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COMMUNICATION

Intracellular Delivery of Therapeutic Proteins through *N*-Terminal Site-Specific Modification

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A versatile strategy for the intracellular delivery of functional proteins/antibodies was developed using *N*-terminal site-specific modification. Adopting orthogonal dual-labeling strategies, a cell-permeable RNase A prodrug was designed complementing *N*-terminal site-specific modification with lysine labeling. Upon successful cytosolic uptake, the prodrug showed reactive oxygen species (ROS)-dependent targeted cancer therapy.

Introduction

Protein-based therapeutics have attracted significant attention as they have the potential to engage targets considered non-druggable by small molecules.[1] Although 20% of today's pharmaceutical drugs are protein- or antibody-based, many act on extracellular or secreted proteins.[2] To reach intracellular targets, various strategies for protein delivery have been developed.[3] While these strategies show promise, inefficient escape from the endolysosomal pathway remains a key barrier.[3a] As such, research is continuously underway to address this limitation. Precise and spatiotemporal modulation of protein functions inside cells are also advantageous for therapeutic applications.[4] Diseased microenvironments provide inherent and non-invasive triggers for achieving stimuli-responsive precision therapy, thus reducing undesirable side effects and ensuring higher therapeutic efficacy. Of late, stimuli-responsive nanocarriers has been utilised in protein delivery to mimic prodrug strategies.[5] Under endogenous triggers such as glutathione (GSH) or hypoxic conditions,[5b-d] the degradation of stimuli-responsive nanocarriers results in the release of functional proteins. However, harsh nanocapsule formation conditions, low cargo encapsulation efficiency and slow release profiles are some intrinsic issues.

Protein chemical modification has become a useful tool for endowing proteins with various functions.[6] Recently, Francis' group reported a one-step *N*-terminal α-amino group site-specific modification under mild conditions.[7] We hypothesised that a dual-labeling strategy complementing the *N*-terminal modification with other orthogonal stimuli-responsive chemical protein labeling approaches[8] would enable us to achieve a cell-permeable protein prodrug. Since *N*-terminal modification occurs exclusively at the *N*-terminal α-amino group, other amino acid residues on the protein such as active-site Lys, Cys or Ser would remain unaffected for subsequent masking by a stimuli-responsive probe. In this work, we first tested the versatility of the *N*-terminal modification strategy in the intracellular delivery of various proteins by using cell-penetrating poly(disulphide)s (CPDs) (Fig. 1).[9] CPDs (structure in SI, Scheme S2) have been shown to facilitate endocytosis-independent cell uptake of various cargos.[10] Once in the cytosol, endogenous GSH catalyses the rapid depolymerisation of CPD, resulting in traceless release of cargos. Such a strategy would be advantageous for the intracellular delivery of proteins i.e. transcription factors, which may require organelle localisation for proper function.[11]

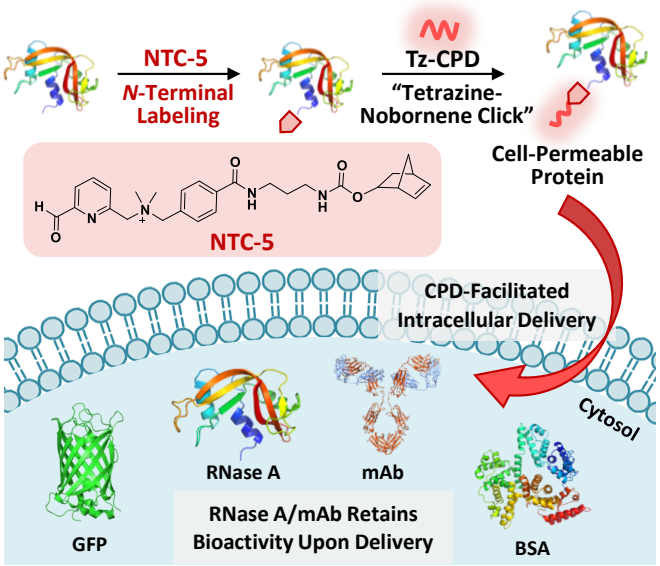

Fig. 1 Single labeling: *N*-terminal site-specific labeling of proteins using **NTC-5**. Subsequent click reaction with Tz-CPD facilitates cytosolic delivery of various proteins such as RNase A, GFP, BSA and IgG.

NTC-5 (our *N*-terminal labeling probe) was designed with a 2-pyridinecarboxyaldehyde (2-PCA) moiety for reacting with the *N*-terminal α-amino group on proteins and a norbornene for

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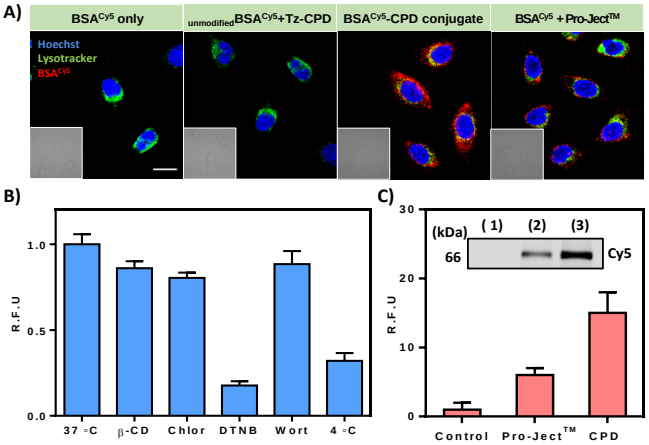

Fig. 2 A) Confocal images of live HeLa cells treated with BSA^{Cy5} only, unmodified BSA^{Cy5} mixed with Tz-CPD, BSA^{Cy5} protein-CPD conjugate and BSA^{Cy5} delivered via Pro-Ject^{TM} approach (100 nM, 2 h). Scale bar = 20 μm. B) FACS uptake analysis of BSA^{Cy5} protein-CPD conjugate (100 nM; 2 h incubation) in HeLa cells treated with different inhibitors, including chlorpromazine (Chlor), wortmannin (Wort), methyl-β-cyclodextrin (β-CD), and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) and at 4 °C. R.F.U = relative fluorescence units. C) Quantitative analysis of Pro-Ject^{TM} and CPD-assisted BSA^{Cy5} delivery to HeLa cells by flow cytometry. (Inset) SDS-PAGE/In-gel fluorescence scanning of lysates from the corresponding treated cells. (1) Control; (2) Pro-Ject^{TM} method; (3) CPD-assisted delivery. See Fig. S4 for Coomassie (CBB)-stained gel. Note: BSA^{Cy5} used in above experiments were **NTC-5**-modified (unless otherwise stated), but for simplicity, they were abbreviated as BSA^{Cy5}.

copper-free tetrazine (Tz)-click ligation to small molecules, as well as cell-penetrating polymers such as CPD (Fig. 1). Using **NTC-5**, *N*-terminal modification was first optimised using BSA as our model protein. To determine the success of the reaction, dye labeling of **NTC-5**-modified BSA was carried out via click chemistry with Tz-Rhodamine (Tz-Rh), followed by SDS-PAGE gel analysis and in-gel fluorescence scanning (Fig. S1). Optimal labeling was achieved when 200 equiv. of **NTC-5** was used, and the reaction was allowed to proceed at 37 °C for 16 h. The reaction condition is mild (50 mM phosphate buffer, *p*H 7.4) and would be compatible with delicate proteins. ESI-TOF mass spectrometry analysis also confirmed the site-specific modification of BSA at the *N*-terminus only (Fig. S2).[7] As negative control, Cytochrome C, a *N*-terminal acetylated protein, was also subjected to the same labeling conditions; no fluorescence band of **NTC-5**-treated Cyt C was observed after SDS-PAGE gel analysis and in-gel fluorescence scanning (Fig. S3); the inability of **NTC-5** to label Cyt C clearly indicated that the 2-PCA reaction with the *N*-terminal α-amino group of a protein was indeed highly chemoselective.

Next, in order to study cellular uptake, **NTC-5**-modified BSA^{Cy5} was clicked with Tz-CPD (1 equiv.) and used directly in a "mix-and-go" protocol.[9c] 100 nM of the protein-CPD conjugate was incubated with HeLa cells for 2 h. Confocal laser scanning microscopy (CLSM) of live HeLa cells indicated the successful cellular uptake of BSA^{Cy5} protein-CPD conjugate within 2 h (Fig. 2A). Without CPD conjugation, no intracellular delivery of **NTC-5**-modified BSA^{Cy5} was observed. To ensure CPD did not compromise the integrity of cell membrane by allowing diffusive entry, unmodified BSA^{Cy5} mixed with Tz-CPD was incubated with HeLa cells as a control. To validate the cellular uptake pathway, HeLa cells were treated at different temperatures and with different endocytosis inhibitors. After which, quantification of protein uptake by fluorescence activated cell sorting (FACS) was carried out (Fig. 2B).[9b,c] As expected, various endocytosis inhibitors did not significantly inhibit protein delivery. Reduced temperature (4 °C) decreased but failed to block protein delivery completely. On the other hand, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) led to a notable suppression in protein uptake. Together, these results suggest that the mechanism of uptake is thiol-dependent and mostly endocytosis-free, but possibly with some small degrees of endocytosis-dependent uptake (as evidenced by small decreases observed in the endocytosis inhibitor experiments) (Fig. 2B).[9-10] We also compared the cellular uptake efficiency of the CPD-assisted strategy with Pro-Ject^{TM} reagent,[12] a commercially available liposome-based protein delivery system. Quantification of protein uptake using FACS as well as SDS-PAGE analysis/in gel-fluorescence scanning of cell lysates indicated that CPD-facilitated protein delivery was more efficient than the conventional Pro-Ject^{TM} approach (Fig. 2C).

Next, to determine if *N*-terminal modification could be applied for the intracellular delivery of proteins of various sizes,

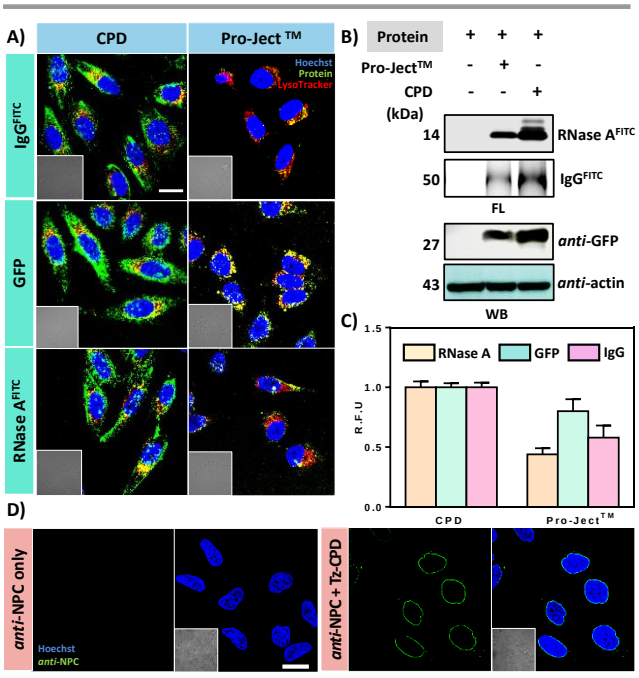

Fig. 3 A) Confocal images of live HeLa cells and B) SDS-PAGE/In-gel fluorescence scanning of HeLa cell lysates incubated with RNase A^{FITC}, GFP and IgG^{FITC} protein-CPD conjugates as well as Pro-Ject^{TM}-assisted intracellular protein delivery (100 nM, 2 h). DTT in loading dye results in the reduction of IgG to form heavy chain (50 kDa) and light chain (25 kDa). Only the 50 kDa heavy chain is shown. Complete FL gel and CBB-stained gel were reproduced in Fig. S6. Western Blotting (WB) was carried out for GFP experiment as lysate treatment with SDS resulted in denaturation of GFP protein, hence fluorescence loss. GFP was successfully detected at ~27 kDa. Loading control = actin. C) Quantitative analysis of Pro-Ject^{TM} and CPD-assisted protein (RNase A^{FITC}, GFP and IgG^{FITC}) delivery to HeLa cells (100 nM, 2 h) by flow cytometry. D) CLSM images of live HeLa cells treated with *anti*-NPC antibody-CPD conjugate (500 nM, 12 h) prior to fixation and incubation with FITC-conjugated goat anti-mouse IgG (H+L) secondary antibody. Cells incubated with *anti*-NPC only (no CPD) were carried out as controls. Scale bar = 20 μm. Note: All proteins used in above experiments were **NTC-5** modified, but for simplicity, they were abbreviated as their protein names, i.e. RNase A^{FITC}, GFP, IgG^{FITC} and *anti*-NPC.

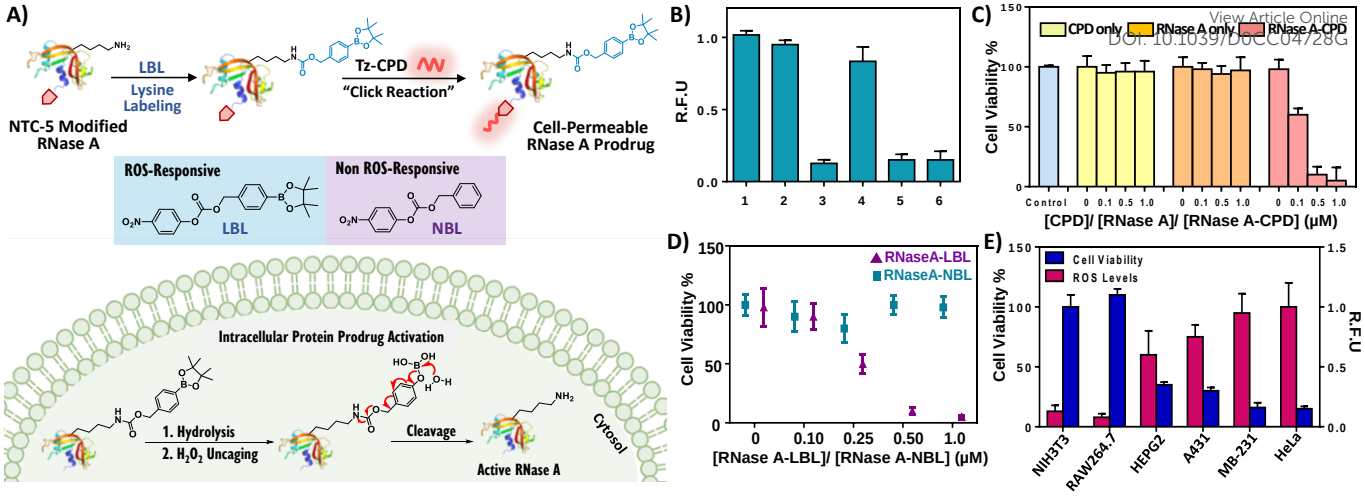

Fig. 4 A) Dual labeling: *N*-terminal site-specific modification followed by **LBL** lysine labeling of RNase A for the intracellular delivery of RNase A prodrug. Upon intracellular activation by reactive oxygen species (ROS), self-immolative cleavage of **LBL** boronate moiety results in restoration of RNase A activity, rendering it useful for targeted cancer therapy. **NBL**, the non ROS-responsive probe, was used as negative control. B) *In vitro* RNase A activity assays. 1: Native RNase A; 2: RNase A; 3: **LBL**-labeled RNase A; 4: **LBL**-labeled RNase A + 5mM H_2O_2; 5: **NBL**-labeled RNase A; 6: **NBL**-labeled RNase A+ 5 mM H_2O_2. C) HeLa cells alone (control) and incubated with CPD only, RNase A only and with RNase A protein-CPD conjugate for 24 h at 37 °C. D) Concentration-dependent cytotoxicity of **LBL**-labeled RNase A and **NBL**-labeled RNase A protein-CPD conjugates against HeLa cells E)Red: FACS analysis of intracellular ROS levels in non-cancerous and cancer cells determined by using 10 μM DCFDA. Blue: **LBL**-labeled RNase A protein-CPD conjugate (500 nM, 24 h) selectively prohibited cancer cells proliferation. Note: All RNase A used in above experiments was **NTC-5** modified RNase A (unless otherwise stated), but for simplicity, they were abbreviated as RNase A.

RNase A (14 kDa), GFP (27 kDa) and IgG (150 kDa) were similarly modified with **NTC-5**. Gratifyingly, SDS-PAGE gel analysis and in-gel fluorescence scanning indicated successful *N*-terminal modification of all the proteins (Fig. S5). In order to image cellular uptake, fluorescently-labeled RNase A and IgG (RNase A^{FITC} and IgG^{FITC}) were used. The **NTC-5**-modified proteins were then clicked with Tz-CPD to obtain the corresponding protein-CPD conjugates. 100 nM of the protein-CPD conjugates were directly used for incubation with HeLa cells for 2 h. CLSM was used to confirm intracellular delivery (Fig. 3A). Most of the fluorescence signals were distributed throughout the cytosol, with a small amount of punctated signals colocalizing with LysoTracker. These results again indicate some small degrees of endocytosis-dependent uptake, which agrees with earlier endocytosis inhibitor experiments (Fig. 2B). Delivery efficiency was compared with Pro-Ject™ reagent and quantification determined *via* FACS (Fig. 3C). SDS-PAGE/In-gel fluorescence scanning/Western-blot of lysates from HeLa cells treated with protein-CPD conjugates (100 nM each; 2 h incubation) also confirmed successful and efficient cellular uptake and subsequent traceless release of proteins (Fig. 3B). As such, *N*-terminal modification is a versatile strategy for CPD-facilitated intracellular delivery of various proteins.

To validate if proteins retained their function after *N*-terminal modification and subsequent CPD-facilitated intracellular delivery, we chose a commercially available *anti*-NPC antibody capable of binding to the nuclear pore complex (NPC) found on the nuclear membrane. The *anti*-NPC antibody was first labeled with **NTC-5** (Fig. S7A), followed by intracellular delivery using Tz-CPD. Subsequently, the cells were fixed and permeabilized, followed by immunofluorescence (IF) staining with FITC-labeled secondary antibody and imaging with CLSM. Fluorescence signals were detected around the nucleus (Fig. 3D), corresponding to results obtained with standard IF on fixed HeLa cells (Fig. S7B). Using this approach, the successful delivery of functional *anti*-NPC antibody was achieved, corroborating Li et al's findings, where *N*-terminal modification of antibodies did not adversely affect their bioactivities.[13] This novel antibody delivery approach might be useful in the development of cell-permeable antibodies for antibody-based therapeutics.[3b]

With success using the *N*-terminal strategy for intracellular protein delivery, we next adopted a dual-labeling approach to make cell-permeable RNase A prodrug. Ribonuclease A (RNase A), a therapeutic protein which has recently entered clinical trials for cancer treatments, can induce cytotoxic effects upon cellular uptake.[14] It has been reported that the lysine residues on RNase A are essential for its activity.[8d] *N*-terminal site-specific modification was complemented with lysine boronate labeling (**LBL**) of RNase A to achieve intracellular delivery of our protein prodrug (Fig. 4A). Upon activation by endogenous reactive oxygen species (ROS), self-immolative cleavage of the boronate moiety on **LBL**-labeled RNase A results in restoration of RNase A function.[8c] As such, targeted cancer therapy can be achieved due to higher levels of ROS present in such cells.

First, *N*-terminal modification of RNase A was carried out with **NTC-5**. Subsequent conjugation with Tz-CPD would render the resulting complex cell-permeable. Next, we labeled the lysine residues on **NTC-5**-modified RNase A with **LBL**, a ROS-responsive probe which can be cleaved by H_2O_2 to endow it with a "prodrug function".[8c] As negative control, non-cleavable **NBL** (Fig. 4A) was used. *In vitro* RNase A activity assay indicated labeling of RNase A with **NBL** and **LBL** significantly inhibited enzymatic activity due to blocking of key lysine residues. Treatment with H_2O_2 (5 mM, 3 h) significantly restored RNase A activity for **LBL**-labeled RNase A *via* a self-immolative cleavage of the boronate moiety, however this was not observed for **NBL**-labeled RNase A (Fig. 4B & S8). To confirm the functionality of RNase A protein-CPD conjugate in cell culture, HeLa cells were

first incubated with CPD only, RNase A only and RNase A protein-CPD conjugate, respectively. Cell viability was determined using an MTT assay. After 24 h treatment, 500 nM of RNase A protein-CPD conjugate exhibited notable cytotoxic effect, while CPD and RNase A controls alone did not significantly lower cell viability (Fig. 4C).

To determine whether the dual-labeled RNase A prodrug could be activated by endogenous ROS upon intracellular delivery, CPD was conjugated to both the **NTC-5-**modified RNase A prodrug (i.e. labeled with **LBL**) and the corresponding control (i.e. **NBL**-labeled RNase A), followed by incubation with HeLa cells. After 24 h, HeLa cells treated with 500 nM of the cell-permeable RNase A prodrug had a much lower viability (~20%) than that of the non ROS-responsive control (Fig. 4D). We further determined if cancer cell-specific cytotoxicity can be achieved using the cell-permeable RNase A prodrug. A panel of cancer and non-cancerous cell lines, including NIH3T3 (embryonic fibroblast cells) and RAW264.7 (murine macrophages) were screened for their intracellular ROS levels by incubation with 2',7'-dichlorodihydrofluorescein diacetate (DCFDA), a ROS-responsive fluorescent probe. FACS analysis indicated elevated levels of ROS in all 4 cancer cells compared to the other 2 non-cancerous cell lines (Fig. 4E). Following the intracellular delivery of the RNase A prodrug, the viability of cancer cell lines (HeLa, MDA-MB-231, A431, HEPG2) decreased to ≤ 30%. On the other hand, non-cancerous cell lines, NIH3T3 and RAW264.7, displayed over 90% viability (Fig. 4E). To eliminate any discrepancy due to uptake efficiency, the cellular uptake of RNase A protein-CPD conjugates for each cell line was determined and found to be similar (Fig. S9). These results suggested that dual modification of RNase A successfully endowed it with both cell-penetrating ability and ROS-responsive prodrug function for targeted cancer therapy.

In summary, we have reported a new approach for CPD-facilitated cytosolic delivery of proteins using *N*-terminal site-specific modification. As shown, proteins such as RNase A and *anti*-NPC antibody retained their functions after intracellular delivery. Hence, this strategy holds great potential in the intracellular delivery of functional monoclonal antibodies for antibody-based therapeutics. Using a dual-labeling strategy, we also successfully generated a cell-permeable ROS-responsive RNase A prodrug which specifically targets cancer cells. We believe that this dual-labeling approach involving *N*-terminal site-specific modification and other orthogonal stimuli-responsive labeling chemistry can be similarly exploited for the development of other therapeutic proteins as prodrugs for precision therapy, such as protein kinases, which require essential Lys needed for catalytic activities, or proteases which have key amino acid residues such as Cys or Ser in their active sites.

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Conflicts of interest

There are no conflicts to declare.

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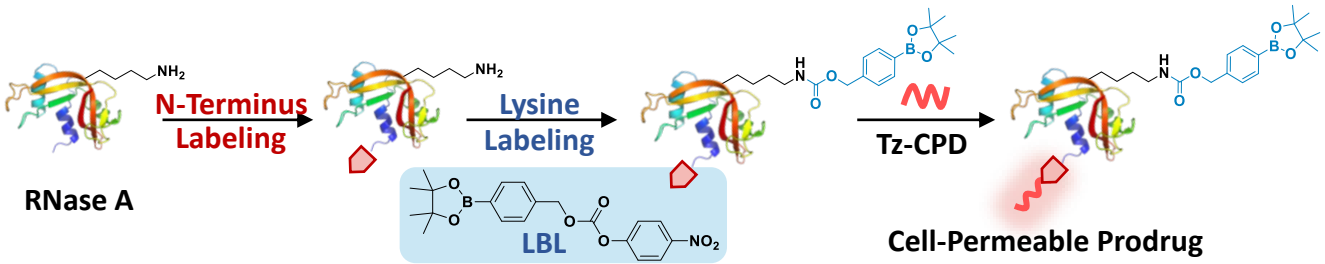

- **Adopting orthogonal dual-labeling strategies, a cell-permeable RNase A prodrug was designed for ROS-responsive targeted cancer therapy.**