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Tunable Thioesters as "Reduction" Responsive Functionality for Traceless Reversible Protein PEGylation

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Supporting Information

ABSTRACT: Disulfide has been the only widely used functionality to serve as a reduction responsive trigger in drug delivery. We introduce thioester as a novel thiol responsive chemistry for drug delivery, whose reactivity can be conveniently modulated by choosing the appropriate steric environment around the thioester. Compared with disulfides, thioesters are facile to synthesize and have an order of magnitude broader kinetic tunability. A novel traceless reversible protein PEGylation reagent is developed based on thioester chemistry.

Bioresponsive materials can modulate their physicochemical properties in response to endogenous microenvironmental triggers via cleavage of covalent bonds or disruption of supramolecular interactions and have been widely applied in drug delivery systems.[1,2] For intracellular delivery, three kinds of biological triggers have been extensively used, including the intracellular enzymatic activities, the acidic environment of the endolysosomes, and the reductive environment in the cytoplasm.[2,3] A large selection of acid sensitive triggers have been developed, including hydrazones,[4] vinyl ethers,[5] acetals,[6] poly(orthoesters),[7] thiopropionates,[8] dimethylmaleic anhydride,[9] and silyl ethers.[10,11] In addition, many peptides can be cleaved by certain intracellular enzymes to achieve controlled release.[3] In sharp contrast, disulfide has been the only widely used functionality to serve as a reduction responsive trigger.[12−14] Even though disulfide groups have been successfully applied in some drug and gene delivery systems, it is well-known that the reaction kinetics of disulfides is not readily tunable.[15] It was recently demonstrated that Au−S bonds can be cleaved by intracellular glutathione (GSH), the endogenous reductive trigger.[16] However, this chemistry is limited to inorganic drug carriers. Thus, there is an urgent need to expand the chemical toolbox for reduction responsive reactions in drug delivery applications.

The reductive nature of the intracellular environment is mainly due to the high concentration of GSH (1−11 mM in cytoplasm, 2−10 μM extracellularly).[17] The cleavage of disulfides by GSH is usually referred to as a "reductive" process; however, based on the detailed theoretical and experimental study, this process is better described as an S_N2 substitution reaction.[18] In this communication, we will take advantage of the nucleophilicity of GSH and introduce thioester as a novel GSH responsive functionality, whose reactivity can be conveniently modulated by choosing the appropriate steric environment around the thioester. Furthermore, we will apply the thioester chemistry to develop a traceless reversible PEGylation strategy to regulate the activity of proteins (Figure 1).

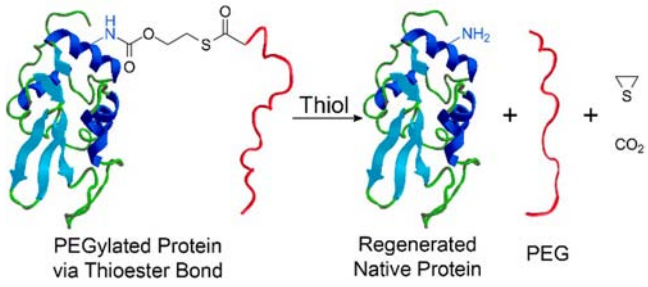
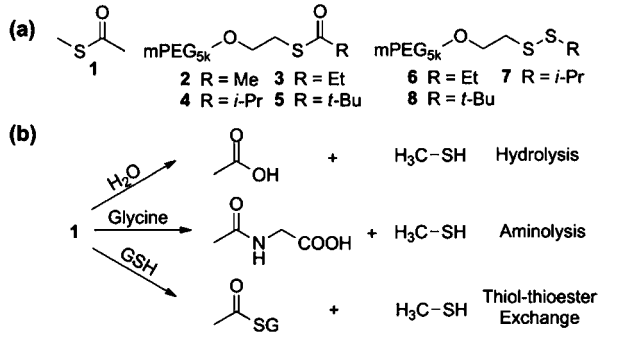


Figure 1. Illustration of traceless reversible PEGylation of proteins based on thioester chemistry.

Despite the fact that thioesters are ubiquitous in biology and the cornerstone of native chemical ligation,[19−23] thiol−thioester exchange has never been applied in controlled drug release to the best of our knowledge. Even though thioesters are usually regarded as highly reactive, their hydrolysis rates are relatively slow. Whitesides et al. recently determined the half-life for hydrolysis of *S*-methyl thioacetate **1** (Scheme 1) to be 155 days at pH 7 and 23 °C.[24] In contrast, the half-life of the

Scheme 1. (a) Structures of Thioesters 1−5 and Disulfides 6−8; (b) Reaction Pathways for Thioester 1



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same thioester shortens to 38 h in the presence of 1 mM of 2-sulfonatoethanethiolate under the same conditions due to rapid thiol−thioester exchange. Motivated by this work and in order to explore the utility of thioesters for drug delivery applications, we therefore set out to reinvestigate the hydrolysis, aminolysis, and thiol−thioester exchange for *S*-methyl thioacetate **1** under physiologically relevant conditions, i.e. pH 7.4 and 37 °C (Scheme 1). Note: all the experiments in this study were carried out at pH 7.4 and 37 °C unless otherwise specified. In order to mimic the intracellular conditions, the thiol source in this study was restricted to GSH.

The half-life of **1** was determined to be 82 h in phosphate buffer saline (PBS) and only 1.3 h in PBS containing 5 mM of GSH (PBS-GSH) (Supporting Information (SI), Figure S1). At pH 7.4, the amino group of GSH is fully protonated (pK_a = 9.08),[25] which suggests insignificant contribution of aminolysis to the degradation of **1** in PBS-GSH. To confirm this assumption, **1** was incubated in PBS containing 5 mM of glycine (PBS-Gly), which was used to mimic the amine functionality in GSH (Scheme 1b). Degradation of **1** in PBS-Gly after 6 h was minimal, while it was fully degraded in PBS-GSH during the same period of time (SI, Figure S1). This indicates that aminolysis does not significantly contribute to the degradation of **1** in PBS-GSH. This observation is also consistent with the basic principle of native chemical ligation, in which thioester reacts with *N*-terminal cysteine in the presence of lysines.[23]

Despite **1** is a convenient model to study the degradation of thioesters, the reactivity of a functional group in small molecules may not extrapolate well to a polymer setting. Poly(ethylene glycol) (PEG) has been widely used in drug delivery.[26] Therefore, we investigated a series of thioester terminated PEGs (M_n = 5000) **2**−**5** (Scheme 1a) with increasing steric hindrance to demonstrate the tunable reactivity of thioesters. We also compared the reactivity of thioesters with the widely used disulfides. Synthesis of thioesters **2**−**5** is very straightforward, using free thiol to react with acyl chloride in one step, which usually completes within a few hours. In contrast, synthesis of disulfides **6**−**8** (Scheme 1a) requires activation of a thiolphile and then formation of the disulfide bonds, whose reaction rate decreases dramatically with increasing steric hindrance (see experimental details in the SI). The synthesis of **8** could not be accomplished below 40 °C and afforded minimal conversion even at 60 °C; therefore it was abandoned for further studies.

As shown in Figure 2a, **2**−**5** degraded monoexponentially in PBS-GSH. The half-life of **2** is 0.40 h in PBS-GSH. Substituting the methyl group in **2** with an ethyl group (i.e., thioester **3**) could extend the half-life by ∼60%. Further increasing steric hindrance around the thioester functionality could prolong the half-lives to 2.2 and 9.7 h for thioesters **4** and **5**, respectively. All PEG thioesters **2**−**5** did not show any appreciable degradation in PBS or PBS-Gly on the same time scale (SI, Figure S2), confirming that thiol−thioester exchange is the main degradation pathway rather than hydrolysis or aminolysis. Similar to PEG-thioesters, **6** and **7** degraded monoexponentially in PBS-GSH. However, their half-lives are both very short (less than 0.5 h, Figure 2b) and have little tunability in kinetics. Based on the degradation study in PBS-GSH, thioesters showed advantages with an order of magnitude broader kinetic tunability compared with disulfides (Figure 2).

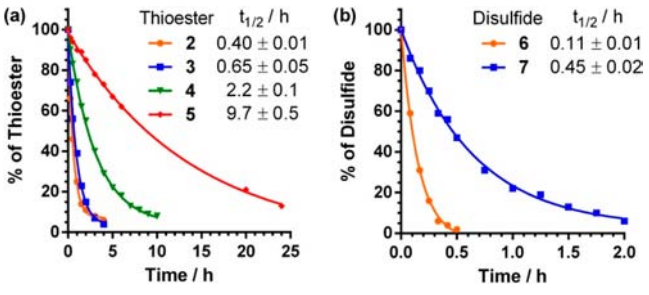
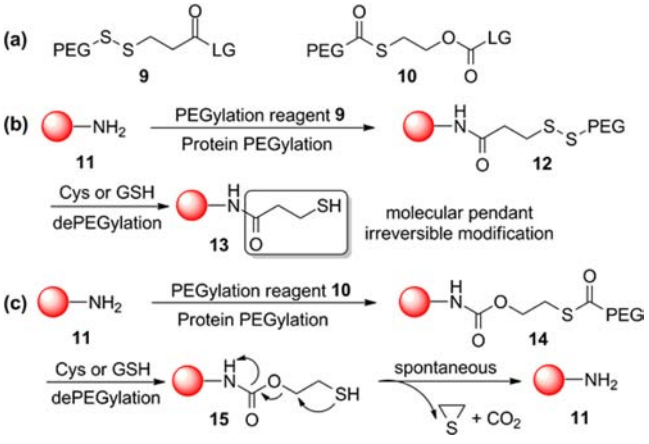


Figure 2. Degradation kinetics of (a) thioesters **2**−**5** and (b) disulfides **6**−**7** in a phosphate buffer saline containing 5 mM of glutathione (pH = 7.4) at 37 °C.

In order to demonstrate the utility of thioester chemistry, we developed a traceless reversible PEGylation strategy to regulate the activity of proteins. Proteins are a kind of promising therapeutic agent. However, the development of protein therapeutics is mainly hindered by its short half-life, immune response, and degradation in serum.[27] PEGylation has been explored as an effective strategy to overcome these problems.[28] Tumor necrosis factor-α (TNF-α) has been investigated in the clinic as an anticancer therapy.[29] Even though PEGylated TNF-α can improve its pharmacokinetics and stability, its activity is also compromised by PEGylation.[27] It was reported that the free thiol concentration in the extracellular environment of tumor cells is more than 10 times higher than that in serum, especially for multidrug-resistant cancer.[30] Therefore, a reductive cleavable PEGylation chemistry will solve this dilemma.[31−33] Conventional disulfide based PEGylation agents, e.g. compound **9** (Scheme 2a), can react with the free lysine

Scheme 2. (a) Structures of PEGylation Reagents Based on Disulfide 9 and Thioester 10 (LG: leaving group); (b−c) Reaction Schemes of Protein PEGylation Using 9 and 10 and Their Corresponding Degradation under Reducing Environment[a]



[a]Red sphere represents lysozyme in this study. The amine group in **11** represents the free lysine residuals in lysozyme.

residual in a protein and be cleaved upon exposure to reductive conditions. However, this reaction usually leaves a chemical pedant on the released protein, which may cause an unexpected immune response (Scheme 2b).[34] In studies by the groups of DeSimone and Wender, disulfide based traceless reversible cross-linkers for protein particles and drug conjugates were reported.[13,35] In this study, we will report a thioester based traceless reversible PEGylation reagent (Scheme 2c), which

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would achieve more facile synthesis and better kinetic tunability compared with disulfide based counterparts.

Lysozyme was initially used as a model protein in this study because (1) its enzymatic activity depends on the intactness of the lysine residuals and can be abolished by single PEGylation and (2) the enzymatic activity can be conveniently quantified using a turbidity assay by lysing bacterial cell walls.[36] PEGylated lysozyme **14** was synthesized by reacting lysozyme with **10** in a borate buffer (pH = 8.0) and followed by purification using high performance liquid chromatography (HPLC). The identity of the PEGylated lysozyme was confirmed by matrix-assisted laser desorption ionization (MALDI) mass spectrometry (SI, Figure S3). The half-lives of PEG cleavage from **14** were determined to be 0.73 h in PBS-GSH (Figure 3a). A

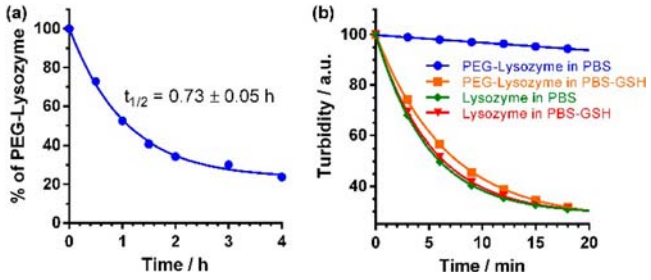

Figure 3. (a) Degradation kinetics of PEG-lysozyme conjugate **14** in a PBS-GSH at 37 °C. (b) Turbidometric assay of the activities of lysozyme and PEG-lysozyme incubated in PBS and PBS-GSH at 37 °C.

turbidometric assay was used to test the enzymatic activities of PEGylated and regenerated lysozyme from dePEGylation. The turbidity was measured at 450 nm using a microplate reader. Faster decay of absorbance at 450 nm corresponds to a higher lysozyme activity. As shown in Figure 3b, the enzymatic activity of PEGylated lysozyme was almost abolished in PBS (blue), while incubating PEGylated lysozyme in PBS-GSH (orange) could restore the enzymatic activity to its native state (green and red). In order to confirm that dePEGylated lysozyme was formed without any chemical pedants (Scheme 2c), the dePEGylation product in PBS-GSH was analyzed using electrospray ionization mass spectrometry (ESI-MS). The M_w of dePEGlyated lysozyme was determined to be 14 304.8, which is the same as that of native lysozyme (M_w = 14304.5) within experimental error (SI, Figure S4). In addition, it is worthy to note that the spontaneous thiol initiated intramolecular reaction for **15** afforded the three-membered ring thiirane and CO_2,[37] instead of the five-membered thiocarbonate as previously proposed.[13,35] Based on the turbidity and ESI-MS experiments, it is safe to conclude that the thioester based reagent **10** can reversibly introduce PEG to proteins and restore the PEGylated protein into its native form with full activity under a reducing environment.

To further demonstrate the utility of the thioester based reagent **10**, tumor necrosis factor-related apoptosis inducing ligand (TRAIL, Figure 4a) was reversibly PEGylated to improve its therapeutic efficacy. TRAIL can selectively induce apoptosis, programmed cell death, in a variety of cancer cells while sparing most normal cells.[38] This unique feature renders TRAIL as a promising protein based anticancer therapeutic, which is currently under phase II clinical trial.[38] However, similar to most of the protein based therapeutics, the efficacy of TRAIL suffers from its poor pharmacokinetics. Reversible

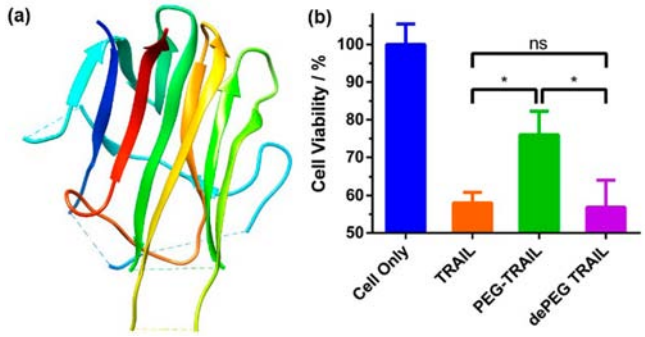

Figure 4. (a) Protein structure of TRAIL. (b) Cytotoxicities of native TRAIL (orange), PEGylated TRAIL (green), and regenerated TRAIL after dePEGylation (purple) on Jurkat cells. Cytotoxicities were determined with CellTiter-Glo assays after 15 h incubation. Graph represents mean ± s.d. (n = 3). *, P < 0.05; ns, nonsignificant.

PEGylation of TRAIL as demonstrated for lysozyme should improve its therapeutic efficacy. PEGylated TRAIL was synthesized by incubating TRAIL with **10** in a borate buffer (pH = 8.0). The completion of the PEGylation reaction was followed by HPLC. The PEGylated TRAIL was characterized using MALDI mass spectrometry (SI, Figure S5). An acute T cell leukemia Jurkat cell line was used as the cell model to test TRAIL induced cell death. As shown in Figure 4b, native TRAIL can induce >40% of cell death at a concentration of 200 nM (orange). The cytotoxicity of PEGylated TRAIL was reduced to ~25% at the same concentration (green). Removing the PEG in PBS-GSH can fully restore the TRAIL activity (purple). The cytotoxicities of regenerated TRAIL through reversible PEGylation and native TRAIL are statistically the same. This experiment further demonstrated the potential of the thioester based reversible PEGylation chemistry in improving protein based therapeutics.

In summary, we demonstrated thioester as a new member in the toolbox of "reductive" responsive chemistry for drug delivery. Thioesters can be conveniently synthesized, and their thiol responsive degradation rates can be conveniently modulated by choosing the appropriate steric environment around the thioester. We also developed a thioester based traceless PEGylation strategy to modulate the protein activity, which could potentially enhance its *in vivo* performance. The rate of dePEGylation of protein can be further tuned by varying the steric hindrance around the thioester moiety as we demonstrated in the model studies. The thioester based traceless reversible chemistry can also be applied in small molecule conjugation, protein cross-linking, and protein–polymer conjugates.[39−41] We expect thioesters to play an important role in future studies on thiol responsive controlled drug delivery.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details on synthesis, thioester degradation studies, protein PEGylation, and *in vitro* cell culture assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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