

Thiolytically Cleavable Dithiobenzyl Urethane-Linked Polymer–Protein Conjugates as Macromolecular Prodrugs: Reversible PEGylation of Proteins

Samuel Zalipsky,* Nasreen Mullah, Charles Engbers, Maria U. Hutchins, and Radwan Kiwan

ALZA Corporation, 1900 Charleston Road, Mountain View, California 94043. Received May 25, 2007;
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New thiolytically cleavable dithiobenzyl (DTB) urethane-linked conjugates of methoxypoly(ethylene glycol) (mPEG) and a model protein, lysozyme, were prepared and thoroughly characterized. In contrast to our earlier communication [Zalipsky, et al. (1999) *Bioconjugate Chem.* 10, 703], in the current study we used a more sterically hindered form of *para*-DTB urethane linkage containing a methyl group on the α -carbon to the disulfide moiety. The new reagent for covalent attachment of mPEG-DTB to amino groups of proteins was synthesized via a seven-step process. As a result of PEG conjugation, the lysozyme was shown to completely lose its bacterial cell wall-lysing activity. However, activity was almost fully restored upon cysteine-mediated cleavage of the PEG component. The conjugate decomposition process was monitored by RP-HPLC and by ion spray LC-MS, which showed the formation of the *p*-mercaptobenzyl urethane–lysozyme intermediate, and ultimately its conversion to the unmodified lysozyme as the sole protein component. Pharmacokinetic evaluation of 125 I-labeled cleavable and noncleavable PEG–lysozyme given intravenously in rats revealed similar clearance patterns; both cleared in a significantly slower manner compared to that of the native protein. However, subcutaneous administration of the same conjugates showed a significantly larger AUC of the cleavable conjugate, indicating that some cleavage of the DTB urethane may have occurred. Although the DTB-linked PEG–lysozyme exhibited almost the same plasma clearance as the noncleavable counterpart, hinting that methyl-DTB linkage might be stable in the bloodstream, SDS–PAGE examination of the conjugate incubated in plasma showed decomposition at least partially mediated by albumin. These results suggest the potential of PEG–DTB–proteins as macromolecular prodrugs capable of generating fully active native proteins under *in vivo* conditions.

INTRODUCTION

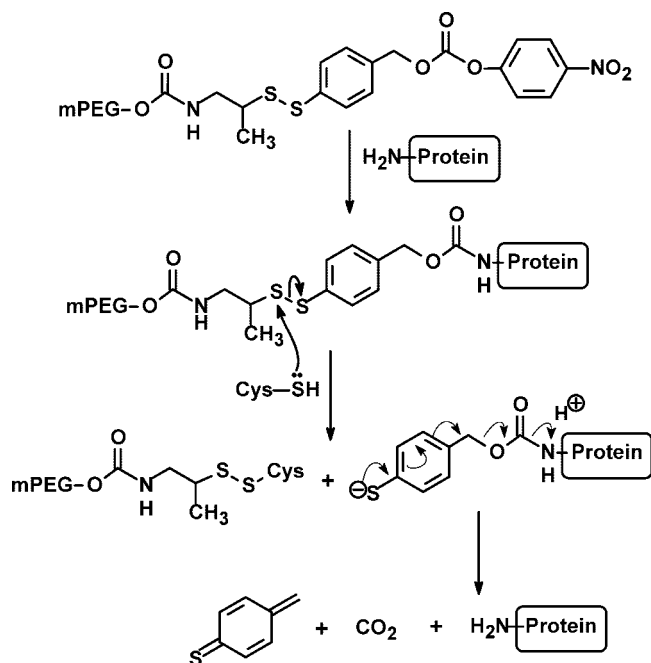
Covalent linking of proteins with water-soluble biocompatible polymers is often used to modulate the properties of the resulting conjugates in order to make them more amenable to *in vivo* applications (1–3). One of the most popular polymers used for this purpose, polyethylene glycol (PEG¹) (4–6), is known to convey a number of useful properties to its conjugates. Among them are prolonged plasma circulation time, reduced immunogenicity and antigenicity, improved solubility, and resistance to proteolytic degradation (3, 5–7). Usually, there is a direct relationship between the amount of the linked polymer and the improvement in the above-listed characteristics. However, covalent attachment of PEG strands to a protein, a process often referred to as PEGylation, is at times accompanied by a substantial loss of the functional biological activity. This is a particularly severe problem for proteins acting on macromolecular substrates (3, 8, 9). Most receptor-binding proteins, for example, cytokines and chemokines, fall into this category. Three approaches were applied to deal with the problem of the loss of biological activity as a result of PEGylation: (i) site specific PEGylation, sometimes in combination with site-specific mutagenesis (10–15); (ii) protection of receptor-binding site during the conjugation (16, 17); and (iii) reversible PEGylation (18–22). The latter is essentially a prodrug approach in which one or several PEG chains are acting as pro-moieties. Each of these approaches has its merits and can potentially be effective

in some specific situations, and each one of them also presents nontrivial technical obstacles.

We recently introduced the new PEGylation chemistry depicted in Scheme 1, which was designed to produce gradual *in vivo* loss of PEG chains from its conjugates (21, 23, 24). It is based on benzyl urethane *para*-substituted with a disulfide, dithiobenzyl (DTB) moiety. The urethane formed between an amino group of a protein and the benzyl is quite a stable linkage. However, reductive or thiolytic scission of the disulfide leads to an unstable *p*-mercaptobenzyl urethane intermediate, which breaks down via 1,6-elimination and decarboxylation, and results in the regeneration of the original amino group. To the best of our knowledge, *p*-aryldisulfanyl-substituted benzyloxycarbonyl mitomycin C described by Senter et al. (25) constituted the first disclosure of a DTB-urethane derivative. In our laboratory, we used this linkage to prepare a lipid-based anticancer prodrug (26, 27) and also thiolytically cleavable lipopolymers (21, 28) that were utilized for liposomal and gene delivery. In addition to the thiolytic recovery of the modified amino group, the relative stability of DTB at neutral pH in the absence of redox agents is important for the convenient formulation and storage of the DTB-based conjugates. This feature offers an advantageous distinction from other published approaches to reversible PEGylation (18–20, 22) that are triggered by hydrolysis or pH changes. Since reductive scission of disulfide-linked conjugates is known to occur in various physiological environments (29, 30), it was envisioned that similarly under *in vivo* conditions DTB urethane would be cleaved, for example, by cysteine or glutathione-mediated thiolysis or perhaps by an enzymatic

* To whom correspondence should be addressed. Intradigm Corporation, 3350 W. Bayshore Rd., Suite 100, Palo Alto, CA 94303. Tel: (650) 855-1517. Fax: (650) 855-9699. E-mail: szalipsky@intradigm.com.

¹Abbreviations: DTB, dithiobenzyl; PEG, polyethylene glycol; mPEG, monomethoxy-PEG; mPEG5K, mPEG of molecular weight 5000 Da; NPC, *p*-nitrophenyl carbonate.

Scheme 1. Reversible PEGylation of Proteins Using Thiolitically Cleavable DTB Urethane Linker^a

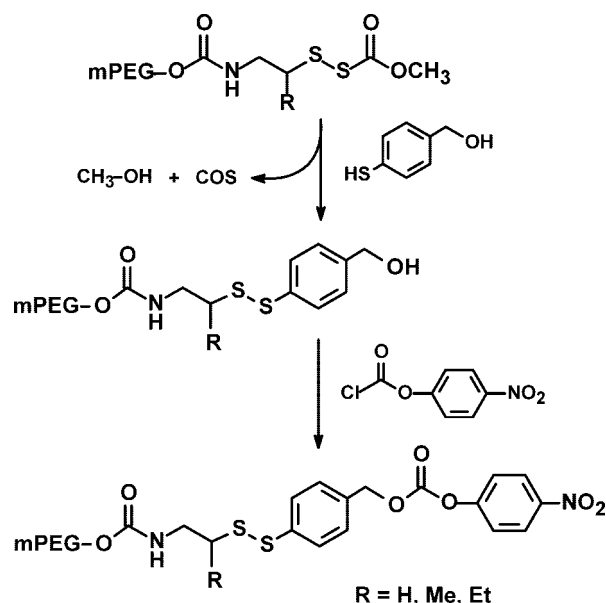
^a Sterically hindered DTB containing a methyl group adjacent to the disulfide was utilized in the current study. Note that the thiolitic cleavage shown here for cysteine is equally applicable to any biologically relevant thiol, e.g., glutathione, albumin, etc.

reduction. Thus, in vivo PEG would be present in these reversible conjugates only temporarily, improving such characteristics as pharmacokinetics and biodistribution. As a result of the PEG-chain detachment, the original native protein and its activity would ultimately be regenerated. In essence, the system is intended to work as a macromolecular prodrug, where each PEG chain is acting as a promoity.

We chose lysozyme as a model protein to perform the initial feasibility studies on conjugate preparation and thiol-mediated decomposition, as well as pharmacokinetic properties of the reversible PEG-DTB-proteins. Lysozyme is a readily available, small protein (14.4 kDa) similar in size to various cytokines and chemokines, and its lytic activity is directed toward a very large substrate, the bacterial cell wall peptidoglycan. Therefore, lysozyme represents a suitable model for a small protein, which is acting on a large substrate to exhibit its biological activity. Herein, we report the results of the lysozyme model studies.

RESULTS AND DISCUSSION

A new reactive polymer derivative, mPEG-DTB-NPC, which is required for the preparation of PEG-DTB-protein conjugates, was synthesized as shown in Scheme 2. This synthesis scheme was applied to mPEG derivatives with molecular weights of 2000, 5000, and 12000 Da with equal success, although the mPEG-2000 derivatives were mainly utilized for the preparation of DTB-linked lipopolymers (21, 28). The R residue, which is intended to regulate the steric hindrance on the disulfide bridge of the DTB urethane linkage and ultimately the rate of the cleavage of this linker was varied from hydrogen to more bulky methyl and ethyl groups. Our earlier experiments with PEG-lipids and PEG-liposomes indicated that when R = Me or Et the liposomes containing these DTB-linked lipopolymers exhibited long persistence in the bloodstream characteristic to STEALTH liposomes, while the analogous preparations with R = H did not (28). On the basis of these observations, the protein modifications described herein were performed with

Scheme 2. Synthesis of mPEG-DTB-NPC, Reagent for the Attachment of mPEG-DTB Moieties To Amino Groups on Proteins^a

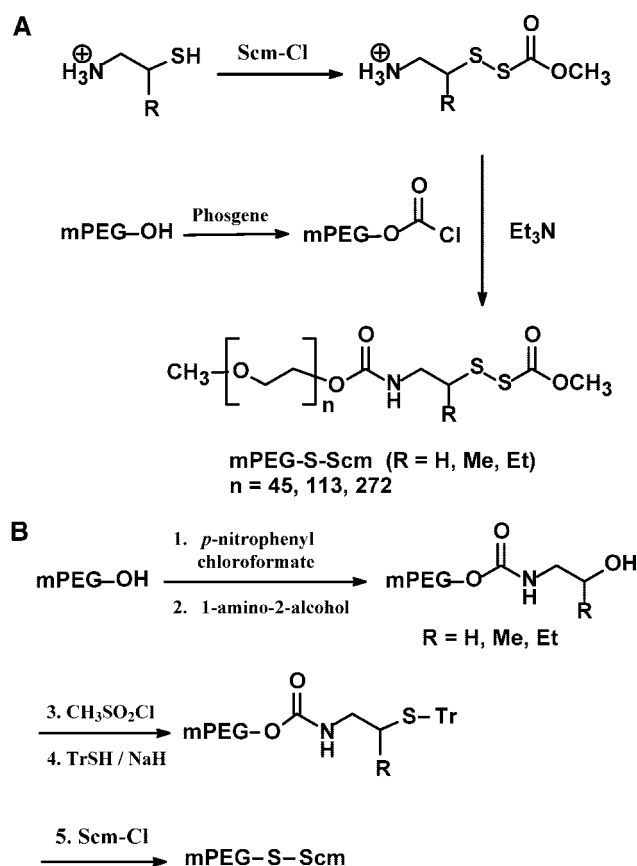
^a Note that the size of the R residue (also shown in Scheme 3) affects the hindrance on the adjacent disulfide and, ultimately, the rate of the PEG detachment from the protein conjugates.

the former more stable mPEG-DTB form of the linkage containing a methyl substituent on the disulfide adjacent carbon (Scheme 1).

For the formation of the DTB-mixed disulfide, we relied on the reactivity of the *S*-sulfenylcarbamate (*S*-Scm) functionality (21) (Scheme 2). Facile formation of asymmetrical disulfides via fragmentation of sulfenyl thiocarbonates is known to proceed even with weakly nucleophilic aromatic thiols (31). The reaction is driven by the release of COS and methanol. The mPEG-S-Scm was assembled from 1-alkyl-substituted 2-aminothiols (32), which were first reacted with methoxycarbonylsulfenyl chloride (Scm-Cl) to form the corresponding ammonium hydrochloride sulfenyl thiocarbonates. The latter were purified and then reacted with mPEG-chloroformate, forming stable carbamate attachments (Scheme 3A). An alternative approach to the *S*-sulfenyl thiocarbonates, prepared by treatment of the corresponding PEG-S-trityl with Scm-Cl by a modification of previously published method (33), also worked well. The corresponding S-Tr derivative was prepared in a clean manner from mPEG-OH in four steps as depicted in Scheme 3B. The reaction of mPEG-S-Scm with *p*-mercaptobenzyl alcohol was carried out in methanol at ambient temperature, yielding mPEG-DTB-OH. This reaction was considerably faster for the less-hindered Scm derivatives (R = H), but nevertheless, it was driven to completion with excess *p*-mercaptobenzyl alcohol over 24–48 h in the case of the more hindered analogues (R = Me and Et). To enable alkoxy-carbonylation of amino groups, the hydroxyl group of mPEG-DTB-OH was converted into a reactive carbonate form by treatment with nitrophenyl chloroformate (Scheme 2).

This new reagent, mPEG-DTB-NPC (Scheme 1), was utilized in a series of lysozyme modification experiments. The aim of these experiments was to prepare lightly PEGylated species and to study their thiolitic decomposition while comparing the properties to those of the noncleavable analogues of the PEG-lysozyme. The latter were prepared with a known alkoxy-carbonylating reagent of proteins, mPEG-NPC (34). Our lysozyme modification experiments demonstrated that both reagents, mPEG-DTB-NPC and mPEG-NPC, had similar reactivity

Scheme 3. Synthesis of mPEG-S-Scm by Two Different Approaches: (A) Reacting the Amino-thiol Compound with Methoxycarbonylsulfonyl Chloride (Scm-Cl), Followed by Coupling To mPEG-Chloroformate, and (B) Generation of mPEG-S-Tr Followed by Its Conversion To mPEG-S-Scm



toward amino groups on proteins and under the same conditions yielded comparable PEGylated lysozyme mixtures with 1–3 polymer chains per protein molecule. We characterized these conjugates by SDS–PAGE, RP–HPLC, and MALDI–TOF–MS (Figures 1, 2 and 3). We purified the PEG–lysozyme conjugates from the unreacted PEG and protein by cation-exchange chromatography. Baseline separations between the reaction mixture components, PEG–lysozyme, unreacted protein, and excess of PEG reagent, were achievable on the CM–HEMA column. Fractionation on the CM–column also allowed us to obtain pure 1:1 PEG–lysozyme conjugates, which proved to be very useful in our mechanistic studies. MALDI–TOF–MS confirmed the expected molecular masses of (mPEG)_{1–3}–lysozyme conjugates in the purified samples. It also showed the presence of the unmodified lysozyme present in the mass spectra of the PEG–DTB–conjugates (Figure 3), even though the preparations were free from the native enzyme by RP–HPLC and SDS–PAGE. This is explained by the partial cleavage of the disulfides linkages under the MALDI conditions, which has been previously reported (21, 35). The conjugate derived from both mPEG–5K and –12K (not shown) produced similar patterns of MALDI spectra.

As illustrated in Scheme 1, the reversibility of the DTB–urethane linkage is based on the thiolytic or reductive cleavage of the disulfide bond. Under *in vivo* conditions, this can occur via a reaction with any of the thiols present in blood plasma or tissues or by an enzymatic reductive scission of the disulfide. Among the low molecular weight thiols present in the physiological milieu, cysteine is the most reactive. Therefore, to examine the PEG detachment reaction, we used cysteine as the cleaving agent. Upon incubation of the (mPEG–DTB)_n–

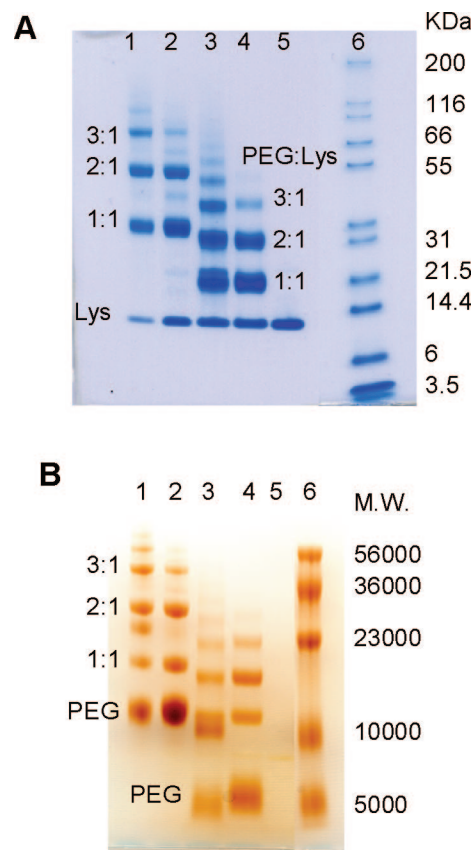


Figure 1. SDS–PAGE showing the content of the conjugate mixtures, using two visualization methods: (A) Coomassie blue detection for protein and (B) iodine staining for PEG species. Lanes: (1) mPEG12K–DTB–lysozyme conjugation, (2) mPEG12K–lysozyme conjugation, (3) mPEG5K–DTB–lysozyme conjugation, (4) mPEG5K–lysozyme conjugation, (5) lysozyme, and (6) molecular weight markers (protein or PEG).

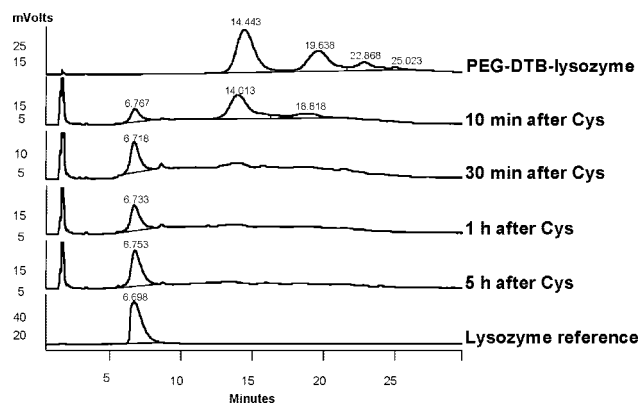


Figure 2. RP–HPLC of (mPEG5K)_{1–3}–DTB–lysozyme conjugates and follow-up of their decomposition in 1 mM cysteine solution.

lysozyme–conjugate mixture with cysteine, gradual decomposition and cleavage of the PEG chains took place. Following the de–PEGylation process with RP–HPLC (Figure 2), it was revealed that the heavier PEG–lysozyme species were converted to lighter ones and ultimately formed a sole protein component. The identity of the regenerated protein showing the same elution time on RP–HPLC as lysozyme was corroborated by ion spray LC–MS. Average spectra taken on the peak eluting at 6.7 min were identical to the spectra of the reference lysozyme (Figure 4A and D). LC–MS also confirmed that the main peak of the conjugate mixture eluting at 14.4 min (Figure 4B) was mPEG5K–DTB–lysozyme (calculated molecular mass of 20

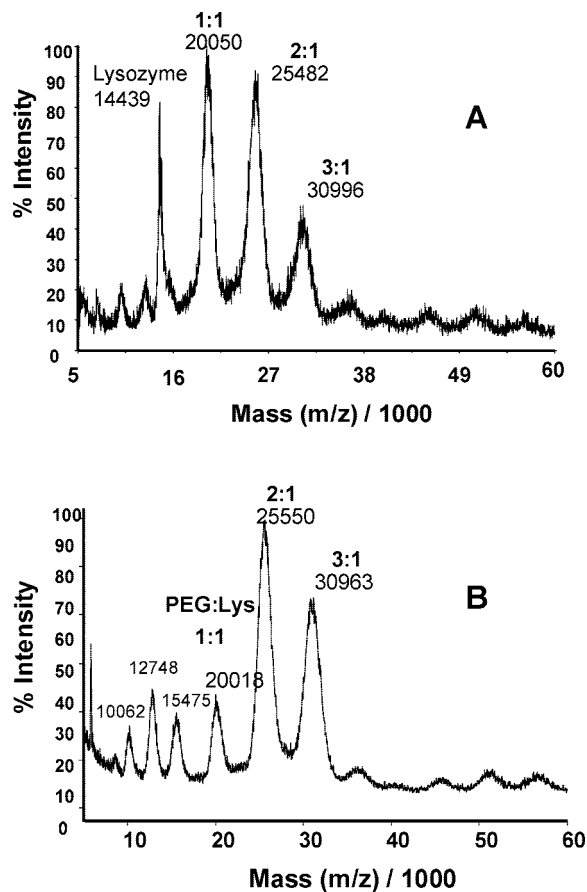


Figure 3. MALDI-TOF spectra of (A) mPEG5K-DTB-lysozyme conjugates and (B) their noncleavable counterparts.

kDa). Two distinct PEG products with bell-shaped distributions centered around 5480 and 5600 Da, differing approximately by the molecular weight of cysteine (121 Da), were observed during the cysteine-mediated cleavage of DTB urethane. The relative amounts of the lighter species (eluting later, less polar mPEG-SH) versus the heavier ones (eluting earlier, more polar mPEG-SS-Cys) increased with the cysteine incubation time, as judged by the ratios of their RP-HPLC peaks (result not shown). This is consistent with the disulfide-linked mPEG-cysteine being the main product (Scheme 1) at the early time points and its conversion to mPEG-thiol with time. For comparison, the ion spray MS of the corresponding mPEG5K-DTB-OH produced a typical bell-shaped distribution of signals centering around 5600 Da. Upon closer examination of the cysteine-mediated de-PEGylation reaction using the 1:1 conjugate of PEG5K-DTB-lysozyme (Figure 5A), we learned that the putative *p*-mercaptobenzyl urethane lysozyme intermediate (confirmed by LC-MS; Figure 4C) was detectable by RP-HPLC. As shown in Figure 5B, the decomposition of this intermediate occurred slower than the initial thiolysis step, which explains why the disappearance of the starting conjugate was faster than the appearance of the free lysozyme product.

One of the characteristics of lysozyme that makes it an excellent model for a large substrate binding protein is the high sensitivity of its cell wall-lysing activity to PEGylation. Consistent with previously reported observations (36, 37), we found that attachment of even one PEG chain to lysozyme completely abolishes its enzymatic activity in a *Micrococcus luteus* lysis assay. As illustrated in Figure 6, both cleavable PEG-lysozyme and its noncleavable counterpart showed no activity in this assay. However, in contrast to the noncleavable conjugate, the treatment of PEG-DTB-lysozyme with cysteine restored the lytic activity of the enzyme.

Pharmacokinetic properties of PEG-proteins are mainly governed by the molecular size of the conjugates (38). In other words, the results obtained with PEG-lysozyme should be roughly representative of conjugates derived from proteins of size similar to that of lysozyme. We studied the pharmacokinetic properties of the ^{125}I -labeled (mPEG) $_{1-3}$ -lysozyme conjugates in rats, comparing the stable urethane analogue to the cleavable DTB-linked counterpart and to the native protein. In order to enhance the contrast between the free protein and its conjugates, we used the higher molecular weight mPEG-12K in these experiments. Following intravenous administration, we observed that both PEG-lysozyme preparations circulated much longer than the native protein (Figure 7A). The DTB-linked conjugate exhibited pharmacokinetic characteristics similar to those of the noncleavable analogue, which hinted at an apparent plasma stability of the DTB-linkage-containing methyl group on the α carbon. This result was consistent with our observations in the liposomal system (28), in which the PEG-liposome containing the same sterically hindered DTB linkage also exhibited the same clearance curves as those of the liposome containing the stable urethane-linked lipopolymer analogue. Administration of the same mPEG12K-lysozyme preparations by the subcutaneous route (Figure 7B) resulted in noticeably larger AUC for the DTB-linked conjugate compared to its noncleavable urethane counterpart. Subcutaneous administration is often considered to be a more attractive and less invasive route compared to the intravenous route. Both conjugates exhibited a much longer presence in circulation than the free protein, which rapidly peaked in the bloodstream after subcutaneous administration and also was rapidly cleared. These route-of-administration differences between the results of the two pharmacokinetic experiments and their implications regarding the stability of DTB linker in the relevant environments might be due to the differences in the thiol species and their concentration between plasma and interstitial fluid. It was reported, for example, that the levels of sulfur-containing amino acids are higher in interstitial fluids compared to those in plasma (39). However, the albumin concentration in interstitial fluid is significantly lower (40) compared to its plasma levels, where it is the most abundant and also the most reactive thiol (41).

The latter fact prompted our investigation of the stability of our DTB-linked conjugates in the presence of albumin or plasma. Note that there is a great deal of homology among rat (RSA), human (HSA), and bovine (BSA) albumin, all of which contain a single reactive thiol group, Cys-34. First, we followed the integrity of purified 1:1 PEG5K-lysozyme incubated in BSA solution (4% in PBS, similar to the albumin concentration in plasma) by SDS-PAGE using PEG-sensitive staining. This staining provides information on PEG-containing species, although proteins at high concentrations are also visualized. Conversely, the free lysozyme present at a relatively low concentration was hardly detectable. The results of these experiments are shown in Figure 8. The band of mPEG-DTB-lysozyme completely disappeared from the gel after overnight incubation with albumin (Figure 8A, lane 9). Under the same conditions, the noncleavable conjugate remained stable (lane 4), excluding the possibility that noncovalent association with albumin was responsible for the disappearance of the cleavable PEG-lysozyme (lane 9). BSA with blocked thiol (pretreated with iodoacetamide) did not react with mPEG-DTB-lysozyme (lane 8), indicating that the thiol group played a role in the decomposition of the conjugate. Finally, when a BSA-decomposed sample of mPEG-DTB-lysozyme was treated with excess DTT, free mPEG5K was readily detectable on the gel (lane 7) to the same extent as cysteine-mediated cleavage product (lane 3). Likewise, mPEG5K-DTB-lysozyme incubations in rat plasma (Figure 8B) produced the same pattern of

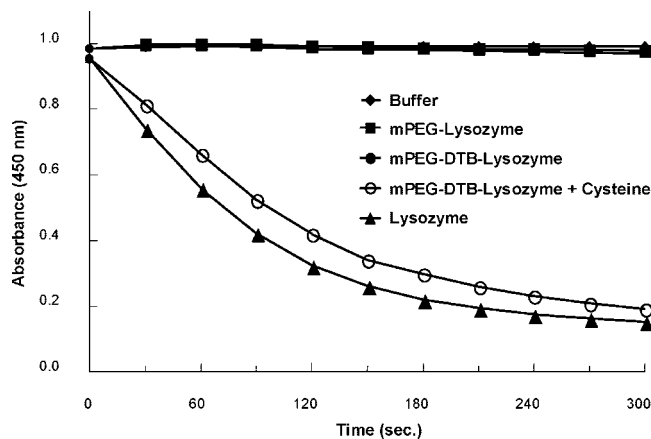


Figure 6. Turbidometric assay of bacterial cell wall lysis of lysozyme and its conjugates. The conjugates and the control samples were treated as described in the Materials and Methods section, under lysozyme activity assay.

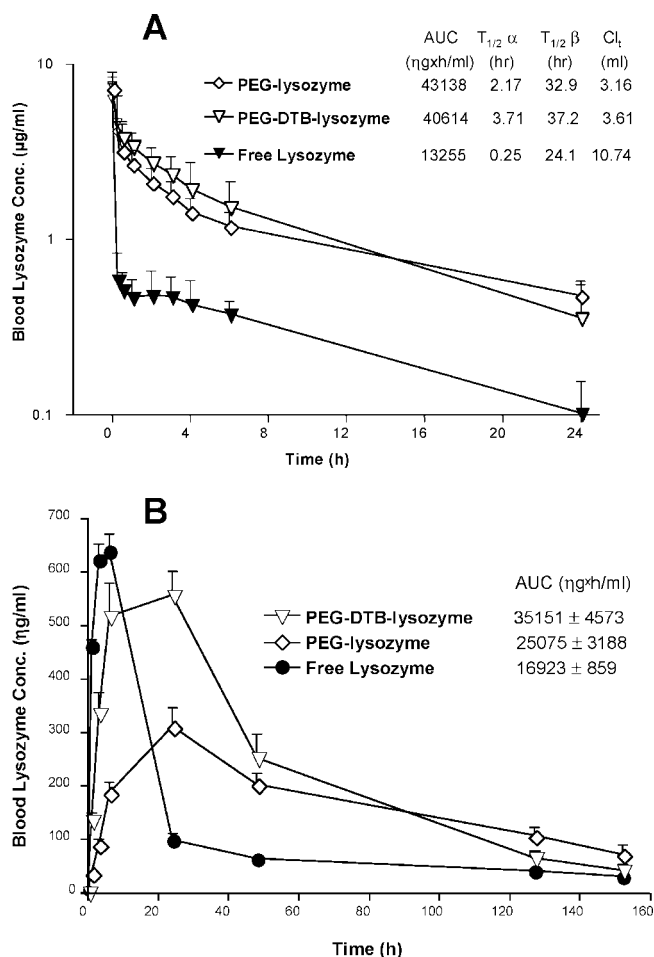


Figure 7. Pharmacokinetics in rats of ^{125}I -labeled mPEG12K-DTB-lysozyme conjugates, its noncleavable analogues, and the free parent protein. Clearance curves were constructed from the radiolabeled detection of ^{125}I after (A) intravenous and (B) subcutaneous administration.

noncleavable counterparts. Thiolytic cleavage occurs under mild conditions achievable in vivo, and most importantly, as expected from a true prodrug, the original native protein is generated as the result. This was demonstrated both by complete recovery of the PEGylation-induced loss of activity and by LC-MS yielding identical spectra of the native and de-PEGylated lysozyme. Furthermore, our studies show that although

mPEG12K-DTB-lysozyme exhibited long circulating characteristics similar to its noncleavable counterpart, the DTB linker is clearly vulnerable to both low and high molecular weight biologically relevant thiols, cysteine, and albumin. The latter thiol is the most likely culprit responsible for the decomposition of the conjugate in plasma. The observation of both DTB cleavage and long circulation of mPEG12K-DTB-lysozyme could be the result of a relatively slow breakdown rate; however, alternative explanations are also possible. We investigated, in further detail, the albumin reaction with several PEG-DTB conjugates, and the results as well as the insight into the possible mechanisms involved and the reasons for the long in vivo circulation are presented in a separate paper (Hutchins, M. U., et al., in preparation).

MATERIALS AND METHODS

Lysozyme was obtained from Roche Molecular Biochemicals, Indianapolis, IN. Amino-2-propanethiol hydrochloride (32) and 4-mercaptobenzyl alcohol (42) were prepared as described elsewhere. All the synthetic procedures below, although written for derivatives of mPEG5K, are equally applicable to the mPEG12K analogues (gifts from Union Carbide and NOF, respectively). All the NMR spectra were acquired in DMSO- d_6 /Me $_4$ Si using 360 MHz spectrometer at Acorn NMR, Livermore, CA.

Methoxycarbonyldisulfanyl-2-propylammonium Chloride.

The title compound was prepared by reacting methoxycarbonylsulfenyl chloride with an equivalent amount of amino-2-propanethiol hydrochloride according to the previously published procedures (43, 44). Amino-2-propanethiol hydrochloride (1.83 mL, 19.44 mmol) in methanol (15 mL) was added dropwise (~ 1.0 mL/min) to a cold (4 °C) solution of methoxycarbonylsulfenyl chloride (Scm-Cl, 2.5 g, 19.6 mmol) in methanol (15 mL). The solution was stirred at room temperature for 3 h and then rotary evaporated. The residue was taken up in acetone and then stripped of the solvent two more times, resulting in the formation of a white solid. Silica-C8 (1 g) was added to the product solution in MeOH, which was then evaporated. The product-containing residue was added to the top of the C8-silica column and was eluted with water under N $_2$ pressure. Fractions containing the pure product were combined, evaporated, and lyophilized from *tert*-butanol (5 mL). It was further dried in vacuo over P $_2$ O $_5$ to give product as a white solid (2 g, 47% yield). ^1H NMR δ : 1.30 (d, CH $_3$ CH, 3H), 2.94 (br t, CH $_2$ NH $_3$ Cl, 2H), 3.37 (q, CH $_3$ CHCH $_2$ NH $_3$ Cl, 1H), 3.88 (s, CH $_3$ OCO, 3H), 8.21 (br s, CH $_3$ CHCH $_2$ NH $_3$ Cl, 3H) ppm.

mPEG5K-Oxycarbonylamino-propyl-S-sulfenyl carbomethoxy (mPEG-S-Scm), Method A. This method is shown in Scheme 3A. mPEG-OH (15 g, 3 mmol) was dried by azeotropic removal of toluene (100 mL). The solution was cooled, diluted with dichloromethane (40 mL), and treated with phosgene (10 mL, 19.3 mmol) at 4 °C. The ice bath was removed, and the reaction was stirred at room temperature overnight and then evaporated to dryness, leaving solid mPEG-chloroformate. Methoxycarbonyldisulfanyl-2-propylammonium chloride (686.7 mg, 3.15 mmol) and then TEA (836.3 μL , 6 mmol) were added to the mPEG-chloroformate in chloroform (30 mL). The reaction mixture was stirred at room temperature overnight and then evaporated. The residue was taken up in warm ethyl acetate (150 mL) and filtered to remove the TEA hydrochloride. The solvent was removed, and the product was purified by recrystallization from ethyl acetate/iso-propanol (100/100 mL, three times) and dried in vacuo over P $_2$ O $_5$, yielding a white solid (14.5 g, 93%). ^1H NMR δ : 1.18 (d, CH $_3$ CHSS $_2$, 3H), 3.10 (m, CH $_2$ NH, 2H), 3.24 (s, CH $_3$ O, 3H), 3.50 (s, PEG, $\approx 520\text{H}$), 3.86 (s, CH $_3$ OCOSSH, 3H), 4.05 (t, OCOCH $_2$, 2H), 7.38 (t, OCONH, 1H) ppm.

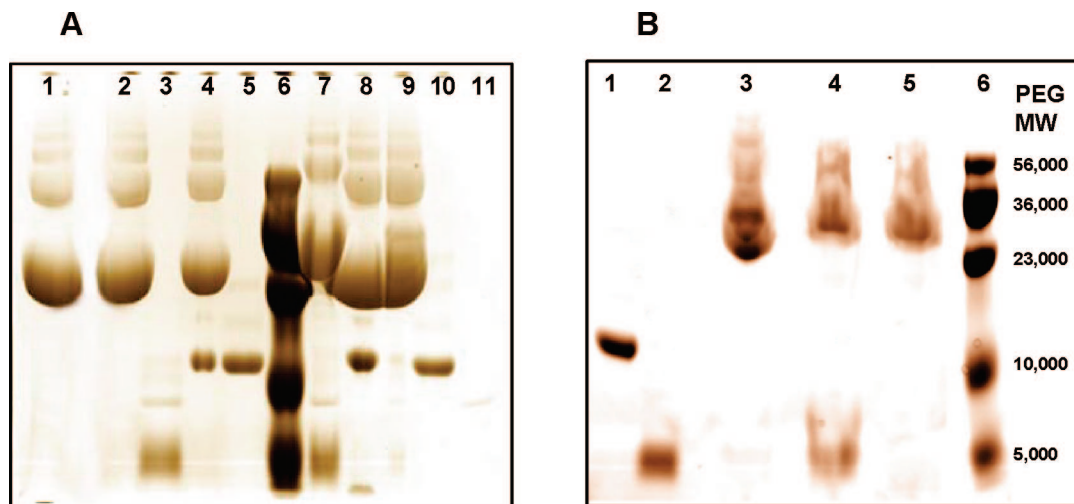


Figure 8. SDS-PAGE gels of samples from an overnight incubation of mPEG5K-DTB-lysozyme and controls in (A) albumin (BSA) solution and in (B) rat plasma. Samples list A: (1) BSA-blocked SH, (2) BSA, (3) mPEG-DTB-lysozyme + cysteine, (4) noncleavable PEG-lysozyme + BSA, (5) noncleavable PEG-lysozyme, (6) PEG MW Markers, (7) mPEG-DTB-lysozyme + BSA, then DTT, (8) mPEG-DTB-lysozyme + BSA-blocked SH, (9) mPEG-DTB-lysozyme + BSA, 16 h, (10) mPEG-DTB-lysozyme, and (11) lysozyme. Samples list B: (1) mPEG-DTB-lysozyme, (2) mPEG-DTB-lysozyme + cysteine, (3) mPEG-DTB-lysozyme in rat plasma, 18 h, (4) mPEG-DTB-lysozyme in rat plasma, then DTT, (5) rat plasma control, and (6) PEG molecular weight markers.

mPEG5K-Nitrophenyl Carbonate (mPEG-NPC). Azeotropically dried mPEG5K-OH (40 g, 8 mmol) in toluene/dichloromethane (20/100 mL) solution was treated with *p*-nitrophenyl chloroformate (2.42 g, 12 mmol) and TEA (3.3 mL, 24 mmol). After the initial cooling (≈ 15 min) on ice-water during the addition of the reagent, the reaction mixture was stirred at room temperature overnight. The reaction solution was evaporated to dryness, and the residue was taken up in warm ethyl acetate (≈ 50 °C). TEA hydrochloride was filtered off and washed with a small volume of warm ethyl acetate. The solvent was rotary evaporated, and the residue was recrystallized from iso-propanol twice and then dried in vacuo over P_2O_5 , yielding 38.2 g (92%) of white powder. 1H NMR δ : 3.24 (s, CH_3O , 3H), 3.50 (s, PEG, 460H), 4.37 (t, $OCOCH_2$, 2H), 7.57 (d, C_6H_5 , 2H), 8.32 (d, C_6H_5 , 2H) ppm.

mPEG5K-Oxycarbonylamino-2-propanol (mPEG-iPr-OH). Amino-2-propanol (1.1 mL, 14.52 mmol) and TEA (2.02 mL, 14.52 mmol) were added to mPEG5K-NPC (25 g, 4.84 mmol) solution in DMF (60 mL) and CH_2Cl_2 (40 mL). The reaction solution turned yellow. It was stirred at room temperature for 30 min. TLC ($CHCl_3/MeOH = 90:10$) showed that the reaction went to completion. Dichloromethane was removed by rotary evaporation, and iso-propanol (250 mL) was added to the concentrated DMF solution. The precipitated product was collected by filtration and further purified by two additional precipitations from iso-propanol. It was then dried in vacuo over P_2O_5 , yielding 22.1 g (90%) of white powder. 1H NMR δ : 0.99 (d, CH_3CH , 3H), 2.90 (m, $NHCH_2$, 2H), 3.24 (s, CH_3O , 3H), 3.50 (s, PEG, $\approx 480H$), 4.04 (t, $OCOCH_2$, 2H), 4.85 (d, CH_3CHOH , 1H), 7.11 (t, NH, 1H) ppm.

mPEG5K-Oxycarbonylamino-2-propyl methanesulfonate (mPEG-OMs). mPEG5K-oxycarbonylamino-2-propanol (22.12 g, 4.34 mmol) was azeotropically dried in its toluene solution (45 mL). The solution was cooled, diluted with dichloromethane (60 mL), and then treated with methanesulfonyl chloride (604.6 μL , 7.81 mmol) and TEA (3.93 mL, 28.21 mmol, 6.5 equiv) while maintaining continuous stirring and cooling (4 °C), and protecting the reaction mixture against air moisture. After 30 min, the cooling bath was removed, and the reaction mixture was stirred at room temperature for 16 h. The solution was rotary evaporated, and the residue was taken up with warm ethyl acetate. TEA salt was removed by filtration. The product was precipitated with iso-propanol and recrystal-

lized with the same solvent two more times, and then dried in vacuo over P_2O_5 yielding 20.27 g (90%) of white powder. 1H NMR δ : 1.28 (d, CH_3CHOMs , 3H), 3.13 (s, CH_3O_2SO , 3H), 3.18 (t, CH_2NH , 2H), 3.24 (s, CH_3O , 3H), 3.50 (s, PEG, $\approx 480H$), 4.08 (t, $OCOCH_2$, 2H), 4.65 (q, $CHOMs$, 1H), 7.51 (t, NH, 1H) ppm.

mPEG5K-Oxycarbonylamino-2-propyl-S-triphenylmethane (mPEG-S-Tr). Sodium hydride (60% oil dispersion, 377 mg, 9.4 mmol, 4.75 equiv) was treated with anhydrous toluene (60 mL) at ≈ 4 °C. Triphenylmethanethiol (3.92 g, 14.6 mmol, 7.15 equiv) was added after 5 min, followed by mPEG-oxycarbonylamino-2-propyl methanesulfonate (10.3 g, 1.98 mmol) 10 min later. The reaction turned yellow. After 45 min, acetic acid (440 μL , 7.7 mmol) was added to the reaction mixture to neutralize the excess sodium hydride. A white precipitate was formed. The solution was evaporated to dryness. Warm ethyl acetate (30 mL) and iso-propanol (70 mL) were added to the residue. The solution was filtered while still warm (≈ 40 °C) and then cooled down to facilitate product precipitation. Then the product was further recrystallized from iso-propanol/*tert*-butanol (100/20 mL) and dried in vacuo over P_2O_5 , yielding 8.87 g (84%) of white powder. 1H NMR δ : 0.75 (d, CH_3CHSTr , 3H), 2.42 (m, $CHSTr$, 1H); 2.77 (m, CH_2N , 2H); 3.23 (s, CH_3O , 3H); 3.50 (s, PEG, $\approx 520H$), 4.01 (t, $OCOCH_2$, 2H); 7.24 (t, *p*-H of $SC(C_6H_5)_3$ & NH, 4H); 7.33 (t, *m*-H of $SC(C_6H_5)_3$, 6H); 7.42 (d, *o*-H of $SC(C_6H_5)_3$, 6H) ppm.

mPEG5K-Oxycarbonylamino-2-propyl-S-sulfonylcarbomethoxy (mPEG-S-Scm), Method B. mPEG5K-oxycarbonylamino-2-propyl-S-triphenylmethane (8.87 g, 1.65 mmol) was dissolved in TFA/ CH_2Cl_2 (10/10 mL) at 4 °C and treated with methoxy carbonylsulfonyl chloride (185.5 μL , 1.99 mmol) while vigorously stirring the solution. The reaction was allowed to proceed at room temperature for 45 min and then evaporated to dryness. The residue was recrystallized with iso-propanol/*tert*-butanol (80/20 mL) twice and then lyophilized from *tert*-butanol (5 mL) and further dried in vacuo over P_2O_5 to yield a white fluffy solid (8.32 g, 97 %). 1H NMR was identical to that of the same derivative obtained in Method A (see above).

mPEG5K-Oxycarbonylamino-2-propyl-dithiobenzyl Alcohol (mPEG-DTB-OH). mPEG5K-oxycarbonyl-2-(aminopropyl)-S-sulfonylcarbomethoxy (8.32 g, 1.6 mmol) was reacted with *p*-mercaptobenzyl alcohol (592 mg, 4 mmol) in methanol/chloroform (25 mL, 90:10) at ambient temperature for 18 h. In

order to drive the reaction to completion, another portion of mercaptobenzyl alcohol (322 mg, 2.2 mmol) was added and allowed to react for 24 h. The reaction solution was evaporated to dryness. The solid residue was recrystallized from ethyl acetate/isopropanol (30/100 mL) three times and then dried in vacuo over P₂O₅, yielding a white powdery material (7.25 g, 94%). ¹H NMR δ: 1.17 (d, CH₃CHS₂, 3H), 3.02 (m, CH₂NH, 2H), 3.24 (s, CH₃O, 3H), 3.50 (s, PEG, ≈470H), 4.05 (br t, CH₂OCO, 2H), 4.48 (d, HOCH₂C₆H₅, 2H), 5.21 (t, HOCH₂C₆H₅, 1H), 7.30(d, C₆H₅, 2H), 7.46 (br t, mPEG-OCONH, 1H), 7.50 (d, C₆H₅, 2H) ppm.

mPEG5K-Oxycarbonylamino-propyl-dithiobenzyl Nitrophenyl Carbonate (mPEG-DTB-NPC). mPEG5K-oxycarbonylamino-2-propyl-dithiobenzyl alcohol (6.75 g, 1.27 mmol) was dissolved in CHCl₃ (30 mL) and treated with *p*-nitrophenyl chloroformate (513 mg, 2.54 mmol) and TEA (531 μL, 3.81 mmol) at 4 °C. After 30 min, the ice bath was removed, and the reaction mixture was stirred at room temperature overnight. The reaction solution was rotary evaporated, and the residue was taken up in warm ethyl acetate. TEA hydrochloride was filtered off, and then the solvent was evaporated. The solid residue was recrystallized with ethyl acetate/iso-propanol (30/100 mL) three times and dried in vacuo over P₂O₅, yielding 6.55 g (94%) of white solid. ¹H NMR δ: 1.18 (d, CH₃CHSSC₆H₅, 3H), 3.16 (m, CH₂N, 2H), 3.24 (s, CH₃O-PEG, 3H), 3.40 (s, PEG, 180H), 4.03 (br t, OCOCH₂, 2H), 5.28 (s, C₆H₅CH₂OCO, 2H), 7.45–7.60 (overlapping two types of C₆H₄ and NH, 7H), 8.31 (d, C₆H₄NO₂, 2H); ¹³C NMR (DMSO-*d*₆) δ: 17.5, 45.1, 45.8, 58.0, 63.2, 68.8, 69.7 (PEG), 71.2, 122.5, 125.3, 126.7, 129.3, 137.8, 145.1, 151.9, 155.2, 156.2 ppm.

Preparation and Purification of PEG-Lysozyme Conjugates. Lysozyme (at final concentration of 10 mg/mL) was allowed to react in borate buffer (0.1 M, pH 8.0) at 25 °C for 2–5 h with either mPEG-DTB-NPC or mPEG-NPC, using the feed molar ratio of 3.5 PEG/lysozyme (0.5 PEG/amino group). The conjugation reactions were quenched by the addition of 10-fold excess of glycine.

PEG-lysozyme conjugates were purified on a CM HEMA-IEC Bio 1000 semipreparative HPLC column (7.5 × 150 mm) purchased from Alltech Associates, Deerfield, IL. First, the conjugation reaction was injected into the HPLC column in 10 mM sodium acetate buffer at pH 6. The elution with this buffer was continued until all unreacted PEG was removed. Then, 0.2 M NaCl in 10 mM sodium acetate at pH 6 was applied for 15 min in order to elute the PEGylated-lysozyme. Finally, the native lysozyme was eluted by increasing the salt concentration to 0.5 M NaCl over 20 min. Fractions (1 mL) were collected and assayed for protein and PEG contents. Thus, aliquots (25 μL) of each fraction were reacted with BCA protein assay reagent (200 μL, Pierce Chemical Company, Rockford, IL) in microtiter plate wells at 37 °C for 30 min, and the absorbance was read at 562 nm. Similarly, for PEG determination (14), 25 μL aliquots were reacted with 0.1% polymethacrylic acid solution in 1 N HCl (200 μL), in microtiter plate wells, followed by absorbance reading at 400 nm. Fractions containing both protein and PEG were pooled. For the isolation of the PEG-lysozyme containing only one PEG moiety, the same cation-exchange chromatography protocol was repeated and the collected fractions were analyzed by the HPLC reversed-phase assay. Fractions containing the single peak of 1:1 PEG-lysozyme conjugate species were pooled.

SDS-PAGE. Polyacrylamide gel electrophoresis under denaturing conditions was performed for conjugate characterization. Precast 4–12% NuPAGE Bis-Tris gels, MES running buffer, molecular weight protein standards (Mark12), and a Colloidal Coomassie G-250 staining kit, were all obtained from Invitrogen, Carlsbad, CA. In a typical gel, 1 to 3 μg of protein

containing sample were loaded per well, then electrophoresed at a constant voltage of 200 mV, and stained for protein according to the manufacturer's instructions. For PEG detection, a duplicate gel was stained with iodine according to Kurfürst (45).

Reversed-Phase HPLC Analysis. RP-HPLC separation of PEG-lysozyme conjugates was achieved on a Vydac Protein C4 (4.6 × 150 mm) column eluted at 1 mL/min with an aqueous acetonitrile gradient as follows. Each chromatography run started with mobile phase A (30% acetonitrile, 5% 0.1 M sodium acetate at pH 5, and 65% H₂O) and over a period of 40 min ended with mobile phase B (50% acetonitrile, 50% 0.1 M sodium acetate at pH 5). A UV detector set to 280 nm was used to monitor the chromatography.

De-PEGylation Experiments. A mixture of purified mPEG-DTB-lysozyme conjugates (2 mg/mL protein), containing 1:1, 2:1, and 3:1 conjugate species, was incubated with 1 mM cysteine in 0.1 M sodium phosphate buffer at pH 7.3 containing 5 mM EDTA at 37 °C on a rocking mixer. Aliquots were taken out from the reaction vial at various time points and analyzed by RP-HPLC as described above. These results are shown in Figure 2.

De-PEGylation experiments of the cation-exchange-purified 1:1 PEG-DTB-lysozyme (0.5 mg/mL protein) treated at room temperature with 0.6 mM cysteine (or glutathione (not shown)) were monitored by a modified RP-HPLC method on the same Vydac Protein C4 column and flow rate but under the following elution protocol, which was also suitable for LC-MS. Each chromatography run was conducted with a gradient of 30% to 40% mobile phase B in 18 min. Mobile phase A was composed of 0.1% TFA in 5% acetonitrile and 95% H₂O, while mobile phase B was composed of 0.1% TFA in 95% acetonitrile and 5% H₂O. The fluorescent detector was set at 295 nm excitation and 360 nm emission. The results of these experiments are depicted in Figure 5.

Lysozyme Activity Assay. The enzymatic activity of lysozyme and its conjugates was assessed as previously described (46). *Micrococcus luteus* cells were suspended, at 0.3 mg/mL in 0.1 M sodium phosphate buffer at pH 7.3. Test samples were diluted to 0.5 mg/mL (lysozyme equivalent) in the phosphate buffer. Cells suspension and test sample were mixed at a v/v ratio of 1:14 in a 1 cm glass cuvette. Absorbance at 450 nm was immediately recorded for the initial time reading and then for the subsequent 30-s time intervals.

LC-MS. Purified PEG-lysozyme containing only one PEG moiety per lysozyme was treated with 1 mM cysteine, while the reaction was monitored by LC-MS. The HPLC system (HP 1100 LC-102) equipped with the same C4 column as that described above was connected to an electrospray mass spectrometer (Micromass QTOF II), permitting peaks identification by their molecular weights. The sodium acetate in the mobile phase was replaced with ammonium acetate in order to minimize salt interference with electrospray ionization.

Preparation of ¹²⁵I PEG-Lysozyme. Lysozyme (66 mg in 100 mg/mL in 0.1 M sodium phosphate buffer at pH 7.3) was mixed with 605 μCi of Na¹²⁵I (ICN Biomedicals, Irvine, CA), in an Iodo-Gen coated tube (Pierce Chemical Company, Rockford, IL), and allowed to react for 1 h at room temperature with mixing at 20 min intervals. The iodination reaction was stopped by removing the free ¹²⁵I on a Sephadex G-25F gel filtration column (17 mL) and collecting the ¹²⁵I-lysozyme, which was then reacted with either mPEG-DTB-NPC or mPEG-NPC, and purified by cation exchange chromatography as described above.

Pharmacokinetic Experiments. Male Sprague-Dawley rats (250–330 g each, 3 animals per formulation per experiment) were dosed either by intravenous (via a lateral tail vein) or by

subcutaneous (dorsally above the right rear leg) routes with ^{125}I -labeled lysozyme or its PEG conjugates (0.35 mL, 0.4 mg protein/mL, 4.6×10^6 cpm/mL). Blood samples (0.4 mL) were collected via the retro-orbital sinus. All injections and blood collections were performed while the animals were under inhaled anesthesia (isoflurane/ O_2). Samples were collected on heparin into polypropylene tubes and stored on ice for no longer than 1 h before being pipetted in triplicate (0.100 mL) into fresh polypropylene tubes. Blood samples were collected at the following times after dosing (though no single rat had blood collected at all of the following times): 30 s, 15 min, 30 min, and 1, 2, 3, 4, 6, 8, 24, 48, 72, 96, 120, and 168 h post-dose. Note that the last four time points were added for the longer subcutaneous experiments. The samples were then counted for ^{125}I in a Packard 5000 gamma counter. The results are summarized in Figure 7.

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