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Intracellular Delivery of Native Proteins Facilitated by Cell-Penetrating Poly(disulfide)s

Linghui Qian,[†] Jiaqi Fu,[†] Peiyan Yuan, Shubo Du, Wei Huang, Lin Li* and Shao Q. Yao*

Abstract: Intracellular delivery of therapeutic proteins is highly challenging and in most cases requires chemical or genetic modifications. Herein, two complementary approaches for endocytosis-independent delivery of proteins to live mammalian cells are reported. By using either a "glycan" tag naturally derived from glycosylated proteins or a "traceless" tag that could reversibly label native lysines on non-glycosylated proteins, followed by bioorthogonal conjugation with cell-penetrating poly(disulfide)s, we achieved intracellular delivery of proteins (including antibodies and enzymes) which, upon spontaneous degradation of CPDs, led to successful release of their "native" functional forms with immediate bioavailability.

Seven of the top-ten selling drugs are protein-based biologics, and one of the largest classes of therapeutic proteins is that of antibodies because of their ability to bind to targets with high affinity and specificity.[1,2] Despite obvious benefits of protein-based therapy, most proteins do not enter mammalian cells directly, which severely limits the number of disease-relevant targets they can target/modulate.[3] In fact, of all existing therapeutic antibodies, almost all of them target cell-surface antigens overexpressed in malignant cells in order to bypass the need for intracellular delivery.[2,4] Therefore, intracellular delivery of functional, native proteins (including antibodies) remains a key challenge in protein-based therapy. Although various approaches for intracellular protein delivery have thus far been reported,[5-9] most rely on endocytosis-based cell uptake mechanisms and necessitate significant genetic or chemical modifications to the protein. Futaki *et al* reported an endosomolytic peptide (i.e., L17E) that forms noncovalent complexes with various proteins, leading to cell entry *via* endocytosis followed by successful cytosolic protein liberation.[10] Although the delivered cargos were unmodified, their slow endosome escape was not ideal for immediate bioavailability. The noncovalent L17E/cargo formulation also limits the type of proteins that could be used and delivery efficiency. Of few reports that showed successful delivery of proteins directly into the cytoplasm of mammalian cells, all of them require genetic and/or chemical modifications to the cargos,[11-13] and almost none allows intracellular release of "native" proteins to enable full restoration of their activities.

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Cell-penetrating poly(disulfide)s (CPDs) are synthetic mimics of poly-arginines in which the polypeptide backbone was replaced with poly(disulfide)s (Figure 1A), and have been shown to facilitate endocytosis-independent cell uptake of various cargos.[14-18] The positively charged guanidinium groups in CPDs help cargo accumulation near the negatively charged mammalian cell surface and their disulfide backbone helps intracellular cargo delivery *via* disulfide-exchange mechanisms with no apparent endosomal trapping.[19] In the cytoplasm, CPDs undergo rapid depolymerization (< 5 min), a process catalyzed by endogenous glutathione (GSH). At present, both non-covalent and covalent conjugations are available to append CPDs to proteins modified either genetically or chemically with suitable "tags" (i.e. histidine tag). Such "artificial" modifications might affect protein activities and conformations. Alternatively, CPD-coated biodegradable nanocapsules were used as carriers to encapsulate native antibodies leading to successful intracellular delivery and on-demand release.[20] This material chemistry-based delivery method is however not applicable to many native proteins which are much more fragile than antibodies and could be readily denatured by the harsh conditions used during nanocapsule formation.[7] More general and practical approaches for endocytosis-independent intracellular delivery of "native" functional proteins (including antibodies) are urgently needed.

Herein, by using protein chemistries,[21] we report two complementary CPD-facilitated approaches for endocytosis-independent delivery of "native" proteins (Figure 1). In the first strategy (named PTM-based tagging), we took advantage that glycosylation (a process in post-translation modification, or PTM) occurs in > 50% of mammalian proteins including all antibodies (i.e. IgG).[22] As shown in Figure 1B (top), different from our previously reported method that relies on genetic engineering to introduce artificial tags into recombinant proteins (Table S1),[15] the glycan naturally present in a glycoprotein was converted into a bioorthogonal tag (Step 1), either chemically (by $NaIO_4$ oxidation to aldehyde[23]) or chemoenzymatically (by β-galactosidase/GalNAz to N_3[24]), followed by bioorthogonal conjugation to the corresponding CPD (oxime-containing **$_{AO}$CPD** or tetrazine-modified **$_{Tz}$CPD**/TCO-PEG_{12}-DBCO, respectively; Step 2). Upon successful intracellular delivery *via* CPD-assisted endocytosis-independent pathways,[19] followed by spontaneous GSH-catalyzed depolymerization inside mammalian cells,[14,15] the resulting CPD-free cargo was essential "native" barring minimal changes to the glycan, which was not expected to alter the protein in any noticeable way. In the second strategy (named traceless tagging; Figure 1B, bottom), in order to label non-glycosylated proteins with CPD, two cleavable linkers (**NBL** and **TCOL**) were developed to reversibly label lysines in a protein (Step 1). These two linkers contain *p*-nitrophenylcarbonate which could readily react with primary amines (e.g. lysines), and a disulfide-bridged norbornene and *trans*-cyclooctene (TCO), respectively, that could be conjugated with **$_{Tz}$CPD** (Step 2).[15]

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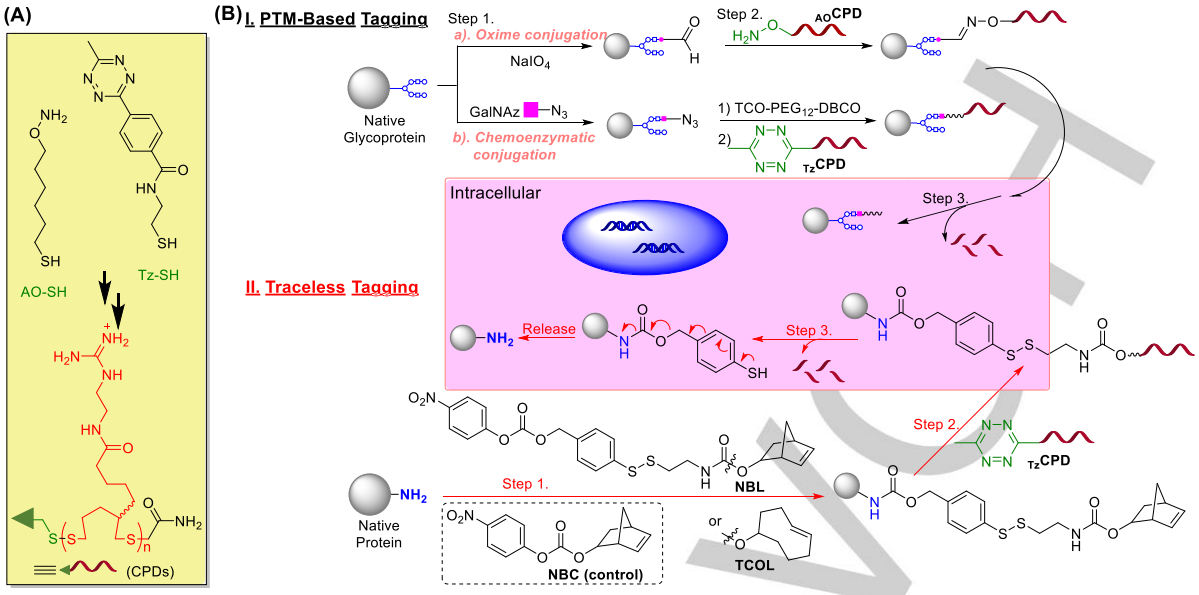

Figure 1. Overview of CPD-facilitated intracellular delivery of native proteins. (A) Structures of AO-SH and Tz-SH used to synthesize $_{AO}$**CPD** and $_{Tz}$**CPD**. (B) Scheme showing the two CPD-facilitated protein delivery approaches: (I) PTM-based tagging, and (II) traceless tagging.

Following intracellular delivery (Step 3), the resulting protein complex could undergo GSH-catalyzed disulfide cleavage followed by spontaneous cleavage of the self-immolative linker,[25] leading to successful release of the "native" protein. It should be noted that while our manuscript is in preparation, Raines *et al* reported diazo compounds derived from (*p*-methylphenyl)glycine for reversible esterification of carboxyl residues in proteins leading to subsequent intracellular delivery.[13] Our traceless approach thus provides an alternative for protein delivery through reversible tagging of lysines instead.

All compounds were synthesized and characterized (Figure S1 and Schemes S1/S2). Subsequently, the two tagging strategies were examined by using model cargos (Figure 2). As antibodies are amongst the most important therapeutic glycoproteins,[2] human IgG, which has a single *N*-linked glycan present in the Fc region and numerous surface-exposed lysines, was chosen as a model to test both strategies. In order to image cellular uptake, IgG was first fluorescently labeled (giving IgG^{Cy3} or IgG^{Cy5}). Bovine serum albumin (BSA) was chosen as a model for non-glycosylated proteins (Figure 2).

In PTM-based tagging with oxime conjugation (Figure 1B, top), IgG^{Cy3} was first oxidized with 10 mM $NaIO_4$ for 0.5 h on ice by following published protocols.[23] The aldehyde formation was successfully monitored by incubating the oxidized product with FITC-ONH_2. Alternatively, IgG^{Cy3} was chemoenzymatically modified by following a previously published protocol with some optimizations (Scheme S3);[24] upon treatment with *β*-galactosidase to trim off the sugar chains, the exposed GlcNAc residue on IgG^{Cy3} was enzymatically incorporated with GalNAz (an azide-containing GalNAc analog) by using a mutant *β*-1,4-galactosyltransferase, providing the N_3-labeled IgG^{Cy3} (i.e. IgG^{Cy3}-N_3). Following bioorthogonal strain-promoted alkyne-azide cycloaddition (SPAAC) with TCO-PEG_{12}-DBCO,[26] the resulting TCO-modified IgG^{Cy3} was ready for subsequent CPD conjugation and intracellular delivery. By comparing with a previously reported antibody conjugation strategy using a commercially available bis-sulfone reagent, ThioLinker-TCO™ (Scheme S3),[27] we found both the oxime and chemoenzymatic strategies were able to modify the antibody with comparable efficiency (Figure S2). With both oxidized IgG^{Cy3} and IgG^{Cy3}-N_3 in hand, we next carried out CPD conjugation by mixing them with $_{AO}$**CPD** and $_{Tz}$**CPD**, respectively. Both conjugations occurred rapidly within 1 h in a "plug-and-play" manner without additional reagents and the resulting CPD conjugates were directly used for intracellular delivery. Live-cell imaging with confocal laser scanning microscope (CLSM) was carried out in HeLa cells co-stained with an endolysosomal tracker and Hoechst nuclear stain (Figure S2B); similar to CPD-conjugated IgG^{Cy3} modified by ThioLinker-TCO™ as previously reported,[15] both oxidized IgG^{Cy3} and IgG^{Cy3}-N_3 were able to successfully enter cells upon CPD conjugation, and the uniformly distributed fluorescent patterns of delivered cargos throughout the cytosol with few overlaps with the endolysosomal tracker indicated successful endocytosis-independent uptake mechanisms.[14-19] More quantitative fluorescence-activated cell sorting **(**FACS) analysis was carried out (Figure 2A); consistent with live-cell imaging, the PTM-based tagging with oxime conjugation showed the highest intracellular IgG^{Cy3} delivery efficiency upon CPD conjugation, followed by that of chemoenzymatic conjugation and the previously reported ThioLinker approach.[15] Without CPD conjugation, however, none of the strategies was effective for intracellular delivery. Consequently, oxime conjugation was chosen as the method-of-choice for PTM-based tagging in subsequent experiments.

We next investigated whether the traceless tagging could be used for CPD conjugation of a model non-glycosylated protein (e.g. BSA) followed by successful cellular delivery and spontaneous intracellular release of the native cargo. As shown in Figure 2B (and Figure S3), following a concentration-dependent labeling of BSA by both **NBL** and **TCOL**, the resulting linker-modified, as well as the subsequent GSH-catalyzed linker cleavage, BSA was visualized by in-gel fluorescence scanning following click chemistry with TER-Tz1 and SDS-PAGE

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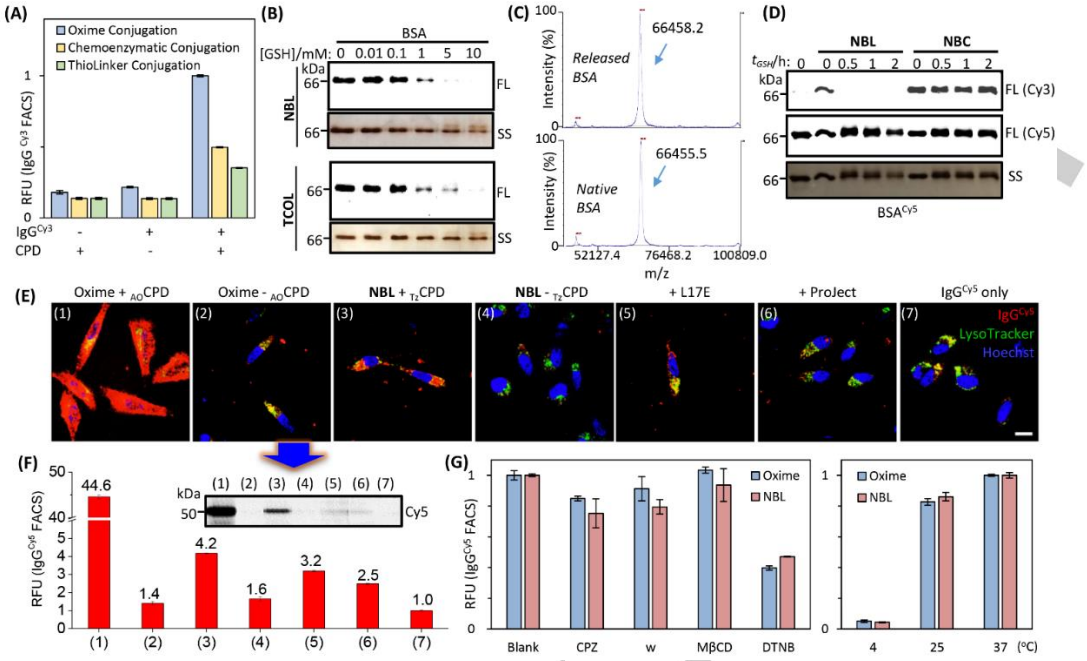

Figure 2. (A) FACS quantification of IgG^{Cy3} uptake in live HeLa cells with "PTM-based tagging" in comparison with ThioLinker conjugation,[15] before/after adding the corresponding CPDs. Relative fluorescence (RFU) in each experiment was normalized to that of cells treated by oxime conjugation (set as 1). (B) GSH-dependent cleavage (0 to 10 mM) of BSA labeled by "traceless tagging". Native BSA was labeled by **NBL**/**TCOL** prior to being clicked with TER-Tz1, followed by GSH treatment (0 to 10 mM; 2 h at 37 °C) and SDS-PAGE before in-gel fluorescent scanning (FL, indicating only labeled BSA) and silver staining (SS, indicating total BSA). (C) MALDI-TOF spectra of native BSA (bottom) and **TCOL**-labeled BSA (top) after GSH cleavage (5 mM, 2 h at 37 °C). (D) Time-dependent GSH cleavage between **NBL**- and **NBC**-labeled BSA^{Cy5}. BSA^{Cy5} was first tagged by **NBL** or **NBC**, clicked with TER-Tz1, then treated with GSH (5 mM at 37 °C) for 0 to 2 h, before SDS-PAGE/in-gel fluorescence scanning. (Cy3): TER-Tz1/linker-labeled BSA; (Cy5): total BSA^{Cy5} intensity. (E) 3D projections of z-stack images showing intracellular delivery of IgG^{Cy5} conjugated to CPDs by using different approaches (oxime/**NBL**). Controls via L17E/ProJect strategies were run. HeLa cells were incubated with IgG^{Cy5} (50 nM for 1.5 h; colored in red) following each delivery formula (Supporting Information), then co-stained with Hoechst (blue) and LysoTracker (green). Cells treated with unmodified IgG^{Cy5} (panel 7) or "tagged" IgG^{Cy5} but without CPD conjugation (panels 2 & 4) were imaged as negative controls. Scale bar = 20 μm. (F) FACS quantification of IgG^{Cy5} uptake in HeLa cells treated with different delivery methods described in (E). The RFU in each sample was normalized to that from cells treated with unmodified IgG^{Cy5} (set as 1), and indicated on top of each bar. (Inset): SDS-PAGE/in-gel fluorescence scanning of lysates from the corresponding treated cells. (G) FACS of CPD-conjugated IgG^{Cy5} uptake in HeLa cells after oxime or **NBL** tagging, in the presence of endocytosis inhibitors (left) or at different incubation temperatures (right). Inhibitors include chlorpromazine (CPZ), wortmannin (w), methyl-*β*-cyclodextrin (MβCD), and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB). Data were normalized against cells treated with no inhibitors (Blank; set as 1; left graph), or cells incubated at 37 °C (set as 1; right graph).

separation. We found both **NBL**- and **TCOL**-labeled BSA were efficiently cleaved by 5 mM GSH in as little as 15-30 min (Figure S3). With 1 mM GSH, however, a minimum of 2-h incubation was needed to afford complete linker cleavage (Figure 2B). Since GSH is present at 1-10 mM in most mammalian cells, we reckoned these linkers would be intracellularly cleaved in a matter of 1-2 h. The "traceless" tagging/release of BSA was further verified by MALDI-TOF mass spectrometry with **TCOL**-labeled BSA as a representative (Figures 2C & S4); virtually identical molecular weights of native BSA and GSH-cleaved BSA following **TCOL** labeling were detected, indicating a complete linker removal from the labeled BSA. No GSH-catalyzed linker release was observed with BSA labeled by a non-cleavable control linker, **NBC** (Figures 1B/2D & S5). Nonetheless following **$_{Tz}$CPD** conjugation, even **NBC**-labeled BSA was successfully delivered to the cytosol of mammalian cells (Figure S5C), underscoring the essential role of CPD in facilitating intracellular cargo delivery.

We next used IgG to compare the delivery efficiency of the newly developed approaches. We were surprised to notice that IgG labeling by **TCOL** was substantially weaker than **NBL**, even though no obvious difference was observed in similar labeling reactions with BSA (Figure S6A). This indicates the efficiency of our traceless tagging approach was protein-dependent. For simplicity, **NBL** was chosen as the method-of-choice for traceless tagging in subsequent experiments. When oxime and **NBL** conjugations were compared side-by-side with the same cargo (IgG^{Cy5}; Figure S6B), the former appeared to label IgG^{Cy5} much more strongly than the latter, which might have been caused by competitive Cy5-NHS pre-labeling on IgG, thus resulting in less available free lysines for subsequent **NBL** labeling. Importantly, upon CPD conjugation, both methods enabled intracellular delivery of IgG^{Cy5} (Figure 2E/F); compared to L17E and ProJect™ (a commercial protein transfection kit),[10,15] both CLSM and quantitative FACS showed our newly developed approaches were more effective (oxime > **NBL** > L17E ≈ ProJect). The endocytosis-independent advantage of the methods was further verified to be similar with previously reported systems (Figure 2G);[14,15] while common endocytosis inhibitors didn't noticeably affect protein delivery, both DTNB and reduced temperature (4 °C) led to significant inhibition in cellular uptake, indicating thiol-mediated uptake mechanisms as previously reported.[14-19]

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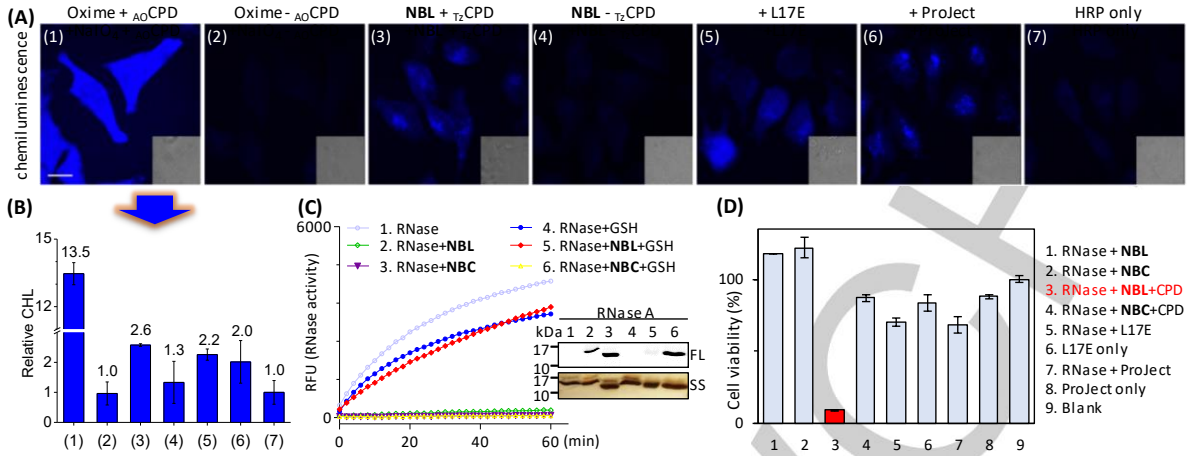

Figure 3. Intracellular delivery of native proteins for immediate bioavailability. (A) HRP delivery to live HeLa cells followed by measurement of enzymatic activities. Cells were treated with HRP (50 nM, 1.5 h) following each delivery formula, before being washed, fixed and incubated with ECL reagents (20 min at RT) then imaged. Scale bar = 20 μm. (B) Relative chemiluminescence intensity (CHL) extracted from different images in (A). The signals were normalized from cells treated with HRP only (panel 7; set as 1), and indicated on top of each bar. (C) *In vitro* RNase A activity assays towards (1) native RNase A, (2) **NBL**-labeled RNase A and (3) **NBC**-labeled RNase A. Upon GSH treatment (5 mM, 37 °C, 15 min), samples (4, 5, 6) were measured again. (Inset): in-gel fluorescence scanning (FL) of the six samples upon click chemistry with TER-Tz1 and SDS-PAGE. (D) XTT cell viability assay of HeLa cells upon intracellular delivery of RNase A (0.52 μg/well; 24 h) via different methods. Error bars were obtained from triplicate.

Having shown both the PTM-based and traceless tagging coupled with CPD conjugation were effective for endocytosis-independent intracellular protein delivery, we next investigated their ability to deliver native functional proteins with immediate bioavailability. We turned to enzymes as the cargos. Horseradish peroxidase (HPR), a glycoprotein whose endogenous accumulation in mitochondria leads to neurodegenerative diseases and cancer,[28] is widely used in biology as a sensor to image endogenous H_2O_2. By catalyzing oxidization of a suitable substrate in the presence of H_2O_2, detectable chemiluminescent or fluorescent signals could be generated. Previous reports indicate chemical modifications on any of the eight glycosylation sites in HRP had negligible effects on its enzymatic activity and our *in vitro* enzymatic assay also supported this (Figure S6C).[29] Upon successful conjugation with either PMT-based or traceless tagging (Figure S6D), followed by bioorthogonal CPD attachment, the resulting conjugates were used for intracellular delivery (Figure 3A/B); by imaging (and quantifying) the enzymatic activity of intracellularly delivered HRP from various delivery methods,[30] we detected significant chemiluminescent signals in HeLa cells treated with both CPD conjugates (e.g. panels 1 & 3) as well as L17E and ProJect™ methods (panels 5 & 6), with the oxime conjugation again showing significantly more superior delivery efficiency (see Figure 3B; compare 1 vs others). This is similar to what was observed with IgG in our earlier experiments (Figure 2F). In the absence of CPD, however, neither tagging produced any noticeable endogenous HRP activity (panels 2 & 4). In another example for intracellular delivery of native, functionally active RNase A (a non-glycosylated protein), we further studied the traceless tagging with **NBL** by comparing it with the non-cleavable **NBC**. It was previously reported that lysine residues in RNase A, a therapeutic protein which has recently entered clinical trials for cancer treatments, are essential to its activity,[31] Upon successful cell uptake, this enzyme causes strong cytotoxicity. We hypothesized that RNase A conjugation with **NBL** should block its activity. Upon subsequent GSH-triggered "traceless" cleavage of **NBL** to release native RNase A, the enzyme's activity should be fully restored. **NBC**-tagging on RNase A, on the other hand, should not be capable of restored activity due to the linker's non-cleavable nature (Figures 3C & S5A). Indeed, when compared to native RNase A, tagging of RNase A with **NBL** and **NBC** significantly attenuated its enzymatic activity (compare line 1 vs 2/3 in Figure 3C). Upon successful GSH-catalyzed linker cleavage, the enzymatic activity of **NBL**- but not **NBC**-labeled, RNase A was fully restored (compare lane 4 vs 5/6). Following CPD conjugation and intracellular delivery of **NBL**-tagged RNase A to live HeLa cells for 24 h, we detected significant cell death (Figure 3D, in red); even positive cells treated with L17E/Project™ methods showed significantly less cell death. This indicates our traceless tagging/delivery strategy was more effective than other existing approaches for intracellular delivery and release of native proteins with immediate bioavailability. Consistent with the *in vitro* assays, **NBC**-labeled intracellularly delivered RNase A showed very weaker cytotoxicity even after 48-h incubation (Figure S6E).

To sum up, we have developed novel conjugation chemistries that enabled convenient tagging with cell-penetrating poly(disulfide)s, endocytosis-independent cellular delivery and intracellular release of "native" functional proteins (including antibodies) with immediate bioavailability. In future, other types of post-translational modifications may be similarly exploited for CPD-facilitated intracellular delivery of other native proteins.

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Keywords: native protein • intracellular delivery • antibody • endocytosis-independent • cell-penetrating poly(disulfide)s • bioorthogonal chemistry

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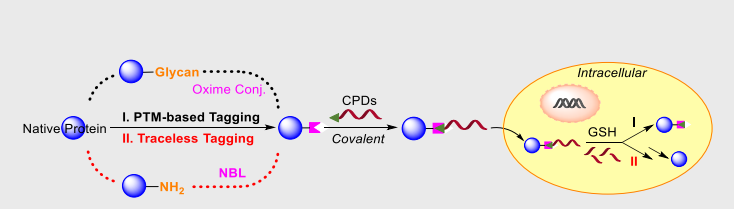

Delivery free of "tag": By making use of either a glycan (for glycoproteins) or lysines (for a a non-glycosylated protein), cell-penetrating poly(disulfide)s were conjugated to the cargo, resulting in endocytosis-independent cellular uptake and spontaneous release of the "native" functional protein in the cytosol.

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