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In Vivo Comparison of Branched vs Linear Pegylation of a Capsule-Degrading Enzyme for Treatment of Anthrax

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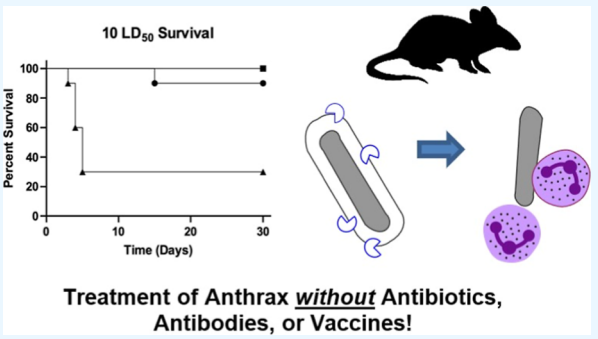
ABSTRACT: Polymers of D-glutamic acid (PDGA) form capsules of virulent strains of *Bacillus anthracis*. PDGA is antiphagocytic and weakly immunogenic; it enables the bacteria to evade innate immune responses. Capsule depolymerase (CapD) catalyzes the covalent anchoring of PDGA to the cell wall. There has been interest in developing CapD as a therapeutic to treat antibiotic-resistant anthrax infections. We previously showed that a His-tagged and circularly permuted (CP) CapD modified with a branched 3-prong polyethylene glycol (PEG) molecule termed PEG-CapD^{S334C}-CP could protect mice with 80% survival against a 5 LD_{50} challenge with virulent *B. anthracis*. In the current study, we compare the efficacy of tag-free CapD^{S334C}-CP modified by either the branched 3-prong or a linear 1-prong PEG. Pharmacokinetic studies in mice showed the branched 3-prong PEG-CapD^{S334C}-CP has a slightly higher, but not statistically different, total exposure in animals than the linear 1-prong PEG variant. The T_{max} of the 1-prong PEG-CapD^{S334C}-CP was 8 times shorter than the 3-prong. Mice infected with spore doses of 10 and 100 LD_{50}, treated 24 h after infection every 8 h for 7 days, were protected by both the 3-prong (10 LD_{50}: $p = 0.0051$; 100 LD_{50}: $p = 0.0463$, log-rank analysis) and 1-prong (10 LD_{50}: $p = 0.0009$; 100 LD_{50}: $p < 0.0001$, log-rank analysis) PEG-CapD^{S334C}-CP when compared with control animals. A second iteration similarly showed statistically significant protection by both variants at both challenge doses, and the combined data from both experiments showed no significant difference in efficacy between the 1- and 3-prong enzymes confirming that tag-free PEG-CapD^{S334C}-CP is protective against experimental anthrax infection.



INTRODUCTION

Therapies utilizing enzymes for the treatment of infections have a long history dating back to the pre-antibiotic era.[1] For example, enzymes such as lysins,[2] autolysins,[3] and lysozymes[4] have been used to treat bacterial infections, and others used to degrade bacterial biofilms,[5−7] slime layers,[8] or lipopolysaccharides.[9] Thus far, enzymes have been tested against *Staphylococcus aureus*,[10] *Klebsiella pneumoniae*,[11−13] *Acinetobacter baumannii*,[10,14] *Pseudomonas aeruginosa*,[15] and *Clostridioides difficile*[10] in animals. However, the use of enzymes in vivo has been limited by their instability, absorption, and excretion because they are subject to cleavage by proteases and clearance by the immune system.

Pegylation, which is conjugation with polyethylene glycol (PEG), is a strategy to improve the pharmacokinetic properties of enzymes by enhancing solubility and stability, increasing circulation times, and decreasing renal clearance and immunogenicity.[16] Examples of successful pegylated enzymes used therapeutically in humans include alpha-galactosidase A for the treatment of Fabry disease[17] and bovine adenosine deaminase for the treatment of severe combined immune deficiency.[18] However, pegylation strategies can compromise the bioactivity. Pegylation often depends upon the presence of a surface cysteine or lysine residue for direct functionalization, and these amino acids are sometimes required for substrate binding or catalysis.[19] In addition, the disordered structure of PEGs makes it difficult to predict which pegylation sites would have the least adverse effects on the enzyme activity. It has also been shown that branched PEG could extend the half-life of proteins more than linear PEG in vivo,[20] but the longer half-life also means prolonged exposure to serum proteases. In vivo activity may also decrease or be lost when conjugated to larger branched PEGs versus smaller linear PEGs.[20,21] Thus, the structure and size of PEGs, such as branched versus linear PEGs, may affect the pharmacokinetics and function of an enzyme.

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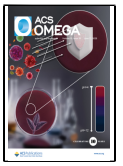

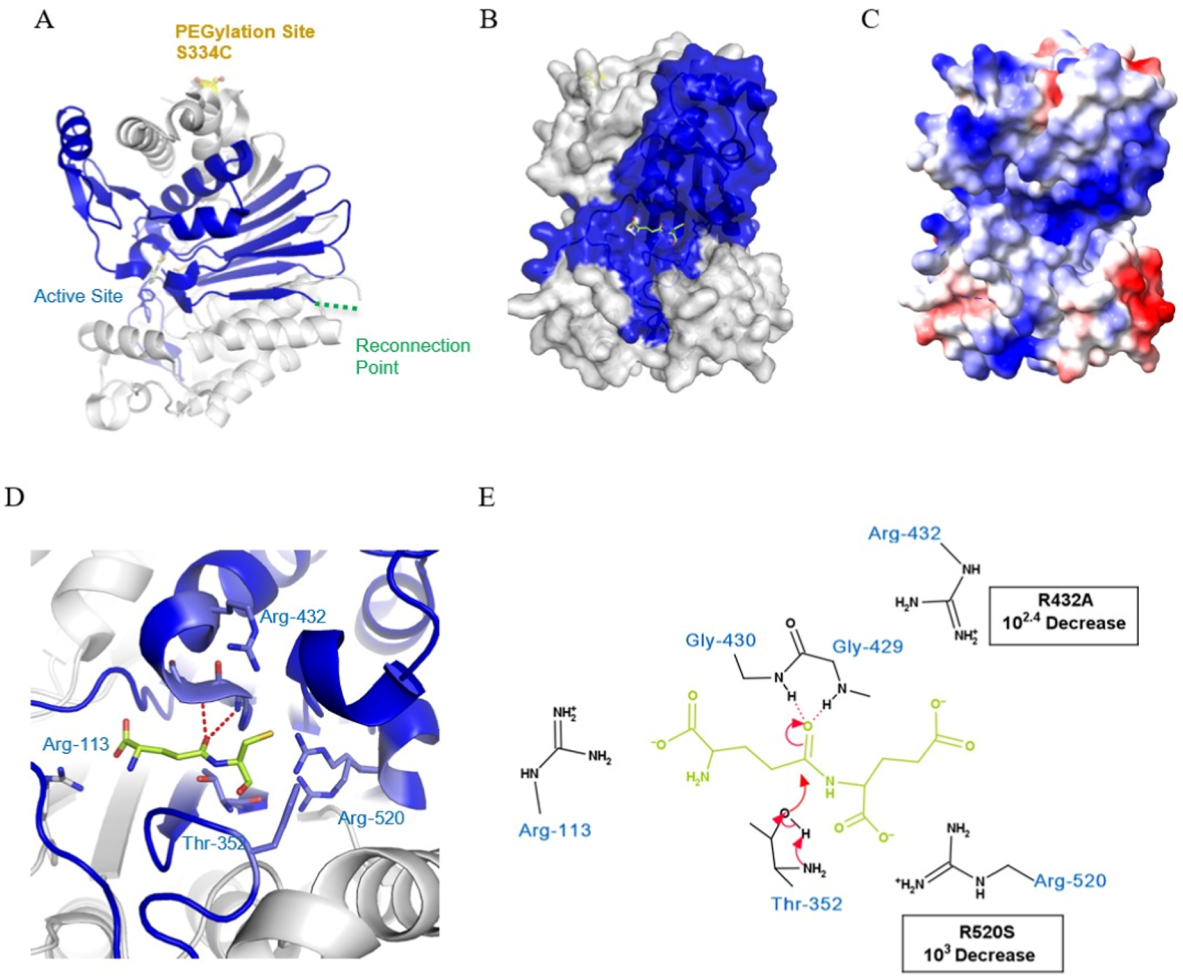

Figure 1. Proposed mechanism of CapD, its pegylation site, and modification present in the engineered circularly permuted PEG-CapD-CP. (A) Structure of CapD-N (PDB 3G9K),[33] showing the pegylation site (S334C, yellow) and where the two polypeptides (gray and blue) were reconnected (dashed line, green) on the rear face of the enzyme to form the circularly permuted CapD-CP. To circularly permute the N and C domains, M1-K46, N527, and K528 were deleted, and a linker (green) was added to produce CapD-CP.[34] The N-terminal signal peptide has also been removed (M1-C27). (B) Surface representation overlaid upon the *Helicobacter pylori* GGT T380A in complex with *S*-(nitrobenzyl)-glutathione bound, shown in lime (PDB 2QMC). After internal cleavage, CapD consists of two polypeptide chains (gray and blue). (C) Electrostatic map showing the positively charged residues in the substrate binding site (blue). (D) Active site of CapD. The nucleophilic Thr-352 is shown in blue. (E) First step of the proposed mechanism of the CapD (capsule depolymerase)-catalyzed reaction.[22,29] Arg-520 coordinates the alpha-carboxyl group of the leaving amino acid as Thr-352 acquires a positively charged amine in the transition state.[29] Figure 1A was adapted with permission from ref[35], 2022 American Chemical Society.

CapD (capsule depolymerase), a γ-glutamyltransferase encoded on the pXO2 plasmid of *Bacillus anthracis* is part of the operon that synthesizes and covalently attaches polymers of D-glutamic acid by γ-glutamyl linkages to the peptidoglycan to produce the capsule.[22−25] The presence of capsule renders *B. anthracis* antiphagocytic; unencapsulated strains lacking the operon or the pXO2 plasmid, such as the vaccine strain Sterne,[26] are quickly internalized by host cells.[24,27,28] CapD when applied externally to the bacterium acts as an anti-infective by degrading and removing the capsule on the surface,[22,29,30] rendering the bacterium susceptible to host phagocytic killing.[24,25,31,32]

We previously showed that circular permutation of CapD (CapD-CP, GenBank No. AEG74409.1), by moving the Thr-352 to the N-terminus of the polypeptide,[33] removed any need for internal cleavage (Figure 1).[22,29,34] The activity of the purified CapD-CP was ∼2-fold higher when compared to the native protein.[32,34] Replacement of the serine with a cysteine at position 334 enabled site-specific pegylation.[32] This His-tagged version of the recombinant enzyme, 3-prong PEG-CapD^{S334C}-CP, was found to be efficacious in mice infected subcutaneously with nontoxinogenic, encapsulated *B. anthracis* ΔAmes spores at 382 LD_{50} (100% survival) and toxinogenic, encapsulated *B. anthracis* Ames spores at 5 LD_{50} (80% survival).[32]

In our current work, we compared the in vitro activity and in vivo efficacy of non-His-tagged CapD^{S334C}-CP pegylated with either a 1-prong or a 3-prong PEG. We found that the pharmacokinetics were not significantly different between the 1-prong and 3-prong PEG-CapD^{S334C}-CP and that both protect against *B. anthracis* in a mouse model of anthrax.

RESULTS

The Enzyme Activity of 1-Prong and 3-Prong PEG-CapD^{S334C}-CP Is Comparable. The size of the PEG molecule could adversely affect the activity of an enzyme if the PEG molecule blocks access to the substrate or active site. The pegylation site, Ser-334, is located on the rear face of the

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Table 1. Kinetic Parameters and Physical Properties for CapD-CP and Variants[a]

mutant	V_{max} (U/mg)	K_m (μM)	k_{cat} (1/min)	no. of amino acids	M_W (kDa)	pI	refs
CapD-CP no PEG His-tagged S334C	0.036	3	2.0	503	55.3	7.22	32,35
CapD-CP 3-prong PEG2k His-tagged S334C	0.041	8	2.3	503	55.3	7.22	
CapD-CP 1-prong PEG tag-free S334C (XAI variant)	0.040	2.3	2.2	487	53.4	8.17	
CapD-CP 1-prong PEG tag-free S334C (XAI variant)	0.070	4	3.6	487	53.4	8.17	this study
CapD-CP 3-prong PEG tag-free S334C (XAI variant)	0.060	3	3	487	53.4	8.17	this study

[a]Steady state kinetic parameters for CapD-CP and its variants were measured in 25 mM HEPES pH 7.4, 0.1% Tween-20 at 22 ± 3 °C using a saturating concentration of L-serine (0.5 mg/mL) as the acceptor substrate. A unit is defined as μmol/min. For reference, our previously reported values[32,35] for other CapD-CP variants are shown.

enzyme and was mutated to Cys to enable pegylation with either the 1-prong or the 3-prong PEG-maleimide (Figure 1A). Pegylation with either variant resulted in steady state kinetic parameters that were similar (Table 1). Slightly higher (<2-fold) specific activity (U/mg) was obtained using the autoinduction (AI) medium than was previously reported.[35] The V_{max} of the 1-prong PEG-CapD^{S334C}-CP using the AI medium was 0.07 U/mg, whereas the V_{max} of that produced in Luria−Bertani (LB) broth was 0.04 U/mg.[32,35] This indicated that the enzymes from the AI medium were slightly purer. The new medium also increased yields by more than 10-fold and reduced the volume of medium needed to produce large quantities of pure enzyme for animal studies.

Improved Thermostability of the PEG-CapD^{S334C}-CP Variant. His-tagged proteins are used for research only, and therefore, the STENLYFQ sequence, which contained a tobacco etch virus (TEV) protease cleavage site, was removed from the original CapD^{S334C}-CP sequence. We constructed the XAI variant by replacing the TEV site with a Factor Xa sequence,[34] which allowed for the removal of the His tag. Higher melting temperature (T_m) indicates more thermostability and less thermal decomposition, which could lead to better therapeutic efficacy in vivo by prolonging the time the enzyme stays active and in circulation. Thus, we measured the T_m of the XAI variants with the His tag and after its cleavage with Factor Xa. The T_m of the original CapD^{S334C}-CP variant with the His tag and the STENLYFQ sequence was 48.9 ± 0.5 °C[32] at pH 7.4. The T_m of the new XAI variant with or without the His tag increased slightly, but they were similar (50.2 ± 0.1 °C vs 50.01 ± 0.05 °C). The 3-pronged pegylation with 2.3 kDa PEG led to a 7 degree increase in T_m for CapD^{S334C}-CP (original variant) (56.2 ± 0.1 °C),[32] whereas the 1-prong pegylation with 2 kDa PEG showed an 11 degree increase (61.0 ± 0.5 °C) for the CapD^{S334C}-CP (XAI variant).

We showed that the CapD^{S334C}-CP (XAI variant) readily loses its secondary structure at pH 4.0.[35] However, the linear 1-prong PEG-CapD^{S334C}-CP (XAI variant) had detectable secondary structure in pH 4 buffer and a T_m = 32.8 ± 0.2 °C. This suggests that the pegylated XAI variant may withstand exposure to low pH better than the nonpegylated variant. The XAI variant is the most robust of the tag-free PEG-CapD^{S334C}-CP variants tested.

The T_m values of the pooled enzyme preparations used for the animal studies herein were lower than what was shown in past preparations due to different sources of PBS used in which the enzymes were buffer exchanged and suspended in. In our previous work, we used 2× PBS pH 7.4 made with in-house deionized water,[35] whereas in this study, we used commercially purchased cell culture grade 1× PBS pH 7.2. In cell culture grade PBS, the T_m of the tag-free 1-prong PEG-CapD^{S334C}-CP was 53.2 ± 0.2 °C and the T_m of the tag-free 3-prong PEG-CapD^{S334C}-CP was 55.3 ± 0.2 °C (Figure 2).

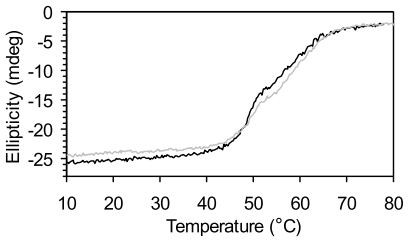

Figure 2. Melting temperature measurement by circular dichroism (CD). The loss of alpha helical structure was monitored at 222 nm in 1× PBS pH 7.2. In black is the 1-prong PEG-CapD^{S334C}-CP tag-free XAI variant and in gray is the 3-prong PEG-CapD^{S334C}-CP tag-free XAI variant. Melts were done in triplicate and averaged. Structures of the PEG-maleimide molecules used herein can be found in the Supporting Information.

The change in the PBS source led to lower amounts of endotoxin and decreased batch-to-batch variability. Also, the calculated pI for the tag free XAI variant is pI = 8.17, whereas the pI = 7.22 for the His-tagged original variant.

Pharmacokinetic Study of 1-Prong and 3-Prong PEG-CapD^{S334C}-CP. To examine the pharmacokinetic parameters of the 1-prong and 3-prong pegylated variants, a single-intraperitoneal dose was given to mice. At particular time intervals, the mice were euthanized, and sera were obtained (Figure 3, Table S1).

The 1-prong PEG-CapD^{S334C}-CP led to a peak concentration (C_{max} = 79.6 ± 43.0 μg/mL, T_{max} = 0.5 h) that was higher and earlier than that observed for the 3-prong PEG variant (C_{max} = 26.8 ± 5.4 μg/mL, T_{max} = 4 h) (Table S1). However, the difference in C_{max} was not statistically significant (p = 0.24). The values shown in Figure 3 are from measured enzymatic activity. The shorter T_{max} of the 1-prong PEG-CapD^{S334C}-CP was also apparent in Western blots using a polyclonal anti-CapD antibody (Figure S2). High levels of the 1-prong PEG-CapD^{S334C}-CP were present in the serum at t = 0.5 h when enzyme activity was high, indicating that active enzyme was circulating, as opposed to dead, degraded, or damaged enzyme. The loss of activity in sera over time could be due to degradation, immune clearance, or excretion. Lower molecular weight degradation products were observed in the 1-prong PEG-CapD^{S334C}-CP sera by the 2 h point; however, the full-length enzyme was still detectable by the 4 h time point, and enzyme activity was measurable (Figure S2, Figure 3). Despite the lower peak concentrations, the 3-prong PEG variant led to higher total plasma exposure (area under the curve $(AUC)_{0-last}$ = 330.6 ± 22.7 μg*h/mL) and a longer half-life ($t_{1/2}$ = 7.7 h) compared to the 1-prong PEG variant

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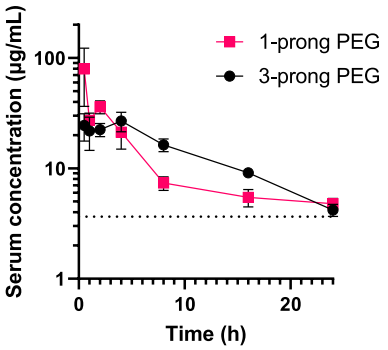

Figure 3. Pharmacokinetic study comparing the 1-prong vs 3-prong PEG-CapD^{S334C}-CP in mice following intraperitoneal injections. Groups of five BALB/c mice were used at each time point (0.5, 1, 2, 4, 8, 16, and 24 h) after injection with 40 mg/kg PEG-CapD^{S334C}-CP. Data points shown are geometric means (n = 5) with standard error. The activity of the enzyme was used to measure the concentration of the enzyme in serum at each point. Aliquots of sera (2 μL) were assayed in triplicate for each mouse at each time point. A standard curve (μg enzyme vs activity) was made using an equal amount of PBS control mouse serum and known amounts of purified enzyme. The dotted line corresponds to the limit of detection. The average AUC_{last} of the 1-prong (285.0 ± 31.4 μg*h/mL) and the 3-prong PEG-CapD^{S334C}CP (330.6 ± 22.7 μg*h/mL) were not statistically different (p = 0.26). The C_{max} (μg/mL) was higher for the 1-prong PEG-CapD^{S334C}-CP enzyme (79.6 ± 43 μg/mL) than the 3-prong (26.8 ± 5.4 μg/mL), but this difference was not statistically significant (p = 0.24). All calculated PK parameters can be found in Table S1.

(AUC_{0-last} = 285.0 ± 31.4 μg*h/mL, $t_{1/2}$ = 5.3 h), although the difference in AUCs was not statistically significant (p = 0.26) (Table S1). Of note, a trend toward higher average concentrations at longer time points (8 and 16 h) was observed in the 3- versus 1-prong PEG variants; 3-prong PEG-CapD^{S334C}-CP was also detectable in Western blots at these time points (Figures 3 and S2). As noted, neither the C_{max} nor AUC_{0-last} parameters were statistically different, which is consistent with the high standard deviations within the time points. However, the exposure extrapolated out from the elimination phase ($AUC_{0-\infty}$) was higher for the 3-prong PEG variant (377.9 versus 296.9 μg*h/mL) and that, along with a trend toward lower variability and higher exposures, is consistent with a target profile for a biological therapeutic.

Protection Afforded by 1- or 3-Prong PEG-CapD^{S334C}-CP after Challenge with *B. anthracis*. Mice were infected by the subcutaneous route with 10 or 100 LD_{50} *B. anthracis* spores. Twenty-four h after infection, 10 mice/group were treated by the intraperitoneal route every 8 h for 7 days with 1- or 3-prong PEG-CapD^{S334C}-CP or bovine serum albumin (BSA) as a control (each at 40 mg/kg). A second iteration of the experiment was also completed (Figure 4A,B).

In a similar experiment, mice (10/group) were challenged subcutaneously with 10 or 100 LD_{50} spores, but treated with ciprofloxacin, instead of CapD. Ciprofloxacin (30 mg/kg) was administered intraperitoneally every 12 h for 7 (Figure 4C) or 14 days (data not shown) beginning 24 h postinfection. Control mice were injected instead with 5% dextrose alone for 14 days beginning 24 h postinfection with *B. anthracis* (Figure 4C).

Mice in the ciprofloxacin-treated control group survived both the 10 and 100 LD_{50} challenges with *B. anthracis* spores (100% survival for each group with treatment for 7 days (Figure 4C) and for 14 days (data not shown)). Mice treated every 8 h with the 1-prong PEG-CapD^{S334C}-CP for 7 days achieved 90% survival in both challenges with 10 LD_{50}. Mice treated with the 3-prong PEG-CapD^{S334C}-CP every 8 h for 7 days yielded 70% and 100% survival in the two 10 LD_{50} challenge experiments. The protection relative to the BSA control was statistically significant for all treatment groups (Table 2) and when results of both experiments were combined (p < 0.0001, log-rank analysis for both 1- and 3-prong enzymes). However, the differences between the 1-prong and 3-prong PEG-CapD^{S334C}-CP enzymes were not significantly different for both iterations of the 10 LD_{50} challenge (p = 0.3228 for the first iteration and p = 0.3178 for the second iteration, log-rank analysis).

In the 100 LD_{50} challenge experiment, significant protection compared to BSA was observed in both iterations for 1- and 3-prong PEG-CapD^{S334C}-CP (Table 2). In the first iteration of the 100 LD_{50} challenge experiment, survival was significantly greater for the 1-prong PEG-CapD^{S334C}-CP than for the 3-prong PEG-CapD^{S334C}-CP (p = 0.0141, log-rank analysis). The 3-prong PEG-CapD^{S334C}-CP had a slightly lower level of enzyme activity than the 1-prong, but the difference was not significant (Table 1). In the second iteration, significant survival was observed with the 1-prong PEG-CapD^{S334C}-CP (p = 0.0015 vs BSA, log-rank analysis) and with the 3-prong PEG-CapD^{S334C}-CP (p = 0.0002 vs BSA, log-rank analysis). This difference in survival between the 1- and 3-prong PEG-CapD^{S334C}-CP enzymes was not statistically significant (p = 0.1914, log-rank analysis). When the results of the two iterations of the 100 LD_{50} challenges were combined, there was no statistically significant difference between the 1- and 3-prong PEG-CapD^{S334C}-CP enzymes (p = 0.3541, log-rank analysis). Notably, the efficacy of the 1-prong and 3-prong PEG-CapD^{S334C}-CP enzymes was not significantly different from ciprofloxacin treatment in the 10 LD_{50} challenge experiment (1-prong vs ciprofloxacin, p = 0.3173, and 3-prong vs ciprofloxacin, p = 0.0675, log-rank analysis, respectively). For the 100 LD_{50} challenge experiment, ciprofloxacin was significantly more efficacious than the 3-prong PEG-CapD^{S334C}-CP (p < 0.0001, log-rank analysis) but not when compared to the 1-prong PEG-CapD^{S334C}-CP (p = 0.0671, log-rank analysis).

DISCUSSION

Activity and Stability of 1-Prong and 3-Prong PEG-CapD^{S334C}-CP. Despite differences in the PEG groups, the measured enzyme activities of the 1-prong and 3-prong PEG-CapD^{S334C}-CP enzymes were comparable. Kinetic parameters, endotoxin levels, and T_m were used as metrics to assess the quality of the protein preparations. Improvements in removing endotoxin and in the expression of the enzyme in *E. coli* using AI medium likely accounted for the higher specific activity of the enzyme observed in this study. However, the activities of the preparations, while slightly higher, were not significantly different from those made earlier in our previous study.[35] A higher measured melting temperature was obtained in 2× PBS at pH 7.4 than in 1× PBS at pH 7.2 for the pegylated tag-free XAI variants. However, in both cases, the T_ms were well above body temperature so they should not denature at physiological temperature.

Effect of the PEG on C_{max} and T_{max}. Whereas, the 1-prong PEG-CapD^{S334C}-CP variant achieved a rapid peak concentration in the serum compared to the 3-prong pegylated

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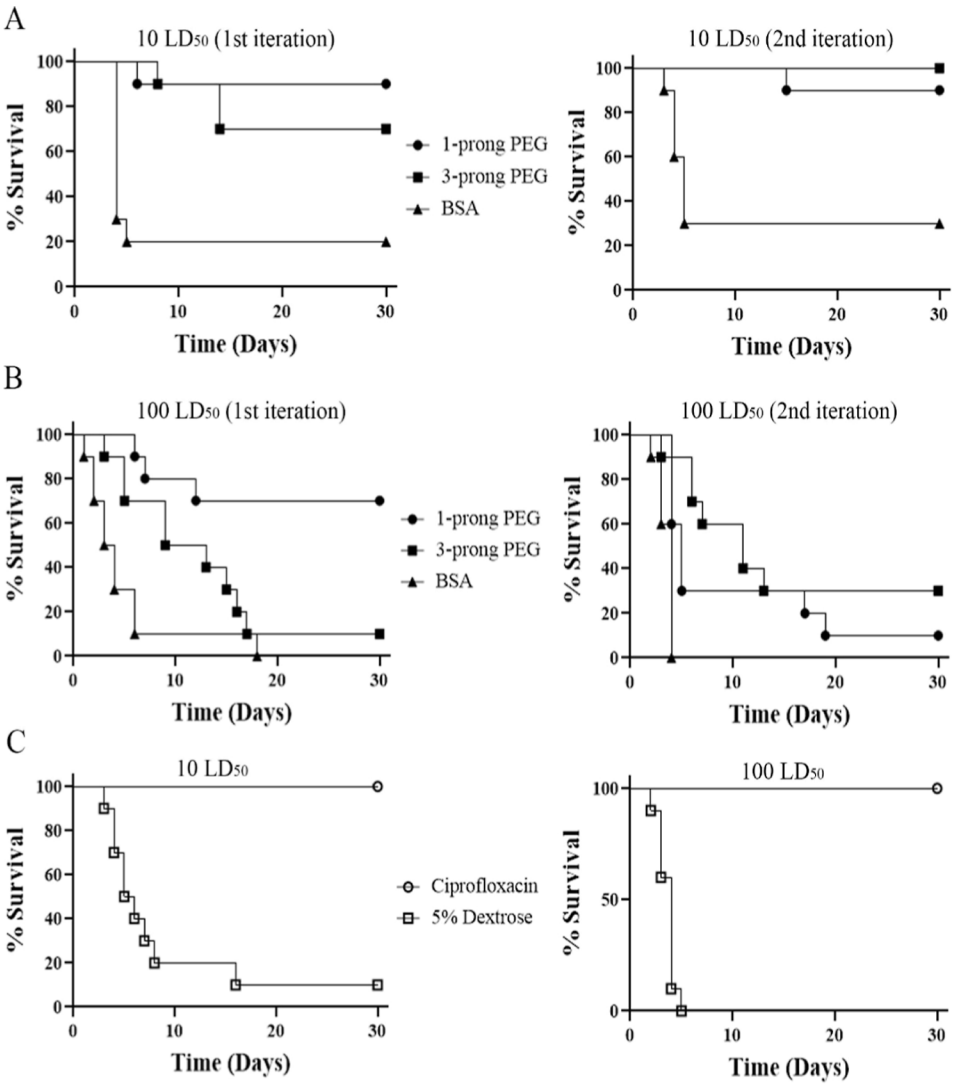

Figure 4. 1-prong and 3-prong PEG-CapD^{S334C}-CP protected mice challenged with *B. anthracis* spores. Ten 12 week old female BALB/c mice per group were challenged with 10 LD_{50} (A) or 100 LD_{50} (B) of *B. anthracis* spores in two iterations (A,B) and then treated starting at 24 h postinfection with either 1-prong or 3-prong PEG-CapD^{S334C}-CP (40 mg/kg) or BSA (40 mg/kg). The treatment schedule for PEG-CapD^{S334C}-CP and BSA is every 8 h for 7 days. Mice were followed for 30 days and scored for survival. (C) Mice (n = 10) challenged with 10 LD_{50} or 100 LD_{50} spores and then treated 24 h postinfection with ciprofloxacin (30 mg/kg) or 5% dextrose every 12 h for 7 or 14 days, respectively.

Table 2. Statistical Analyses of Efficacy for Each Experiment (n = 10) and for the 2 Iterations Combined at 10 and 100 LD_{50} Challenge Experiments (n = 20 mice)[a]

	1st iteration (n = 10)		2nd iteration (n = 10)		combined (n = 20)	
	10 LD_{50}	100 LD_{50}	10 LD_{50}	100 LD_{50}	10 LD_{50}	100 LD_{50}
1-prong PEG vs BSA	0.0009	<0.0001	0.0043	0.0015	<0.0001	<0.0001
3-prong PEG vs BSA	0.0051	0.0463	0.0014	0.0002	<0.0001	<0.0001
1-prong PEG vs 3-prong PEG	0.3228	0.0141	0.3178	0.1914	0.6432	0.3541
1-prong PEG vs ciprofloxacin	0.3173	0.0671				
3-prong PEG vs ciprofloxacin	0.0675	<0.0001				

[a] p value, log-rank analysis of Kaplan−Meier survival curves.

variant ($C_{max}^{1-prong} > C_{max}^{3-prong}$), a lower total exposure and elimination half-life was observed compared with the 3-prong PEG-CapD^{S334C}-CP (Table S1). Differences in variant stability in vivo might be factors affecting this difference. Supporting this hypothesis, increased degradation products were observed in the sera of 1-prong PEG-CapD^{S334C}-CP- but not in the 3-prong PEG-CapD^{S334C}-CP-treated animals at <2 h (Figure S1). The higher AUC and longer half-life observed with the 3-prong PEG variant are consistent with the predicted therapeutic target profile of an antibacterial therapeutic enzyme of this class where higher total exposure and higher trough concentration are likely to correlate to improved efficacy.

The effects of linear vs branched pegylation are not very well documented, although Yoshioka et al. showed that the

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molecular shape and weight of PEG strongly influence the in vivo antitumor activity of mTNFα.[21] Li et al. have also noted longer half-lives for branched versus linear pegylated rhG-CSF.[20] Here, the T_m of the 3-prong PEG-CapD^{S334C}-CP was slightly higher and there was less evidence of its degradation in sera, but the effect of its greater in vivo stability was minor when compared to the 1-prong pegylated variant. The 1-prong PEG-CapD^{S334C}-CP achieved rapid high-level bursts of enzyme activity in the serum immediately after injection. This was evident in both activity assays of the sera and in Western blots (Figures 3 and S2).

In an attempt to determine if a lower dose of PEG-CapD^{S334C}-CP would be efficacious, a dose of 4 mg/kg was also tested (Figure S3). With the 4 mg/kg dose, the 1-prong PEG-CapD^{S334C}-CP only achieved 20% survival ($p = 0.3168$ vs BSA, log-rank analysis), whereas the 3-prong PEG-CapD^{S334C}-CP gave 50% protection in the 10 LD_{50} challenge ($p = 0.0653$ vs BSA, log-rank analysis). Similarly, in the 100 LD_{50} challenge, protection was not observed with the low dose of either the 1-prong or 3-prong pegylated variants ($p = 0.9973$ and $p = 0.1075$, respectively, vs BSA, log-rank analysis). Ultimately, a dose of 40 mg/kg gave significant protection against higher challenge doses than were previously tested.[32]

Pegylation has long been used to reduce the immunogenicity of therapeutic foreign proteins by masking antigenic determinants and reducing proteolysis, resulting in inhibition of antigenic presentation to the immune system. Nevertheless, the development of IgG to pegylated CapD could occur and would result in its increased elimination and reduced efficacy.[36−38]

Ciprofloxacin is an effective antibiotic against *B. anthracis*; however, a ciprofloxacin-resistant *B. anthracis* strain has been described in the literature.[39] Indeed, the ongoing concern for infection with naturally occurring or genetically engineered antibiotic-resistant anthrax strains is recognized and reflected in the latest treatment guidelines from the CDC.[40] Our results showing the effectiveness of both 1- and 3-prong PEG-CapD^{S334C}-CP enzymes in treating experimental anthrax suggest this therapy may prove useful in infections due to multidrug-resistant strains for which no effective treatment is available. Moreover, these positive results in mice support performing further studies in the nonhuman primate, the best model for the human disease.

CONCLUSION

Here, we examined the effects of pegylating CapD^{S334C}-CP with either a linear or a branched ∼2 kDa PEG molecule. The two pegylated enzymes were tested for efficacy in 10 and 100 LD_{50} challenges with virulent *B. anthracis* spores. A dosing regimen of 40 mg/kg every 8 h for 7 days was used. Lower doses (4 mg/kg) were less effective. The 10 LD_{50} challenge experiment was performed twice. With the 1-prong PEG-CapD^{S334C}-CP, 90% survival was achieved in both experiments. With the 3-prong PEG-CapD^{S334C}-CP, 70% and 100% survival was achieved. Both treatments showed significant protection relative to BSA controls and were not significantly different from each other in performance. In the 100 LD_{50} challenge experiments, the 1-prong PEG-CapD^{S334C}-CP led to 70% and 10% survival and the 3-prong PEG-CapD^{S334C}-CP led to 10% and 30% survival. In all cases, protection was significant relative to BSA controls. Thus, both the 1-prong and 3-prong PEG-CapD^{S334C}-CP provided protection in 10 and 100 LD_{50} challenges and were not significantly different.

MATERIALS AND METHODS

Materials. The CapD substrate containing EDANS/DABCYL and 5 residues of D-Glu linked by gamma-glutamyl linkages was used for continuous assays and was purchased from Biopeptide Co., Inc. (San Diego, CA). Phosphate buffered saline (PBS, #P5368), IPTG (#420291), MW Standards (#MW-GF-200), Amicon ultrafiltration units (#UFC901096), and BugBuster were purchased from Millipore Sigma (St. Louis, MO). PD-10 columns, G200 Superdex 10/300 column GL 17-5175-01, Chelating Sepharose, Benzamidine Sepharose, and Q-Sepharose were purchased from Cytiva Inc. (Marlborough, MA). Monofunctional 1-prong PEG-maleimide (MF001022-2K) was purchased from Biochempeg Scientific Inc. (Watertown, MA). The 3-prong PEG-maleimide (#QBD-10406) was purchased from Quanta Biodesign Ltd. (Plain City, OH). Factor Xa was purchased from New England Biolabs (Ipswich, MA). BL21 (DE3) pLysS One Shot *E. coli* were from Novagen, Inc. Gel extraction and plasmid purification kits were purchased from Qiagen (Germantown, MD). Mustang E filters (catalog no. MSTG25 × 10^3) 0.2 μm were from Pall Inc. (Port Washington, NY).

Purification of CapD^{S334C}-CP and Its Variants. Plasmids encoding the His-tagged CapD-CP were transformed into BL-21 (DE3) pLysS *E. coli*. Six liters at a time of Luria−Bertani broth with kanamycin were grown at 37 °C to an OD_{600} ∼1.0, induced with 0.3 mM IPTG, and grown overnight at 17 °C. AI medium[41,42] produced significantly higher yields and only 4.5 L of media were used per prep. Bacteria were lysed in 100 mL of buffer A (50 mM Tris pH 7.3, 500 mM NaCl, 2 mM β-mercaptoethanol) with 25% BugBuster, ∼30 mg of lysozyme, and 25 U DNase. Bacteria were stirred at ambient temperature for 1 h and sonicated 4−6 times in 60 s intervals while submerged in ice water. The lysate was clarified by centrifugation at 20,500*g* for 30 min at 4 °C. A second extraction of the pellet with 100 mL of buffer A was performed to increase the yields. Both supernatants were combined and loaded onto a nickel-charged Chelating Sepharose column equilibrated with buffer A. The column was washed with buffer A containing 60 mM imidazole to remove contaminants, and the protein was eluted with the same buffer containing 300 mM imidazole. Protein was concentrated with ultrafiltration units and filtered with Mustang E filters to remove the endotoxin. Factor Xa was added (225 μg) to the combined fractions to remove the His tag, and the protein was dialyzed against 50 mM Tris pH 7.2, 300 mM NaCl, and 2 mM BME at room temperature (23 ± 3 °C) for ∼4 h and then at 4 °C for 2 days. Protein and flow through were subsequently loaded onto (1) two 0.25 mL benzamidine columns, (2) a nickel column, (3) a Q-Sepharose column, and (4) an SP-Sepharose column, sequentially. Q and SP-Sepharose columns were equilibrated with 50 mM Tris at pH 7.2 and 2 mM BME. Protein eluted in the flow through of the Q-Sepharose column and was loaded onto the SP-Sepharose column. Protein elution from SP-Sepharose was performed using the same buffer containing 300 mM NaCl. The protein was filtered at least twice with Mustang E filters prior to pegylation. The protein was pegylated prior to storing in 1× PBS at pH 7.4 with 50% glycerol at −20 °C.

Pegylation. Prior to pegylation with either the 1-prong or 3-prong PEG, DTT was added to the CapD-CP variants (25 mM final concentration). The DTT was then removed using a PD-10 column equilibrated with 2× PBS pH 7.4. A 20- to 40-

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fold molar excess of each of the 1-prong or 3-polyethylene glycol (PEG)-maleimide molecules was added to 1−10 mL volumes of concentrated protein. Reactions were rocked at room temperature (23 ± 5 °C) for 2 h and then at 4 °C for 1−2 days. The unreacted PEG-maleimide was removed by a PD-10 column equilibrated with 2× PBS at pH 7.4. Pegylated protein was stored in 1× PBS pH 7.4 and 50% glycerol at −20 °C.

Endotoxin Removal and Quantification. The enzyme was buffer exchanged into 1× PBS pH 7.2 using ultrafiltration units. Mustang E filters were prewetted with water and then 1× PBS pH 7.2 in a cell culture hood. The enzyme was then passed over 0.2 μm Mustang E filters 2 to 5 times to remove endotoxin. Removal of endotoxin before and after pegylation was more effective than using multiple filters after pegylation. Buffer exchange using ultrafiltration units and cell-culture grade 1× PBS pH 7.2 reduced endotoxin to 0.1 to ≤2 EU/mg. Prior to injections into animals, all CapD preparations were tested for endotoxin levels using the Pierce Chromogenic Endotoxin Quant Kit (Thermo Scientific, Rockford, IL) according to the manufacturer's protocol. All CapD preparations used in animals had <1 EU/mL endotoxin.

Enzyme Assays. CapD-CP was assayed before and after pegylation using the 5-mer D-Glu)$_5$ FRET-substrate containing EDANS and DABCYL.[35] Measurements were taken using a Molecular Devices SpectraMax M5 Microplate Reader. Cleavage was monitored for 5 min using an excitation wavelength of 340 nm and an emission wavelength of 490 nm. An unquenched control peptide was used to make a standard curve. Inner filter effect corrections were applied to all data.[43] L-Serine was used as an acceptor substrate.[22] The enzyme was assayed in 25 mM HEPES pH 7.4, 0.1% Tween-20, and 0.5 mg/mL L-serine at room temperature.

Circular Dichroism. Thermal denaturation was monitored at 222 nm (2 °C/min) between 10 and 95 °C using a Jasco 810 CD spectropolarimeter fitted with a Peltier temperature controller. A 1 mm path length cuvette was used for the CD experiments. The T_m were determined from 4-parameter fits to the data. Protein was buffer exchanged into 1× PBS pH 7.2, diluted to 3.0−3.5 μM, and then scanned.

Animal Pharmacokinetic Studies. Female BALB/c mice (12−13 weeks, ∼20 g) were purchased from Charles River Laboratories, Raleigh, NC. Animals were given a single 1 mL intraperitoneal injection with either the 1-prong or 3-prong PEG-CapD^{S334C}-CP in PBS. Cohorts were given 1 mL of PBS alone as controls. The amount of enzyme was 0.7 mg per mouse (40 mg/kg) of PEG-CapD^{S334C}-CP (XAI variant) with 1-prong PEG or 3-prong PEG. Animals were euthanized (n = 5/time point/condition) at 0.5, 1, 2, 4, 8, 16, and 24 h postinjection and blood collected into BD Microtainer SST tubes (Becton Dickinson, Franklin Lakes, NJ). Blood was subsequently spun at 6000 rpm for 5 min, and sera were collected. Sera were stored at −80 °C until assayed.

For pharmacokinetics analysis, 2 μL of the sera were assayed at room temperature for each mouse in 100 μL reaction volumes containing 25 mM HEPES pH 7.4, 0.1% Tween-20, 20 μM FRET substrate, and 0.5 mg/mL L-serine. For each sample, averages from two technical replicates were calculated and used to calculate the relevant pharmacokinetic parameters. A standard curve was used to correlate the protein concentration and measured activity in 2 μL of mouse serum (from the PBS-treated mice). For the standard curve, the activity was measured using 0.1, 0.2, 0.3, 0.4, and 0.5 μg of enzyme in the presence of 2 μL of control serum. The activity linearly increased with a concentration within this range. The limit of detection was defined by the average assay response to control serum from mice injected with PBS. Pharmacokinetic parameters were calculated based on total enzyme (μg/mL) in sera determined using the enzyme assays.

Pharmacokinetic parameters for the enzymes were calculated using noncompartmental analysis with sparse sampling in Phoenix WinNonlin 8.3 (Certara USA, Inc., Princeton, NJ). Selection of time points used for the terminal elimination calculation was 2−16 h and 4−24 h for the 1-prong and 3-prong enzymes, respectively. t tests (GraphPad Prism 9.4.0, Boston, MA) were used for statistical comparison to compare the C_{max} and AUC_{0-last} between the 1-prong and 3-prong pegylated variants.

To examine the amount of protein in sera relative to the measured enzyme activity in sera and to analyze the time-dependent appearance of degradation products in sera, we used Western blots and a polyclonal antibody against CapD. Four standards were loaded for each blot. To detect the pegylated and nonpegylated proteins, a polyclonal rabbit anti-CapD-CP antibody at 0.4 mg/mL (GenScript Biotech Corp, Piscataway, NJ) was used after being diluted 1:4000 in 1× PBST (1× PBS pH 7.4 with 0.1% Tween-20). Proteins were separated by SDS-PAGE and transferred to nitrocellulose (80 V, 30 min), blocked with 1× PBST containing 5% milk, incubated overnight with the same buffer containing the antibody, then washed 3× with 1× PBST, and exposed to the HRP goat antirabbit secondary antibody (#A16110, Invitrogen Inc., Carlsbad, CA) diluted 1:1000 in 1× PBST containing 5% milk. After 5 thorough washes with 1× PBST, the blot was developed and imaged using chemiluminescent detection.

Animal Efficacy Studies. Ten mice per group were challenged with a mean dose of 10 and 100 LD_{50} (10 LD_{50}: first iteration—1.41×10^2 CFU/mouse, second iteration—1.55×10^2 CFU/mouse; 100 LD_{50}: first iteration—1.41×10^3 CFU/mouse, second iteration—1.55×10^3 CFU/mouse) of *B. anthracis* spores in a 200 μL volume through a subcutaneous route of infection. The target dose for 10 LD_{50} was 1.4×10^2 spores/mouse, whereas that for 100 LD_{50} was 1.4×10^3 spores/mouse. Twenty-four hours postinfection, the mice were treated intraperitoneally with the different variants of PEG-CapD^{S334C}-CP or BSA (Sigma-Aldrich, St. Louis, MO) in 1× PBS pH 7.2 every 8 h for 7 days. The amounts of protein used were either 0.07 (4 mg/kg) or 0.7 mg (40 mg/kg). To compare the efficacy of CapD to an antibiotic, ciprofloxacin (30 mg/kg) in 5% dextrose or 5% dextrose alone[44] was used to treat challenged mice 24 h postinfection and every 12 h for 7 days (ciprofloxacin) or 14 days (5% dextrose alone). Animals were followed for 30 days postchallenge. Efficacy studies using CapD were performed twice for the 40 mg/kg dose, once for the 4 mg/kg dose, and once for the ciprofloxacin study in conjunction with iteration #1 of the 40 mg/kg efficacy studies. The differences in survival between treatment groups were determined by log-rank analysis of Kaplan−Meier survival curves (SAS Ver 9.4, SAS Institute, Cary, NC).

The animal work was carried out in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011, and The United States Army Medical Research Institute of Infectious Diseases Institutional Animal Care and Use Committee. The protocol was approved by the United States Army of Medical Research Institute of Infectious Diseases

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under an Institutional Animal Care and Use Committee in compliance with the Animal Welfare Act, PHS Policy, and other Federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

Spore Preparation. *B. anthracis* Ames was from the U.S. Army Medical Research Institute of Infectious Diseases collection. Spores were grown on nutrient broth with 8% yeast extract (BD Difco, Becton Dickinson, Franklin Lakes, NJ) for 8 days at ambient temperature, purified through Omnipaque (GE Healthcare, Chicago, IL), and stored in Water for Injection (WFI, Gibco BRL). For animal challenges, spores were heat shocked at 65 °C for 45 min and diluted in WFI. Spores were plated before and after injection on Sheep Blood Agar plates (Remel Products, Thermo Fisher Scientific, Lenexa, KS) to determine colony forming units (CFUs).

■ ASSOCIATED CONTENT

Data Availability Statement

A. Approved for public release: distribution unlimited

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.5c02119.

Immunoblots, results of challenge experiments using a low dose (4 mg/kg) of PEG-CapD^{S334C}-CP, sequence of the protein construct tested, structures of the PEG-maleimides used in this study, and pharmacokinetic parameters (PDF)

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#J.C. and P.M.L. contributed equally. A.M.F., J.C., and P.M.L. designed and directed the studies. J.C., D.J.C., and P.M.L. performed the experiments. J.C., J.M.M., P.M.L., and A.M.F. were involved in analysis and interpretation of data. P.M.L., A.M.F., J.C., and J.M.M. wrote the manuscript. All authors contributed to the revision and final approval of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AI, Auto Induction; AUC, area under the curve; BME, beta mercaptoethanol; CapD, *B. anthracis* capsule depolymerase; CapD-CP, circularly permuted CapD; CD, circular dichroism; C_{max}, maximum concentration; CP, circularly permuted; HRP, horseradish peroxidase; IPTG, isopropyl β-D-1-thiogalactopyranoside; K_m, Michaelis constant; LD_{50}, median lethal dose; OD_{600}, optical density at 600 nm; PBS, phosphate buffered saline; PBST, PBS containing 0.1% Tween-20; PDGA, poly-D-glutamic acid; PEG, polyethylene glycol; TEV, tobacco etch virus; T_m, melting temperature; T_{max}, time to peak drug concentration; V_{max}, the maximum rate of reaction; U/mg, μmol per min per mg of enzyme

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