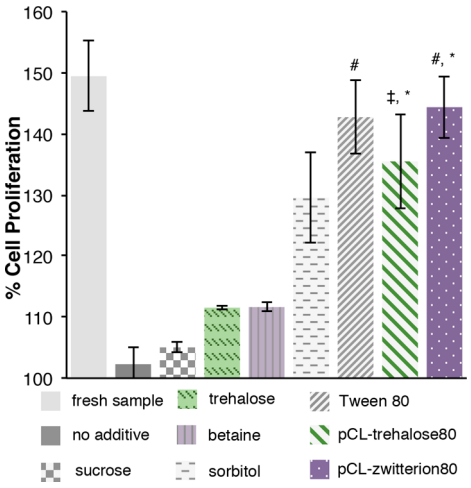

Figure 5. Stabilization of G-CSF against thermal stress at 60 °C for 30 min and comparison of pCL-trehalose$_{80}$ and pCL-zwitterion$_{80}$ with relevant small molecule controls. Data shown as the average of three experimental repeats and six well repeats. # = no statistical difference from the fresh control ($p > 0.05$). ‡ = no statistical difference from sorbitol ($p > 0.05$). * = no statistical difference from Tween ($p > 0.05$).

were chosen to represent the materials present in the high-performing pCL scaffolds, with the addition of sorbitol and polysorbate 80 (Tween 80), which are used industrially in the formulation of Neupogen (therapeutic GCSF)[63] and sucrose, which is a widely used excipient.[5] The pCL-trehalose$_{80}$ and pCL-zwitterion$_{80}$ polymers were selected because they were the highest-performing pCL polymers in the experiments described above and were added at 100 wt equiv. Small molecules were added to be equivalent to the concentration of stabilizing units in the pCL-zwitterion$_{80}$ polymer except for Tween 80, which was added at 100 wt equiv because of its larger molecular weight, similar to the CL polymers. After heating to 60 °C for 30 min, sucrose, betaine, and sorbitol had little stabilizing effect and the cell proliferation was low. However, the sorbitol and Tween 80 maintained high protein activity that was statistically equivalent to pCL-trehalose$_{80}$ and pCL-zwitterion$_{80}$, respectively. The results show that the degradable polymers with DP of 80 are as good as the currently utilized additives for therapeutic G-CSF and better than other common protein excipients at the concentrations tested.

Additionally, the half-life of G-CSF at 60 °C was tested with the DP80 polymers as excipients (Figure S39). When the pCL-trehalose$_{80}$ polymer was added, G-CSF retained 50% of the native activity until 48 min of heating, whereas when the pCL-zwitterion$_{80}$ polymer was used, the half-life was calculated to be 90 min, almost double. In contrast, with no additive G-CSF was already inactive after 30 min (first time point tested). These data show that that both pCL scaffolds, especially the zwitterion-substituted polymer, provide a significant increase in thermal stability.

Degradation, Biological Compatibility, TEM, and DSC Analysis of the Polymers. To confirm that the polycaprolactone was still degradable, pCL-trehalose$_{40}$ and pCL-zwitterion$_{40}$ were treated with 5% KOH to hydrolytically cleave the backbone esters (Scheme S2). The molecular weight of the polymeric materials was determined post-cleavage by aqueous SEC (Figure S41). In both cases, a complete shift in molecular weight toward a lower molecular weight species was observed after 24 h, confirming hydrolytic degradation. No hydrolytic degradation was observed under more moderate degradation conditions (cell media at 37 °C) for up to 49 days, consistent with the slow hydrolysis rates observed for polycaprolactone in vivo.[64] Additionally, experiments were carried out to confirm that the pCL polymers remained stable under the acidic conditions of the cell assay. pCL-trehose and -zwitterion polymers were heated to 60 °C for 30 min to mimic the thermal stress conditions, then buffer was removed and the materials were analyzed by GPC (Figure S42). No shift was observed in the chromatogram, confirming that the polymers were intact throughout the experiment.

Cytotoxicity and biocompatibility of the trehalose and zwitterion based polycaprolactone polymers were also assessed in human umbilical vein endothelial cells (HUVECs) as a primary, noncancerous cell line. HUVECs were cultured in the presence of pCL-trehalose$_{20}$, pCL-zwitterion$_{20}$, and their polymeric degradation products. Compared to the control, no reduction in cell viability was observed upon addition of either polymer (pCL-trehalose and pCL-zwitterion) or polymeric degradation products, up to 1 mg/mL, confirming that the substituted polyesters and their eventual degradation products are noncytotoxic (Figure S43).

Analysis of the substituted pCL polymers using transmission electron microscopy (TEM) indicated the presence of aggregated structures in both samples of pCL-zwitterion$_{80}$ and pCL-trehalose$_{80}$ alone and in the presence of G-CSF (Figure S40). This self-assembly may play an important role in the mechanism of stabilization and shows that the polymers are nonionic surfactants, an important class of excipients.[5] Similar aggregates have been observed for tyloxapol, a polymeric material with an aryl backbone and poly(ethylene oxide) side chains that is structurally similar to the pCL polymers.[65] We additionally investigated the osmolyte character of the synthesized materials using differential scanning calorimetry (DSC). Both polymers changed the enthalpy of melting and crystallization of water (Table S1) suggesting the polymers are able to depress ice formation.[26]

■ DISCUSSION

We have demonstrated that a biodegradable backbone can be transformed into a library of potential protein stabilizers using

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thiol−ene chemistry. This approach allowed us to survey the effectiveness of various side chains without complications due to differences in the number of backbone repeat units. We tested five relevant side chains: three sugars, oligoPEG, and a zwitterion. Yet, one can readily envision the synthesis of larger libraries of side chains using this approach. To investigate side chain effectiveness, the therapeutic protein G-CSF was stabilized against refrigeration and heat, two stresses that are relevant to the storage and shipping of many proteins; for example, nearly 80% of current protein therapeutics need to be refrigerated or frozen.[66] This temperature requirement causes inconvenience and increased costs to patients and may make some therapeutics impossible for use in parts of the world that do not have an effective cold chain. In addition, in some industries such as personal care where products are stored at room temperature, the instability of some proteins of interest may preclude their use.

We found that pCL with trehalose- and zwitterion-substituted side chains were the most effective stabilizers to G-CSF to refrigeration and heating, with the zwitterion polymers as the most effective over different time and molecular weight ranges. Activity loss in G-CSF has been reported to be a result of both methionine oxidation and aggregation.[55,67,68] Trehalose as an excipient has been shown to have no effect on methionine oxidation of G-CSF, presumably because it is preferentially excluded from the protein surface, but has been shown to broadly inhibit aggregation of various proteins.[68,69] Zwitterionic materials are known to be nonfouling and to repel proteins due to their high hydrophilicity and strong hydration.[70,71]Additionally, the nonionic surfactant Tween has been shown to reduce G-CSF aggregation through micelle formation.[72] Initial analyses by TEM and DSC suggest that the trehalose- and zwitterion-substituted polymers form structured aggregates alone and in the presence of G-CSF likely due to the nonionic surfactant character of the polymers. Additionally, the materials have the capability to reduce the enthalpy of water crystallization and melting, equivalent to the thermodynamic effects that have been previously observed for their constituent side chain materials. Many current studies of osmolyte−protein interactions hypothesize that their stabilizing effect is in fact due to water−osmolyte interactions,[73,74] and the pCL materials are likely to be similar to small-molecule osmolytes in this manner. The materials therefore combine two different classes of known excipients, namely osmolytes and nonionic surfactants. We also found the polymers to be as good as excipients currently used in the formulation for Neulasta, a therapeutic G-CSF. However, as has been previously noted, sorbitol and polysorbate both present downsides to large-scale and repeated applications in therapeutics. Namely, sorbitol has been linked to GI tract problems and polysorbate has been shown to undergo auto-oxidation.[32,33] The substituted pCL polymers offer equivalent stabilities and may be potential alternatives to the clinically used additives for G-CSF.

Although we looked at G-CSF, it should be possible to utilize this library approach to investigate a wide variety of proteins, and the outcome may be different depending on the individual protein degradation mechanism and the stress imposed. Using the versatile thiol−ene strategy it should be possible to readily alter the polymer side chains to identify stabilizers for a wide variety of stressors. The use of controlled ring-opening polymerization allows for the rapid synthesis of a variety of molecular weights to compare to commercially available additives, which may also be available in multiple molecular weights. As we have demonstrated, the effect of molecular weight on stabilization can be quite important, and the ability to add molecular weight variation to a library of polymeric stabilizers is significant. Additionally, the excellent control provided by ROP conditions allows for delicate tuning of the hydrolytic stability and degradability through selection of a variety of cyclic monomers or even using copolymerization. We anticipate this will greatly expand the possible applications for these materials, and this work is underway.

Polymers have the additional advantage that they may be processed as bulk materials and are widely used in biomedical applications. For example, polycaprolactone is FDA-approved as a copolymer with glycolide in the absorbable suture Monocryl.[75] We have previously shown that polystyrene with trehalose side chains stabilizes proteins in the solid state.[76] Therefore, it may also be possible to utilize these substituted pCL polymers as solid-state protein stabilizers for a myriad of applications where degradability is required. Thermal gravimetric analysis (TGA) has shown that pCL-trehalose$_{20}$ is stable to over 250 °C when heated (Figure S27), permitting use of these materials at high temperature. Furthermore, polymers such as PEG have been conjugated to proteins to increase their in vivo stability via enhanced pharmacokinetic effects.[34,77] It should be possible to conjugate these polymers to a variety of proteins to additionally stabilize them to environmental stressors, and this work is underway.

CONCLUSIONS

A series of alkene-functionalized polyesters were synthesized by organocatalyzed ring-opening polymerization. Postpolymerization thiol−ene modification with a series of thiols led to well-defined trehalose-, lactose-, glucose-, PEG-, and zwitterion-based biodegradable polyesters. These biodegradable stabilizers were investigated as to their ability to protect the therapeutic protein G-CSF from storage and heat stressors. Side chains containing trehalose and a zwitterionic carboxybetaine were found to be the most effective at maintaining G-CSF activity. Molecular weight studies of pCL-trehalose and pCL-zwitterion were explored, and the polymers were shown to have moderate molecular weight dependence to refrigeration, where larger polymers (DP40 and DP80) demonstrated greater protein stabilization to heat. Both high-performing polymer scaffolds and their degradation products were also not cytotoxic up to at least 1 mg/mL. These materials could be used for stabilization of protein activity in therapeutic and industrial applications, leading to improved performance and lowered cost.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b10776.

Experimental details and characterization of small molecules and polymers, experimental protocol of stability studies and results not in the main paper, polymer stability, degradation studies, TEM data, DSC data, and in vitro cytotoxicity (PDF)

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Notes

The authors declare the following competing financial interest(s): A patent application for these materials is pending.

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