Protein Modifications

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Mix to Validate: A Facile, Reversible PEGylation for Fast Screening of Potential Therapeutic Proteins In Vivo**

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A number of novel proteins with high therapeutic potential are being discovered every year. Once a potent protein is verified, the next step towards clinical studies includes validation in an appropriate animal model. Unfortunately, most candidate protein drugs are inadequate for direct testing in a high-throughput fashion in vivo because of inherently short biological half-lives from non-specific proteolysis and renal clearance.[1] It is well known that proteins with short half-lives do not exhibit similar potency in vivo as they do in vitro.[1b] For decades, PEGylation, the chemical attachment of poly(ethylene glycol) (PEG), has been considered the gold standard for enhancing stability, half-life, and aqueous solubility of protein drugs.[2] In particular, site-specific PEGylation improves protein stability in vivo while minimizing the loss of activity associated with conventional random PEGylation.[3]

Herein, we introduce a facile technique that allows for efficacy testing of protein drugs in animal models by extending the half-life in the blood of any selected protein candidate without compromising bioactivity. This technique offers the benefits of site-specific PEGylation without time-consuming and costly chemical modification and purification processes, enabling high-throughput testing of protein drugs in vivo. The general concept used is to PEGylate proteins through a complementary interaction between an oligohistidine tag (His-tag) and a Ni^{2+} complex of nitrilotriacetic acid (NTA), which is now widely used in protein research.[4] For example, protein immobilization techniques[5] and protein labeling with fluorophores[6] use the properties of a His-tag binding to NTA.

In spite of numerous applications based on the His-tag/NTA pair, including a couple for therapeutic protein research,[7] no studies have successfully used this specific and strong interactive pair to improve the potency of therapeutic proteins in vivo after systemic administration. We hypothesized that PEG analogues with an NTA moiety could be selectively labeled at specific sites of His-tagged proteins by simple mixing and exhibit the benefits of site-specific PEGylated proteins. Then, in vivo efficacy of any protein candidate can be tested in a facile fashion. Herein, with a rationally designed Ni-NTA-PEG analogue and a biologically relevant His-tagged protein, we show a practical technique for use in vivo.

The tumor necrosis factor-related apoptosis inducing ligand (TRAIL) was chosen as a model protein drug. TRAIL selectively induces apoptosis in a variety of cancer cells by delivering apoptotic signals through binding to cancer cell death receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5), while showing negligible toxicity to non-malignant cells.[8] These unique features make TRAIL one of the most promising and versatile anticancer protein drugs. However, it suffers from inherent instability, requiring stabilizers at TRAIL concentrations greater than 200 $\mu g\,mL^{-1}$, and short half-lives of approximately 3, 5, and 30 minutes in mouse, rat, and human, respectively.[9] Our previously reported covalently N-terminal PEGylated TRAIL analogues demonstrate superior pharmacokinetic (PK) and pharmacodynamic (PD)

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profiles.[10] Such analogues involve considerable time in synthesis and optimization to validate bioactivity in vivo. By applying the technique reported herein, novel protein candidates such as TRAIL can be immediately screened and/or validated in vivo for their therapeutic efficacy. Selected proteins could be further optimized using established formulation techniques, like PEGylation, for translation into the clinic.

The general design of the reactive Ni-NTA-PEG analogues, **1** and **4**, and its binding to a His-tag, hexahistidine (H_6), and fused protein are illustrated in Figure 1 A. Binding affinities, that is the dissociation constant (K_D) between the fluorophore-labeled mono-NTA or multivalent NTA analogues and H_6, have been reported.[6b] The K_D of a monoNTA with H_6 remains weak (10–18 μM), however bisNTA exhibits a significantly lower K_D (0.27 μM) towards the H_6 moiety. TrisNTA lowered the K_D tenfold compared to bisNTA, but tetraNTA showed no further improvement.[6b,11] To avoid complicated synthetic processes and potential toxicity that may be induced by multiple Ni^{2+} ions, we designed PEG analogues having monoNTA **1** and bisNTA **4** and investigated their applications in vitro and in vivo, compared with PEG without the NTA functionality.

Compound **1** was synthesized by reacting methoxy PEG *N*-hydroxysuccinimide ester (PEG-NHS) and NTA-Lys, N_α,N_α-bis(carboxymethyl)-L-lysine, followed by chelation with Ni^{2+} ions in a $NiCl_2$ solution. Unlike other reported multivalent NTA analogues, **4** was synthesized using a combination of commercially available compounds including NTA-Lys, Traut's reagent (2-iminothiolane), bis(maleimide) amine, and PEG-NHS, without the need for harsh protection/deprotection schemes. Briefly, a sulfhydryl group was first introduced to the NTA-Lys by incubating NTA-Lys with the amine-reactive Traut's reagent. Thiolated NTA-Lys, NTA-Lys-SH **2**, was then reacted with a bis(maleimide) amine resulting in a bisNTA analogue **3**. Compound **3** was finally conjugated with PEG-NHS and chelated with Ni^{2+} ions to produce **4**. A methoxy PEG with a molecular weight of 5 kDa was used as the backbone. Details of the synthesis and characterization of **1** and **4** are described in the Supporting Information.

The formation of interaction complexes between TRAIL and the Ni-NTA-PEGs were confirmed by size exclusion chromatography (SEC). An active TRAIL including an N-terminal H_6 and trimer-forming zipper sequence, H_6-ILZ-hTRAIL (114–281; MW = 22 kDa), was purified and used as previously reported.[10a] As shown in Figure 2 A, TRAIL mixed with PEG without the NTA moiety (TRAIL/PEG) did not form complexes. However, the addition of **1** or **4**, producing TRAIL/**1** and TRAIL/**4**, showed an increased hydrodynamic radii owing to the interaction of the H_6 tag of TRAIL with the NTA moiety. TRAIL/**4** showed a similar SEC profile compared to that of the covalently bound N-terminal PEGylated TRAIL-PEG_{5K} at a feed molar ratio of TRAIL/**4** above 1:5. In contrast, TRAIL/**1** failed to reach complete complexation at any ratio. To explore the substantial difference in complexation profiles between TRAIL/**1** and TRAIL/**4**, the binding constants of **1** and **4** with TRAIL were studied using BIAcore. The K_D value of **1** with TRAIL was 41.6 μM and **4** was 17.7 μM (Supporting Information, Figure S3). These results demonstrate that the incomplete complexation of TRAIL/**1** is probably because of the higher K_D value and the induced steric hindrance of **1** in the buffer. After fixing the ratio at 1:5, the stability of each formulation was investigated in 20 mM PBS, pH 7.4, at 37 °C, without any stabilizing agents such as Tween 20, concentrated glycerol, or sucrose.[1b] Native TRAIL and TRAIL/PEG at a concentration of 400 $\mu g\,mL^{-1}$ (based on the protein concentration) showed rapid aggregation and precipitation, losing more than 70 % of the protein in an hour (Figure 2 B) because of its low stability and solubility at physiological pH. In contrast, both **1** and **4** improved stability and reduced precipitation of TRAIL under the same conditions. More than 50 % of TRAIL/**4** was found to be stable after twelve hours incubation, however, it gradually lost stability. The bioactivity of each formulation was examined based on tumor-cell-specific

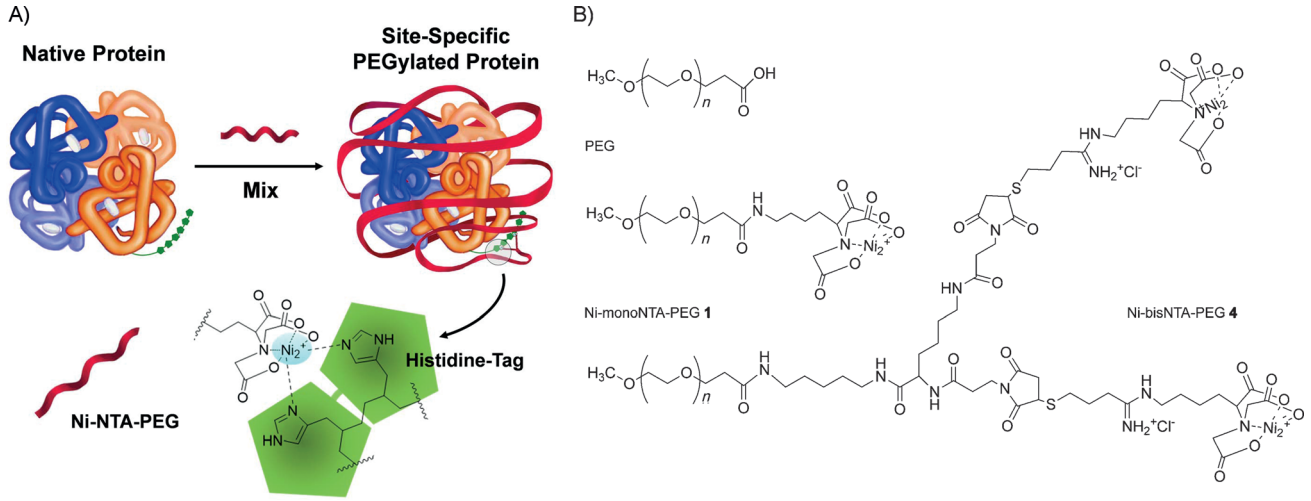

Figure 1. A) Site-specific pseudo-PEGylation of a His-tag-fused protein. Schematic illustration of PEGylating a His-tagged protein with the reactive Ni-NTA-PEG such as **1** or **4**. B) Chemical formulas of methoxy PEG (PEG, MW = 5 kDa) and Ni-NTA-PEG analogues. Synthetic details are described in the Supporting Information.

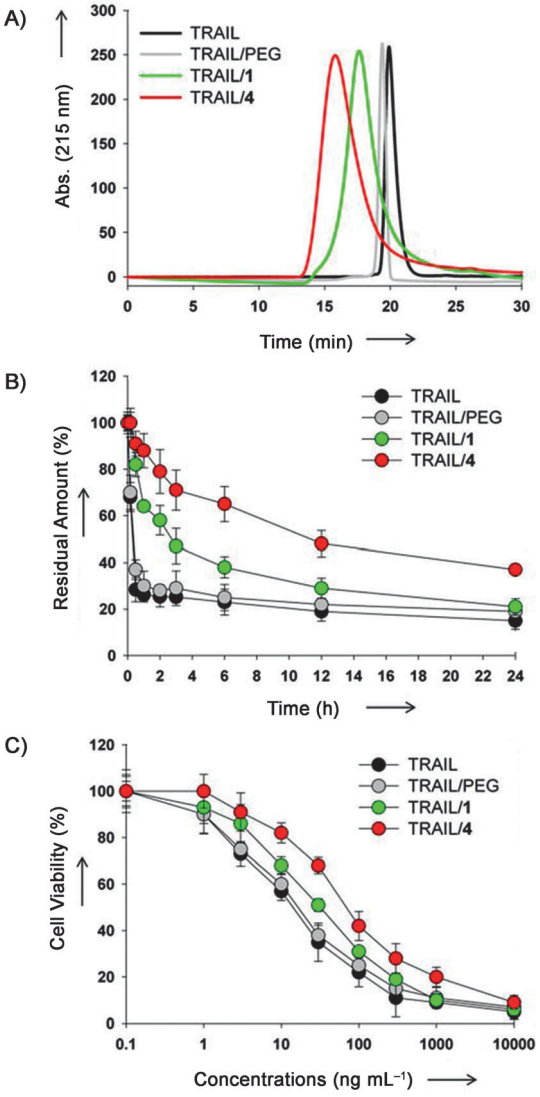

Figure 2. The effects of Ni-NTA-PEGs on the stability and bioactivity of TRAIL. A) Size exclusion chromatograms of TRAIL (100 μg mL^{-1}) and TRAIL associated with PEG, **1**, and **4** at a TRAIL/PEG molar ratio of 1:5 in 20 mM acetate buffer, pH 6.0. B) Time-dependent stability of TRAIL (400 μg mL^{-1}) and its mixtures relative to the stability at time = 0 h in 20 mM PBS, pH 7.4, at 37 °C. C) In vitro biological activity of TRAIL and its mixtures on HCT116 cells. Cytotoxicities of the formulations were determined by performing MTT assays after incubating for 24 h. Graph represents mean ± s.d. ($n = 4$).

cytotoxicity measured by MTT assays, following incubation of TRAIL-based formulations (protein concentration from 10^{-1}–10^{4} ng mL^{-1}) in human colon cancer HCT116 cells (Figure 2C and Table 1). TRAIL and TRAIL/PEG showed a marked apoptotic effect on HCT116 cells. The cytotoxicity of TRAIL/**1** and TRAIL/**4** decreased slightly with increasing NTA affinities but retained 43.7 ± 7.1 % and 20.3 ± 1.6 % of the bioactivity of the native TRAIL, respectively. The observed IC_{50} value of TRAIL/**4** was similar to that of the previously reported TRAIL-PEG_{5K}.[10a] To confirm the tumor cell specificity of TRAIL, the same TRAIL formulas were tested in normal cells (CCD-986sk fibroblasts) and showed no toxicity (Supporting Information, Figure S4A). In terms of the cytotoxicity of Ni-NTA-PEGs, **1** and **4** alone were nontoxic both to normal fibroblasts (CCD-986sk) and HCT116 cells (Supporting Information, Figure S4). Taken together, the in vitro assays demonstrated that simple addition of **1** and **4** to TRAIL was able to improve the stability in solution and reduce aggregation, while salvaging the bioactivity of TRAIL.

Following the validation of TRAIL/Ni-NTA-PEGs in vitro, we analyzed the PK of TRAIL/**1**, TRAIL/**4**, and TRAIL-PEG_{5K} (TRAIL covalently conjugated with the same molecular weight PEG) in rats after intravenous (IV) injection. Total plasma levels of active TRAIL were measured using an enzyme-linked immunosorbent assay (ELISA). All PK parameters are summarized in Table 1 and illustrated in Figure 3A and Figure S5 (Supporting Information). It has been reported that TRAIL has a short half-life of 5–10 minutes in rats, mainly because of rapid renal clearance.[9a] In accordance with the reported values, intravenously injected TRAIL and TRAIL/PEG were rapidly eliminated from rats within one hour (Figure 3A). In contrast, TRAIL/**4** showed a prolonged half-life and maintained activity up to six hours post-injection. Furthermore, the bioavailability of TRAIL/**4**, as determined by the area-under-the-curve (AUC) analysis, was enhanced by 3.8 ± 0.6-fold and 2.1 ± 0.3-fold compared to that of native TRAIL and TRAIL/**1**, respectively (Figure 3B). Because **4** showed significantly extended stability in solution and improved PK parameters of TRAIL both in vitro and in vivo compared to **1**, **4** was chosen for further PD testing in HCT116 tumor-bearing mice. To demonstrate the utility of **4** for in vivo applications, the effect was investigated in tumor models by continuous monitoring of tumor volumes while treating mice with TRAIL every five days. As shown in Figure 3C, all formulations, TRAIL, TRAIL/PEG and TRAIL/**4**, suppressed tumor growth. However, mean tumor growth, quantified as tumor growth inhibition (TGI) values, were only maintained by TRAIL/**4** throughout the study period (at day 20, TRAIL, TRAIL/PEG, and TRAIL/**4** had TGI values of 15.1 ± 7.6 %, 19.2 ± 5.3 %, and 51.8 ± 7.1 %, respectively), and a rebound in tumor size was observed in all other formulations. The TGI value was calculated using the formula $1-(V_T/V_C)\times 100\,\%$, where V_T and V_C are the tumor volume of the drug-treated and control groups, respectively. At the end of the study, tumor tissues were harvested and apoptotic cells in tumor sections were visualized by TdT-mediated dUTP nick end labeling (TUNEL) assays (Figure 3D). As expected, TRAIL/**4**

Table 1: Pharmacokinetic parameters.

	TRAIL	TRAIL/PEG	TRAIL/**1**	TRAIL/**4**	TRAIL-PEG_{5K}[d]
IC_{50}[a]	14.1 ± 4.2	16.7 ± 3.9	32.8 ± 5.3*	69.8 ± 5.6*	66.7 ± 3.9
CL[b]	1.17 ± 0.21	1.12 ± 0.12	0.84 ± 0.08*	0.61 ± 0.05*	0.1 ± 0.01
$t_{1/2}$[c]	11.4 ± 3.1	11.5 ± 2.2	24.1 ± 1.3*	43.3 ± 3.7*	337.3 ± 16.5

[a] The half maximal inhibitory concentration, (ng mL^{-1}); [b] clearance, (mL min^{-1}); [c] elimination half-life, (min). *$p < 0.001$ versus TRAIL alone and TRAIL/PEG. [d] TRAIL covalently conjugated with PEG_{5K}.

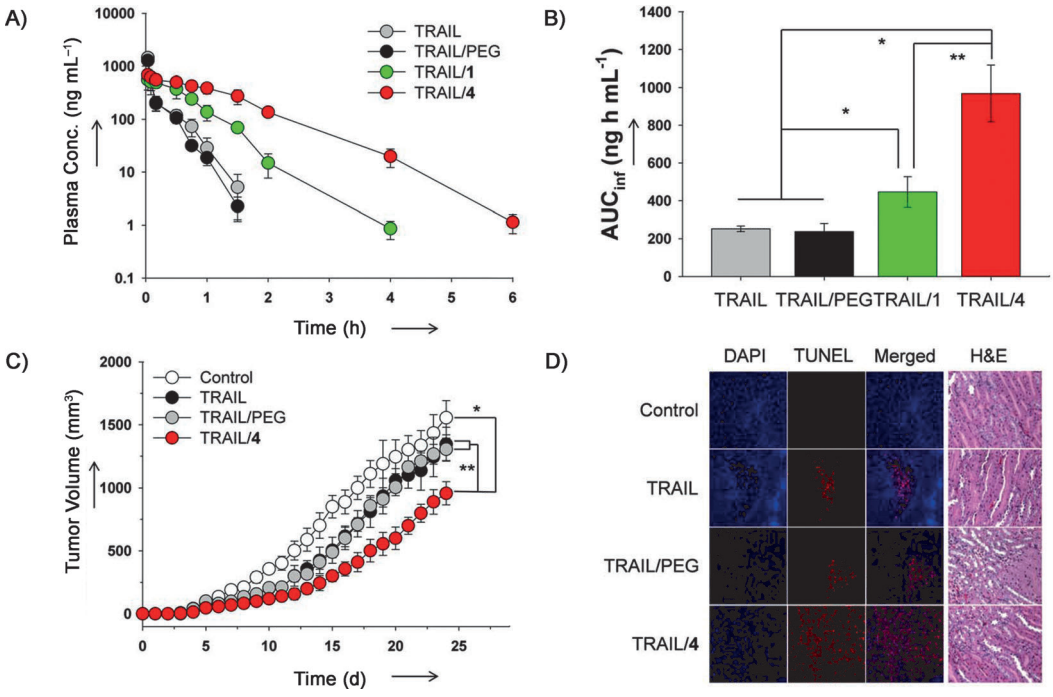

Figure 3. The effects of Ni-NTA-PEGs on the pharmacological efficacy of TRAIL. A) PK profiles of TRAIL, TRAIL/PEG, and TRAIL with Ni-NTA-PEG analogues. Cannulated Sprague–Dawley rats were given an IV injection of TRAIL (100 μg kg^{-1}, based on the TRAIL conc.) and plasma concentrations were monitored by ELISA assay ($n=4$). B) Area under the curve (AUC) values from zero to infinity derived from the PK analysis. *$p<0.001$ versus TRAIL alone and TRAIL/PEG, **$p<0.001$ versus TRAIL/**1**. C) Antitumor activity of TRAIL formulations in HCT116 human colon cancer-bearing mice. Tumor growth suppression was monitored while treating mice with TRAIL (150 μg per mouse, based on the TRAIL conc.) by IV injection every 2 days starting at 5 days after tumor inoculation ($n=6$). *$p<0.001$ versus control, **$p<0.001$ versus TRAIL alone and TRAIL/PEG. D) Fluorescent microscopy images of apoptotic cell death in tumors from mice treated with TRAILs (nucleus stained with DAPI, blue; TUNEL positive apoptotic cells, red): Right) histological images of kidney stained using hematoxulin and eosin (H&E). Graphs represent mean ± s.d.

treated tissues demonstrated increased tumor-cell apoptosis compared to those of native TRAIL and the other formulations. At any injected dose of native TRAIL (50–1000 μg per mouse), TGI of 50% was not achieved under our experimental conditions. Because the major human adverse event related to high PEG exposure is renal toxicity,[1a] acute renal toxicity of **4** was examined by histological investigation of renal tissues after PD studies. No sign of toxicity was observed for any of the formulations (Figure 3D). The experimental results consistently show that the appropriate Ni-NTA-PEG molecule can significantly and positively affect the physicochemical properties of His-tagged proteins in vitro and in vivo, while maintaining bioactivity. Collectively, TRAIL/**4** demonstrated three- to fourfold improved efficacy over native TRAIL in terms of solution stability, in vivo half-life, and bioavailability. This is a significant effect but still not as effective as our previously reported TRAIL-PEG$_{5K}$, which retained more than 80% stability in physiological buffer for 24 hours and showed a tenfold increase in bioavailability compared to TRAIL alone after intraperitoneal (IP) injection.[10a] Surprisingly, however, based on the TGI value, TRAIL/**4** demonstrated similar antitumor efficacy to TRAIL-PEG$_{5K}$ in the same tumor model. This is probably because of the different in vivo bioactivities between TRAIL-PEG$_{5K}$ and TRAIL/**4**; TRAIL-PEG$_{5K}$ has reduced activity (50% versus TRAIL) for all time points, whereas TRAIL/**4** can fully recover its bioactivity once the Ni-NTA-PEG is released from it in the blood. Because the administration route (IP versus IV) and dosing profiles are different, the results cannot be directly compared. Note that our technique offers all of the benefits of site-specific PEGylation without further chemical modification and purification processes. Once **4** is added to protein in solution, the protein can be highly concentrated and freeze-dried. Because an excess of **4** does not interfere with the bioactivity and PK of the protein, one can easily add our analogues as stabilizers. Moreover, the observed pseudo-PEGylation effect can be achieved by a simple incubation, with the total preparation time less than 30 minutes.

We initiated this project to produce a facile and versatile technique that could increase in vivo stability of any protein while maintaining the bioactivity of the protein. Using TRAIL as a model protein, we demonstrated that a unique Ni-NTA-PEG analogue associated with a His-tagged protein is able to provide outstanding physicochemical stability without compromising the bioactivity. Importantly, the Ni-NTA-PEG analogue maximized the pharmacological efficacy of the protein drug in vivo. Significant efforts are under way to develop novel biologics with improved efficacy and reduced dosing profiles and toxicity compared to protein drugs. This current technique can contribute to the development of biologics by reducing the cost of screening proteins and streamlining their evaluation in animal models.

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