Cell-Permeant Bioadaptors for Cytosolic Delivery of Native Antibodies: A “Mix-and-Go” Approach

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ABSTRACT: Antibodies are powerful tools that may potentially find wide applications in live-cell bioimaging, disease diagnostics, and therapeutics. Their practical applications have however remained limited thus far, owing to their inability to cross the cell membrane. Existing approaches for cytosolic delivery of functional antibodies are available, but they are constantly plagued by the need for chemical/genetic modifications, low delivery efficiency, and severe endolysosomal trapping. Consequently, it is of paramount importance to develop new strategies capable of highly efficient cytosolic delivery of native antibodies with immediate bioavailability. Herein, we report a modification-free, convenient “mix-and-go” strategy for the cytosolic delivery of native antibodies to different live mammalian cells efficiently, with minimal endolysosomal trapping and immediate bioavailability. By simply mixing a cell-permeant bioadaptor (derived from protein A or TRIM21) with a commercially available off-the-shelf antibody, the resulting noncovalent complex could be immediately used for intracellular delivery of native antibodies needed in subsequent cytosolic target engagement. The versatility of this approach was successfully illustrated in a number of applications, including antibody-based, live-cell imaging of the endogenous protein glutathionylation to detect oxidative cell stress, antibody-based activation of endogenous caspase-3, and inhibition of endogenous PTP1B activity, and finally TRIM21-mediated endogenous protein degradation for potential targeted therapy. Our results thus indicate this newly developed, “mix-and-go” antibody delivery method should have broad applications in chemical biology and future drug discovery.

INTRODUCTION

The high affinity and exquisite selectivity of antibody—antigen interaction are extensively exploited in basic research and biomedical applications.1–4 For example, as powerful imaging reagents, fluorescently labeled antibodies are used in immunofluorescence (IF) to detect endogenously expressed antigens, but the technique is mostly limited to fixed cells and tissues. Because of the superior selectivity and potency over small-molecule drugs, antibody-based drugs have become the largest and fastest growing class of therapeutics on the market.2 The recent dramatic advances in protein engineering and medicinal chemistry have indeed expanded the utility of antibodies in the clinic.5–7 Accordingly, antibodies or their fragments could also be directly fused to cell-penetrating peptides (CPPs) by using approaches such as chemical or chemoenzymatic labeling, protein transfection, and tumor-specific endolysosomal escape.7 In another approach, a genetically engineered immunoglobulin G (IgG) was internalized into living cells through clathrin-mediated endocytosis, subsequently escaped from early endosomes through pore formation caused by pH-induced conformational changes and selectively bound to oncogenic Ras mutants, resulting in effective blocking of protein—protein interaction (PPI).8 Antibodies or their fragments could also be directly fused to cell-penetrating peptides (CPPs) by using approaches such as chemical or chemoenzymatic labeling, protein trans-
splicing, and native chemical ligation (NCL), all of which led to their successful cell uptake by endocytosis-dependent mechanisms.9−11 All of these methods, however, have limited applications, due to the need for chemical modifications or genetic engineering, as well as the complexity and inefficiency.

As for the delivery of native antibodies into mammalian cells, several approaches have also been reported.14−17 Commercially available protein transfection reagents typically involve the use of lipid micelles to encapsulate native antibodies and to achieve cytosolic delivery, but often with low delivery efficiency and severe endolysosomal trapping.18 Painstaking optimizations of lipid composition and micelle formulation may lead to an improvement in protein delivery efficiency, but the process is extremely laborious and only works with certain cargos (i.e., highly charged proteins).14,15 Futaki et al. reported a strategy in which common monoclonal antibodies were successfully delivered to mammalian cells by using an endosomolytic peptide (i.e., L17E, Supplementary Figure 1);15 subsequent liberation of the antibody from endosomes rendered it cytosolically available for target engagement. Another strategy involved the use of IgG-binding proteins genetically fused to CPPs, and upon binding to the Fc domain of IgG, the resulting noncovalent complex could readily cross the cell membrane.16,17 Nevertheless, the above-mentioned strategies are constantly plagued with issues such as low cytosolic delivery efficiency and high cytotoxicity resulting from the membrane lytic properties of these delivery agents. Consequently, cytosolic delivery of native functional antibodies with high delivery efficiency and minimal endolysosomal trapping remains a key limitation currently, impeding further intracellular applications.

Cell-penetrating poly(disulfide)s (CPDs; Supplementary Scheme 1), originally developed by Matile et al., are rapidly emerging as a class of highly promising cell-penetrating polymers. CPDs are synthetic mimics of poly arginine CPPs, in which the polypeptide backbone has been replaced with poly(disulfide).20,21 Both the positively charged guanidiniums and the disulfide backbone of CPDs promote their cell
membrane accumulation and intracellular delivery via thiol-exchange mechanisms, with minimal endolysosomal trapping. Once inside the cytoplasm, CPDs are rapidly depolymerized by endogenous glutathione (GSH), rendering them less cytotoxic than CPPs. Upon suitable chemical modifications followed by subsequent covalent conjugations to different CPDs via bioorthogonal reactions, native antibodies have been successfully delivered into mammalian cells, but such modifications often lead to a potential loss of antibody activities. Alternatively, CPD-coated biodegradable nanocapsules have been used as nanocarriers to encapsulate native antibodies for successful intracellular delivery and on-demand release (Supplementary Figure 2). This method required exposure of the cargo to harsh conditions during nanocapsule formation and could cause antibody denaturation. Moreover, both strategies necessitate extensive chemical expertise and careful optimizations. Notwithstanding such shortcomings, various strategies based on CPDs and their derivatives for intracellular biomolecular delivery have become increasingly popular in recent years.

We sought general and practical approaches for highly efficient cytosolic delivery of native functional antibodies that require no intervention from chemists, are noncytotoxic, and can render the delivered cargo immediately bioavailable with minimal endolysosomal trapping. Herein, we report one such method through the use of antibody-binding bioadaptors (e.g., Staphylococcal protein A, and tripartite motif-containing protein 21 or TRIM21) to “link” native antibodies indirectly with CPDs (Figure 1a,b). Protein A (PrtA) is a bacterial protein derived from Staphylococcus aureus, commonly used as an affinity matrix for purification of IgG via strong binding to its Fc region (CH2–CH3 site, with $K_d \approx 2 \times 10^{-9}$ M). TRIM21 is an E3 ubiquitin ligase found in the intracellular antibody-mediated proteolysis pathway. During cell infection by a pathogen, TRIM21 recruits the ubiquitin-proteasome system to the cytosolic antibody-bound pathogen and causes it to undergo proteasome-mediated destruction. A recent report shows the use of TRIM21 in a so-called “trim-away” approach (Figure 1c), whereby the rapid degradation of endogenous target proteins in mammalian cells was successfully achieved upon intracellular delivery of appropriate antibodies together with TRIM21 through microinjection or electroporation. Similar to protein A, TRIM21 also binds to the Fc domain of IgG (CH2–CH3 site) via its carboxyl-terminal PRYSPRY domain with high affinity ($K_d \approx 3.7 \times 10^{-8}$ M). Therefore we envisaged that both protein A and TRIM21 could be made cell-permeant and subsequently serve as potent bioadaptors in our strategies for efficient cytosolic delivery of native functional antibodies (Figure 1a,b); since various forms of protein A and recombinant (His)$_6$-tagged TRIM21 are commercially available, they could be conveniently “bioconjugated”, either covalently or noncovalently, to appropriate CPDs by using our previously reported strategies. The resulting cell-permeant bioadaptors (named CpA1, CpA2, and CpT), upon simple mixing with a native antibody (i.e., IgG) by using our “mix-and-go” approach, would allow the immediate formation of a tight ternary complex (CPD-adaptor-Ab) via noncovalent interactions between PrtA/IgG or TRIM21/IgG. Upon successful cellular uptake and endogenous GSH-triggered CPD depolymerization, the adaptor-Ab complex would be released in the cell cytosol. Since the bioadaptor (i.e., CpA1, CpA2, or CpT) only bound to the Fc domain of the delivered antibody, subsequent antibody–antigen interaction required for intracellular target engagement would not be compromised. The entire “mix-and-go” process could be carried out under aqueous conditions at neutral pH, with no prior need of genetic or chemical modifications on the native IgG cargo, and hence this strategy would be highly versatile and applicable with a wide range of off-the-shelf, commercially available antibodies, including those from different hosts and classes. Given that the cell-permeant property of these bioadaptors were built within themselves, easy exchange of different IgGs for cargo delivery could be readily accomplished. In the current work, the practicality of this antibody delivery approach has been successfully illustrated in a number of applications, including antibody-based live-cell imaging of endogenous protein glutathionylation for the detection of oxidative cell stress, and antibody-based activation of endogenous caspase-3 activity and inhibition of endogenous PTP1B activity. Finally, by combining this novel “mix-and-go” delivery strategy with “trim-away”, we have shown that successful degradation of an endogenous protein target (i.e., α-synuclein) could be achieved, thus enabling significant expansion of the proteolysis targeting chimeras (PROTACs) for potential targeted therapy.

**RESULTS**

**Preparation of Cell-Permeant Bioadaptors.** The three cell-permeant antibody-binding bioadaptors, CpA1, CpA2, and CpT, were prepared by “bioorthogonal” conjugation of suitable CPDs to chemically modified PrtA or (His)$_6$-tagged TRIM21 (Figure 1a, Supplementary Tables 1–2 and Figures 2–3). PrtA was modified at its surface-exposed lysine residues with a self-immolative linker NBL to generate NBL-PrtA (traceless tagging approach), or at its glycosylated site by using site-specific oxime conjugation to yield OXPrtA (PTM-based tagging). Following “click” conjugation with CPDs (C$_6$CPD and AOCPD, respectively), t$_2$CPD-NBL-PrtA (also named CpA1) and AOCPD-OXPrtA FITC (also named CpA2) were obtained, respectively. The former labeling method could be used to produce CPD-PrtA conjugates from naturally occurring or recombinant PrtA (as well as Protein G and Protein L), while the latter labeling approach allowed for dual-functionalization of native PrtA with CPD and a lysine-reactive fluorescent dye (i.e., fluorescein isothiocyanate, or FITC). In our antibody delivery experiments, CpA1 was used in most general applications, while the fluorescently labeled CpA2 was used to study cell-uptake and subcellular localization of both the bioadaptor and the antibody cargo by confocal laser scanning microscopy (CLSM). Both CpA1 and CpA2 were generated prior to use without any purifications and characterized by SDS-PAGE and in-gel fluorescence scanning (Supplementary Figure 3). The TRIM21-derived cell-permeant bioadaptor, CpT (also named Ni-NTA-CPD (His)$_6$-TRIM21), was obtained by attaching Ni-NTA-CPD to a commercially available (His)$_6$-tagged TRIM21 protein via noncovalent interaction ($K_d$ of Ni-NTA/(His)$_6 < 10^{-7}$ M). Subsequent “mix-and-go” complexation of a desirable antibody cargo with the respective cell-permeant bioadaptor was carried out by simple mixing, followed by direct cell incubation for cytosolic delivery. The CPD-adaptor-Ab complexes prepared in this work, together with various controls are summarized in Supplementary Tables 1 and 2. Model antibody delivery experiments as well as mechanistic studies were first carried out with PrtA-based, cell-permeant bioadaptors (CpA1 and CpA2; Figure 2). Following which, the optimum conditions were
Figure 2. Cellular uptake of IgG using cell-permeant bioadaptors. (a) CLSM images showing cellular uptake of CpA2-IgGCy5 (50 nM, 1 h). (IgGCy5): red; (CpA2, labeled as OXPrtAF/ITC): green; (Hoechst): blue. Inset: DIC images. Scale bar = 15 μm. (b) Flow cytometry (FACS) quantification of IgGCy5 fluorescence following delivery with CpA2 (CpA2-IgGCy5, 50 nM, 1 h). (Left) Representative flow cytometry histograms. (Right) Percentage of IgGCy5-positive cells (blue bars) and fold-increase in mean IgGCy5 fluorescence (gray bars) over negative controls. (c) Western blotting (WB) analysis of HeLa cells upon incubation with TzCPD, CpA1, IgG, or CpA1-IgG (50 nM, 1 h). Total lysates were immunoblotted with antihuman IgG antibody that recognizes its heavy chain. (d, e) Transmission electron microscope (TEM) images and dynamic light scattering (DLS) measurements of different IgG complexes. Scale bar = 100 nm. Data are presented as mean ± s.d. (n = 3). (f) CLSM images of HeLa cells incubated with CpA1-IgGCy5 (50 nM, 1 h) in the presence or absence of DTNB (4.8 mM). Inset: DIC images. Scale bar = 15 μm. (g) FACS quantification of IgGCy5 uptake (50 nM of CpA1-IgGCy5, 1 h) from HeLa cells treated with different inhibitors. Data were normalized to those of HeLa cells treated with CpA1-IgGCy5 only (Blank). (h) Temperature-dependent CpA1-IgGCy5 uptake by HeLa cells (50 nM, 1 h), as determined by flow cytometry. Data were normalized to those obtained at 37 °C. (i) CLSM images of HeLa cells after 1-h incubation with CpA1-IgGCy5 (50 nM, in red), and colocalization with TMR-Dextran to track endosomes (Global Pearson coefficient R = 0.27), LysoTracker Green DND-26 to track lysosomes (R = 0.15), MitoTracker Green to track mitochondria (R = 0.38), and CellMask Orange to track plasma membrane (R = 0.42). Scale bar = 15 μm. All tracker channels were colored in green. (j) FACS quantification of IgGCy5 (50 nM, 4 h) uptake in HeLa cells treated with different CPD-based delivery methods: bioadaptor approach (CpA1) and direct conjugation approaches (NBL/Oxime/ThioLinker). Cells treated with unmodified IgGCy5 (normalized as 1) or without CPD conjugation were run as negative controls. Delivery methods with L17E and Pro-Ject were tested concurrently under identical conditions. (k) HeLa cells were incubated with IgGCy5 or CpA1-IgGCy5 (final concentration 50 nM) for 16 h and imaged with confocal microscope (“mix-and-go” approach). Alternatively IgGCy5 or CpA1-IgGCy5 (working concentration 1.67 μM) were mixed with HeLa cells, and electroporation was performed with the Neon Transfection System. Electroporated cells were grown in an eight-well imaging dish (concentration of IgGCy5 in medium equivalent to 50 nM) for 16 h for complete adherence and imaged with confocal microscope. Cells were then fixed, extensively washed, and imaged again (panels 2 and 4, fixed cell). Scale bar = 15 μm. (Right) Corresponding line-scanning fluorescence intensity profiles. (n) NPC/cytoplasm and NPC/nucleus fluorescence ratio in HeLa cells treated as described in (m) (bars 1–4 correspond to panels 1–4 in m) compared to standard immunofluorescence (bar IF, Supplementary Figure 14a). Error bars were obtained from five different measurements.
applied to other experiments with CpA1 (Figures 3, 4, and 5) and finally to TRIM21-related experiments with CpT (Figure 6).

Cytosolic Delivery of IgG Using Cell-Permeant Bioadaptors. By using human IgG as our model cargo, we first investigated whether cellular uptake by mammalian cells was possible after complexation with CpA1 or CpA2. In order to image cellular uptake of both IgG and PrtA, a Cy5-labeled antibody (IgG-Cy5; Supplementary Figure 4) and the fluorescently labeled CpA2 were used. As shown in Figure 2a (and Supplementary Figure 6), successful intracellular delivery of both IgG-Cy5 (pseudocolored in red) and PrtA-FITC (pseudocolored in green) was observed for CpA2-IgG-Cy5. As expected, without CPD, CpA2-IgG-Cy5 alone could not enter cells. To further confirm our results, flow cytometry was used to quantify uptake efficiency of IgG-Cy5 by determining either the percentage of cells with fluorescence (IgG-Cy5-positive cells) or the amount of protein delivered (fold-increase in IgG-Cy5 fluorescence; Figure 2b). CpA2 exhibited excellent translocation efficiency for the delivery of IgG-Cy5 (99% IgG-Cy5-positive cells), even when only 50 nM of CpA2-IgG-Cy5 was used (right graph in Figure 2b). This is significantly higher than previously reported strategies for IgG delivery.15,19

CLSM and flow cytometry were used to confirm the successful intracellular delivery of IgG-Cy5 by CpA1 as well (Supplementary Figures 6f,g and 7). In addition, we analyzed the successful cytosolic delivery of unmodified human IgG by Western blotting (WB) analysis of total lysates from cells treated with CpA1-IgG (Figure 2c and Supplementary Figure 7c); results showed the IgG delivery was completely CpA1-dependent (see lane 5 in Figure 2c).

In our imaging experiments, we noted persistent punctate fluorescence signals arising from successfully delivered, fluorescently labeled antibodies, despite minimal entrapment in the endolysosomal vesicles. Such observations had also been reported previously in other antibody delivery systems by using IgG-binding proteins genetically fused with CPPs,17,38 as well as in some CPP-mediated biomolecular delivery systems.12,24,27,28 This phenomenon is atypical, as in other protein delivery systems, where green fluorescent protein (GFP) was typically used as cargo, diffuse fluorescence signals could be observed upon successful cytosolic delivery.15,19 We noted, however, this punctate fluorescence signal pattern was reminiscent of nanoparticle-based protein delivery systems.15,24

Since protein A contains multiple binding domains that bind to the Fc region of an antibody,12 we hypothesized that this might have led to nanoparticle formation in CPD-PrtA-IgG complexes (i.e., CpA1-IgG or CpA2-IgG), even-
tually causing the punctate intracellular signals observed in the delivered cargos. Both transmission electron microscopy (TEM) and dynamic light scattering (DLS) measurements were carried out on the 1:1 mixture of both PrtA-IgG and CpA1-IgG complexes (Figure 2d,e); apparent protein nanoparticle formation (~80–90 nm in size) was successfully observed in both cases. In sharp contrast, the native IgG alone showed a typical protein size distribution (<10 nm) by DLS and was free of any protein aggregation or formation of higher-order nanoparticles in both TEM and DLS experiments.

**Cellular Uptake Mechanism and Efficiency.** In order to evaluate IgG cell uptake mechanisms, endocytosis inhibition experiments were carried out with CLSM and quantitative FACS analysis (Figure 2f–h, Supplementary Figure 8). Similar to previous reports for all CPD-facilitated delivery,20–26 with the CpA1-IgG<sup>Cy5</sup> complex, we observed robust cell uptake of IgG<sup>Cy5</sup> in HeLa cells (in red; Figure 2f, left panel), which was subsequently determined by FACS and CLSM to be inert to most endocytosis inhibitors but significantly inhibited by SS'-dithioobis-2-nitrobenzoic acid (DTNB, a thiol blocker) and temperature-dependent (Figure 2g,h and Supplementary Figure 8), indicating thiol-mediated uptake without significant endosomal capture.21 CLSM was also employed to image the distribution of delivered cargos in subcellular organelles of live HeLa cells by using organelle-colocalizing trackers (Figure 2i, Supplementary Figure 9 and 10); intracellularly delivered IgG<sup>Cy5</sup> (in red) showed minimal colocalization with endosomes and lysosomes (in green). Three-dimensional projections of z-stack images from treated HeLa cells and real-time imaging experiments further showed mostly cytosolic distribution of the delivered IgG<sup>Cy5</sup> (Supplementary Figures 9 and 10). In experiments where free IgG was supplemented in the cell culture medium, the internalization of IgG<sup>Cy5</sup> was not affected (Supplementary Figure 11d).

We next used IgG<sup>Cy5</sup> to compare the cytosolic delivery efficiency of this newly developed “mix-and-go” biodaraptor approach with our previously reported covalent conjugation methods,5,24 as well as the L17E and standard lipid-based protein transfection (Pro-Ject) methods.15,39 As shown in Figure 2j (and Supplementary Figure 12a), all CPD-facilitated methods enabled robust cytosolic delivery of IgG<sup>Cy5</sup> with good efficiency. We were pleased to find that, in both CLSM and flow cytometry experiments, our “mix-and-go” strategy with CpA1-IgG<sup>Cy5</sup> conferred the highest delivery efficiency than other CPD approaches (via direct CPD conjugations). Both the L17E and Pro-Ject approaches on the other hand, showed minimal antibody delivery under identical conditions (50 nM, 1 h). The effect of CpA1-IgG<sup>Cy5</sup> on cell viability was next examined (Supplementary Figure 12c); at 50 nM cargo concentrations (4 h incubation), HeLa cells treated with various CPD-based reagents showed negligible cell toxicity after 12 h, whereas those treated with either L17E or Pro-Ject reagents showed noticeable cell death. No significant cytotoxicity was observed on HeLa cells treated with 1<sub>g</sub>CDP or CpA1-IgG (up to 1 μM) for 24 h (Supplementary Figure 12d). We further tested the “mix-and-go” method on other mammalian cell lines (Supplementary Figure 12e); in all cases, IgG<sup>Cy5</sup> was successfully delivered in a CPD-dependent manner, albeit with varying degrees of efficiency.

When developing intracellular protein delivery systems, it is important to confirm the fraction of the cargo that actually reaches the cytosol, which is typically the material that will confer a biological effect. Physical methods (such as micro-injection and electroporation) are considered the most effective approaches to achieve direct cytosolic delivery of proteins as plasma membrane is reversibly permeabilized to allow the transport of proteins across the membrane.6 Therefore, we have also compared our “mix-and-go” approach with electroporation. Electroporation is used here as a “gold standard” of cytosolic delivery to assess the fluorescent signal pattern, uptake efficiency, and antibody binding. To deliver antibodies into cells by electroporation, we used a device for gene transfection that does not involve classical cuvettes (Neon Transfection System), which has been adapted to the electrotransfer of monoclonal antibodies to cultured cells.34,35 IgG<sup>Cy5</sup> or CpA1-IgG<sup>Cy5</sup> were delivered into HeLa cells through a “mix-and-go” approach or electroporation, respectively, and cells were assessed for fluorescence 16 h postdelivery with flow cytometry and confocal microscopy. For protein delivery by electroporation, fluorescence microscopy revealed diffuse fluorescence with IgG<sup>Cy5</sup>, but fluorescence signals were punctate with CpA1-IgG<sup>Cy5</sup> (Figure 2k, Supplementary Figure 13c). When CpA1-IgG<sup>Cy5</sup> was delivered by electroporation, it is reasonable to assume the cargo directly reached the cytosol without going through endosomal compartments. In this case, the punctate signals still existed, so the main cause for such signals was not a result of endosome trapping. This finding further supported our earlier mechanistic and colocalization studies which also showed minimal endosome trapping of delivered antibodies (Figure 2f–i). The overall intracellular uptake efficiency of CpA1-IgG<sup>Cy5</sup> upon delivery with the “mix-and-go” approach or electroporation was further examined by flow cytometry, which quantitatively determined either the percentage of cells with fluorescence (IgG<sup>Cy5</sup>-positive cells) or the total amount of cargo delivery (fold-increase in IgG<sup>Cy5</sup> fluorescence; Figure 2l); percentages corresponding to IgG<sup>Cy5</sup>-positive cells reached up to more than 95% for both a “mix-and-go” approach and electroporation, while the total amount of IgG<sup>Cy5</sup> delivered by electroporation was twice as much as that for a “mix-and-go” approach (judging from the fold-increase in mean fluorescence intensity).

Furthermore, the ability of CpA1 for delivering functional antibody into cytosol was evaluated by using an antineural pore complex (NPC) labeled with Cy5 (Ab<sub>NPC-Cy5</sub>). When HeLa cells were treated with 50 nM CpA1-Ab<sub>NPC-Cy5</sub> for 1 h, localization of Ab<sub>NPC-Cy5</sub> at the peripheral of the nucleus was observed, in a fashion consistent with the standard IF (Figure 2m panel 1, Supplementary Figure 14). This result was further quantified by the corresponding line-scanning fluorescence intensity profiles (right graph); significant overlaps between Ab<sub>NPC-Cy5</sub> fluorescence (red line) and the cell nucleus (blue line) were observed, with high spikes at the peripheral of the nucleus region (yellow arrows). After fixation and extensive washing, the signal pattern remained (Figure 2m, panel 2). These results are in accordance with electroporation results (Figure 2n, panels 3 and 4). The NPC/cyttoplasm and NPC/nucleus ratios were calculated as an indicator for efficiency of cytosolic delivery (Figure 2n, see section 4.4 in Supporting Information for details), as only true cytosolic delivery would result in Ab<sub>NPC-Cy5</sub> binding to NPC. For a “mix-and-go” approach at 50 nM (bars 1/2 in Figure 2n), the NPC/cyttoplasm ratio was comparable to standard IF (bar “IF”) and better than electroporation (bars 3/4). The NPC/nucleus ratio was lower than the corresponding NPC/cyttoplasm ratio, because Ab<sub>NPC-Cy5</sub> signals not only localized on the nuclear membrane, but also within the nucleus, upon delivery with...
both the “mix-and-go” approach and electroporation. We are aware that, in order to accurately and quantitatively compare the amount of \( \text{AbNPC-GS} \) bound to NPC between “mix-and-go” approach and electroporation, careful titration of \( \text{AbNPC-GS} \) concentration and number of cells would be required, and we need to determine the number of NPC molecules and \( \text{AbNPC-GS} \) per cell. However, with our current experiments, we could reasonably conclude that a sufficient amount of functional antibodies delivered by our “mix-and-go” approach had successfully reached cytosol and actively engaged their intracellular targets, in a fashion most consistent with electroporation.

**Delivery of Commercial Antibodies.** We next investigated whether the current approach could be used for cytosolic delivery of different commercially available antibodies. Commercial antibodies derived from different host species and classes have varying degrees of affinity toward protein A (PrtA), protein G (PrtG), and protein L (PrtL), and may contain different stabilizing additives such as BSA and gelatin. In previous covalent approaches, laborious buffer exchange and BSA/gelatin removal were needed prior to antibody modification/cellular delivery, without which antibody labeling efficiency would be significantly compromised. Our current bioadaptor approach on the other hand was hassle-free (that is, once the cell-permeant bioadaptors were prepared) and generally applicable to different off-the-shelf IgGs. We first took commercial HRP-conjugated antibodies from different species (goat, rabbit, and mouse) and showed they were successfully delivered into HeLa cells with CpA1 (Supplementary Figure 1A); by imaging the enzymatic activity of intracellularly delivered HRP, we detected strong chemiluminescent signals in live HeLa cells treated with rabbit or mouse IgG-HRP (panels 3 and 4, respectively) and moderate signals from goat IgG-HRP (panel 2). Protein A was previously reported to bind to rabbit/mouse IgGs and goat IgG with strong and moderate affinity, respectively. These results thus show that antibody delivery efficiency with our “mix-and-go” bioadaptor approach was intimately dependent upon the relative affinity between the cell-permeant bioadaptor (i.e., CpA1) and the antibody cargo. We further showed that, in addition to PrtA, both recombinant PrtG and PrtL chemically modified with NBL and “clicked” with \( \text{γ-CPD} \) could be successfully used as additional bioadaptors for cytosolic delivery of different antibodies (Supplementary Figure 1B). Moreover, by adopting a reversible permeabilization protocol upon cytosolic delivery of fluorescently labeled anti-GAPDH antibody (named \( \text{AbGAPDH-Cy} \)) with CpA1-Ab\( \text{GAPDH-Cy} \), we examined the cellular distribution of \( \text{AbGAPDH-Cy} \) in HeLa cells before/after live-cell permeabilization (Supplementary Figure 1C). By incubating live HeLa cells with low dosage of Triton X-100 (0.015%, 3 min), reversible permeabilization allowed effective removal of excessive unbound \( \text{AbGAPDH-Cy} \), which caused severe background fluorescence, thus enabling direct imaging of only positive antibody–antigen interaction. Collectively, these results thus show that, with our “mix-and-go” antibody delivery method by using cell-permeant bioadaptors (CpA1 and CpA2), (1) commercial antibodies could be used off-the-shelf, without the need for laborious buffer exchange and/or removal of additives; (2) the delivered antibodies remained biologically functional upon entering the cell cytosol, and (3) they became immediately bioavailable.

Having successfully established that this antibody delivery strategy could be used for the effective delivery of a variety of commercially available antibodies, we next evaluated whether it enables easy exchange of cargo IgGs in order to expand its potential applications. We used three different biologically relevant systems, (1) sensing endogenous changes in post-translational protein S-glutathionylation (PSSG) under stimulated conditions, (2) activation of endogenous caspase-3 activity, and (3) inhibition of endogenous PTP1B activity.

**Cytosolically Delivered Anti-GSH Can Detect Protein Glutathionylation.** Imaging tools capable of detecting endogenous protein S-glutathionylation (PSSG) from live mammalian cells have drawn significant interests in recent years. When cells are under oxidative stress, PSSG occurs, whereby cysteines in affected proteins form disulfide bonds with endogenous GSH resulting in the formation of glutathionylated proteins (protein-SSG) (Figure 3a). This process is catalyzed by redox enzymes together with reactive oxygen species (ROS) and is closely associated with the progression of many diseases. We previously showed that a nanomaterial-based biosensor containing a functional anti-GSH antibody (named \( \text{AbGSH} \)) immobilized on nanoparticles was able to detect endogenous PSSG. By using our “mix-and-go” bioadaptor approach, we wondered whether a cytosolically delivered, fluorescently labeled \( \text{AbGSH} \) (named \( \text{AbGSH-AF647} \)) could also be used to detect endogenous PSSG in live HeLa cells (Figure 3b).

As shown in Figure 3c, successful cytosolic delivery of \( \text{AbGSH-AF647} \) was observed by CLSM in cells treated with CpA1-Ab\( \text{GSH-AF647} \) under our earlier optimized delivery conditions (top panel, in red); no fluorescence signal was detected in cells treated with \( \text{AbGSH-AF647} \) alone (i.e., without CpA1, bottom panel). Upon further treatments with phenylarsine oxide (PAO) and diamide to induce endogenous PSSG upregulation, the resulting HeLa cells were directly fixed, extensively washed, and then imaged again (i.e., standard IF protocol shown in Figure 3b - “Fix first, permeabilize later” strategy). As shown in Figure 3d, we were able to successfully detect elevated levels of PSSG in cells treated with PAO/diamide (top panel), and for control cells (no PAO/diamide treatment, and therefore no PSSG upregulation), no fluorescence was detected (bottom panel). Next, we investigated whether this bioadaptor-based antibody delivery could also be used to image the entire endogenous PSSG process under live-cell conditions, by using the earlier described reversible permeabilization protocol. Upon successful cytosolic delivery of CpA1-Ab\( \text{GSH-AF647} \) to HeLa cells followed by PAO/diamide treatment to induce endogenous PSSG upregulation, the resulting cells were permeabilized (while still alive; Figure 3b), washed (to remove free \( \text{AbGSH-AF647} \)), and directly imaged. In this way, only \( \text{AbGSH-AF647} \) that was still tightly bound to endogenous glutathionylated proteins would be retained in the resulting cells, thus providing PSSG-dependent positive signals with a low background fluorescence. As shown in Figure 3e, we successfully detected positive fluorescence signals only in cells treated with PAO/diamide (top panel), but not in control cells (no PAO/diamide treatment; bottom panel). The same red fluorescence signals in these PSSG-upregulated cells persisted even after cell fixation (Figure 3f, top-left panel; i.e., the “Permeabilize first, fix later” strategy shown in Figure 3b). Unequivocal confirmation of these positive fluorescence signals (in Figure 3d–f, top panels) as a result of PSSG upregulation was further established by imaging the same cells with a secondary antibody (antimouse-FITC; Figure 3f, top-right panel); strong green fluorescence was detected (bottom panel)
fluorescence signals were again detected in PAO/diamide-treated cells, but not in PAO/diamide-free control cells (Figure 3f, bottom panels). Similar quantitative results on these treated cells were obtained by flow cytometry (Figure 3g), which further corroborated relative changes in endogenous PSSG levels with AbGSH-AF647 fluorescence. The levels of protein glutathionylation in these cells were confirmed by standard IF techniques (Supplementary Figure 17).

Finally, electroporation experiments were performed to compare the fluorescent signal pattern with the "mix-and-go" approach (Supplementary Figure 18). Electroporation of AbGSH-AF647 resulted in a diffuse signal in cytoplasm (Supplementary Figure 18a). After PSSG induction and live cell permeabilization, the cells were probed with antimouseFITC (Supplementary Figure 18b). We successfully detected positive fluorescence signals only in cells treated with PAO/diamide (top panel), but not in control cells (no PAO/diamide treatment; bottom panel). The electroporation experiment further validated that the protocol (antibody delivery, PSSG induction followed by live cell permeabilization) can be used for PSSG detection. Hence, we have successfully demonstrated that the cytosolic delivery of AbGSH-AF647 by using the "mix-and-go" bioadaptor approach, was indeed capable of sensitively imaging PSSG from live mammalian cells. Live-cell imaging of changes in protein post-translational modification (PTM) with small molecule-based biosensors is highly challenging and has thus far been achieved mostly with genetically encoded biosensors but with very limited success.47 Our antibody-based biosensor approach reported herein may thus provide a potentially powerful strategy under live-cell settings (i.e., live-cell imaging).
cell IF), to image a variety of PTMs, e.g., phosphorylation/dephosphorylation, methylation/demethylation, lipidation, acetylation/deacetylation, and others, all of which are commonly known to be dysregulated in cancer signaling pathways.48

Cytosolically Delivered Anticaspase-3 Can Activate Endogenous Caspase-3. Caspases are aspartate-specific cysteine proteases that have important therapeutic implications because of their vital roles in apoptosis and inflammation.49,50 Among them, caspase-3 is of particular interest since it is a well-known executioner caspase in apoptosis.51 This enzyme is endogenously expressed as the inactive full-length zymogen (procaspase-3; 35 kDa). Activation of caspase-3 results in proteolytic cleavage of the proenzyme leading to generation of a small fragment (p12; ∼12 kDa) and eventually a catalytically active large fragment (p17; ∼17 kDa). While many small-molecule inhibitors of caspase-3 are known, few caspase-3 activators have been reported thus far.52−54 To the best of our knowledge, no antibody-based activator of caspase-3 has been documented at present. Recently, progress has been made in the discovery of antibody-based enzyme activators; e.g., an engineered synthetic antibody capable of rescuing the enzymatic activity of a cancer-associated isocitrate dehydrogenase 1 (IDH1) mutant was reported.55 With the intention of discovering potential antibody-based inhibitors as well as activators of caspase-3, we took two commercially available anticaspase-3 antibodies, CST9664 and CST9662,56,57 and evaluated their endogenous caspase-3 inhibition/activation properties upon the “mix-and-go” intracellular delivery. On the basis of the product information provided by the vendor, these two antibodies bind to the enzyme at different binding sites; CST9664 can specifically detect the large fragment (p17/19) of cleaved caspase-3 but not the full-length zymogen,56 while CST9662 recognizes both procaspase-3 and its large fragment (p17/19).57 As shown in Figure 4a,c (and Supplementary Figure 19), HeLa cells treated with staurosporine (STS, 2 μM) showed a clear sign of morphological changes (i.e., cell rounding; see Figure 4c, panel 6), which were typical of cells undergoing apoptosis. This was subsequently confirmed by successful detection of endogenous caspase-3 activation upon treating the cells with a cell-permeable fluorogenic caspase-3 substrate, Ac-DEVD-AMC (Figure 4a, panel 6). In contrast, a lower dose of STS (0.5 μM) barely caused any detectable increases in caspase-3 activation under similar cell growth conditions (compare panels 1/2 in Figure 4a, and bars 1/2 in Figure 4b). In a different set of experiments done concurrently, HeLa cells upon successful cytosolic delivery of CST9664-treated cells (without or with 0.5 μM

![Figure 5](image-url)

Figure 5. Cytosolically delivered anti-PTP1B antibody inhibits endogenous PTP1B activity. (a) Schematic representation of how anti-PTP1B might cause cellular effects on insulin pathway. +p: phosphorylation; −p: dephosphorylation; +py: tyrosine phosphorylation; −py: tyrosine dephosphorylation. (b) In vitro inhibition efficiency of MABS197 (2, 100 nM), compound 3 (3, 200 μM) or Na3VO4 (4, 10 μM) toward PTP1B. Relative fluorescence (RFU) in each experiment was normalized to that of PTP1B with no inhibitor (1, 20 ng) (set as 1). (c) Concentration-dependent in vitro inhibition of PTP1B. PTP1B (20 ng) was preincubated with MABS197, compound 3, Na3VO4 or IgG according to indicated concentrations for 30 min, followed by addition of DiFMUP (40 μM). RFU was normalized to that of PTP1B with no inhibitor (set as 1). (d) Phosphorylation upregulation of IRS1 in MABS197-treated HeLa cells. Serum-starved cells were incubated with CpA1-MABS197 (100 nM, 4 h), compound 3 (200 μM, 1 h), Na3VO4 (10 μM, 30 min) or insulin (10 nM, 30 min, in the presence of 10 μM Na3VO4). (e) Phosphorylation upregulation of EGFR in MABS197-treated A431 cells. A431 cells were similarly incubated with CpA1-MABS197 (100 nM, 4 h), compound 3 (200 μM, 1 h), Na3VO4 (10 μM, 30 min) or EGF (50 ng/mL, 10 min) upon serum starvation.
STS in panels 3/4, respectively), and interestingly, the presence of low-dose STS (0.5 μM) apparently accelerated this process (compare bars 3/4 vs bars 1/2 in Figure 4b). In sharp contrast, neither caspase-3 activation nor inhibition was detected in CST9662-treated cells (panel 5 in Figure 4a, even with 0.5 μM STS). Since CST9664 only recognizes the cleaved p17/19 of caspase-3 and not the full-length procaspase-3, we hypothesized its apparent caspase-3-activating property might be similar to that of some reported small-molecule caspase-3 activators (Figure 4d).52−54 By binding to specific regions in p17/19, it caused conformation changes in procaspase-3 and subsequently led to the enzyme’s eventual activation.58 The fact that cells cotreated with CpA1-CST9664 and STS (0.5 μM) showed an accelerated caspase-3 activation might indicate the presence of an elevated level of the p19/p12 complex (in active conformation) being stabilized upon recognition/binding by CST9664 (but not CST9662), whose autocatalysis process could be further sped up upon initial caspase-3 cleavage/activation (Figure 4d). Surprisingly, no significant morphological changes were observed in cells treated with CpA1-CST9664 alone (albeit with caspase-3 activation), and opposite effects were observed for cells treated with low-dose STS (0.5 μM) (i.e., significant morphological changes but with minimal/lower caspase-3 activation; compare panels 2/3 in Figure 4a and 4c). It is known that STS, a small-molecule general kinase inhibitor that does not directly act on caspase-3, could induce cell apoptosis by both caspase-dependent and caspase-independent pathways.59,60 Our results thus indicate CST9664, upon its successful cell entry, became immediately available for direct binding to caspase-3 and quickly activated its enzymatic activity, before ensuing activation of caspase-dependent apoptosis pathways. Notwithstanding further experiments that will be needed to unequivocally confirm our hypotheses, the discovery of CST9664 herein, as a rare antibody-based reagent capable of both specific caspase-3 binding and activation, might indicate a much more widespread presence of other antibodies (similar to small molecules) as potential enzyme activators.54

Cytosolically Delivered Anti-PTP1B Can Inhibit Endogenous PTP1B Activity. PTP1B is a key intracellular protein tyrosine phosphatase and a validated target for type-2 diabetes and obesity.61 By directly dephosphorylating insulin receptor (IR) and insulin receptor substrates (IRS), it negatively regulates insulin signaling pathways (Figure 5a). Small-molecule inhibitors of PTP1B have been intensively pursued for years, but few are sufficiently selective to be developed into potential drugs, due to the highly conserved active site in most protein tyrosine phosphatases (PTPs) and the low cell permeability of these inhibitors which in many cases are highly negatively charged phospho-tyrosine (pTyr) mimics.62 With their unique ability to bind to virtually any immunogenic targets, antibodies are considered ideal “magic bullets” against many diseases, if they can cross the cell membrane and target intracellular antigens.1−3 Antibodies raised against the active site of PTP1B therefore could offer a clear advantage of high specificity and potency over small-molecule PTP1B inhibitors. In fact, conformation-sensor intrabodies have been reported to stabilize oxidized PTP1B, leading to subsequent inhibition of enzymatic activity.63 Efficient cytosolic delivery of such intrabodies, however, remains an unsolved problem. We therefore envisaged the “mix-and-go” method could allow cytosolic delivery of antibody-based drugs and provide new therapeutic avenues. MABS197 is a commercial antibody that binds to the catalytic domain of human PTP1B and may inhibit its enzymatic activity.64 Our hypothesis was that, upon successful cytosolic delivery of MABS197, the in situ inhibition of endogenous PTP1B activities might occur as a result of antibody binding, thereby leading to subsequent upregulation in the phosphorylation status of PTP1B’s two direct targets, IRS1 and EGFR (Figure 5a and Supplementary Figure 20). By using an in vitro PTP1B activity assay, we first confirmed that indeed this antibody was able to potently inhibit PTP1B enzymatic activity when compared to small-molecule PTP1B inhibitors. Compound 3 is a highly selective, small-molecule allosteric inhibitor of PTP1B, and sodium orthovanadate (Na3VO4) is a general phosphatase inhibitor.65,66 As shown in Figure 5b, complete inhibition of PTP1B activity by MABS197 was achieved at a 5:1 antibody/PTP1B molar ratio (corresponding to 100 nM of the antibody), while similar inhibition was observed at a significantly higher concentration of compound 3 (200 μM) or Na3VO4 (10 μM). Further comparison of the PTP1B inhibitory activity by MABS197 at three different concentrations (20, 40, and 100 nM) with compound 3, Na3VO4, and a control antibody (i.e., human IgG) was carried out (Figure 5c); both MABS197 and Na3VO4 showed comparably potent inhibition, while neither compound 3 nor IgG showed any inhibition. Our results thus showed that MABS197, as a potential antibody-based PTP1B inhibitor, could offer clear advantages over the two small-molecule inhibitors owing to the antibody’s potency as well as selectivity. The selective inhibition of MABS197 on PTP1B was associated with its specific antigen recognition, as human IgG showed no inhibition under similar conditions (Figure 5c). Upon successful cytosolic delivery of MABS197 (with CpA1-MABS197, 100 nM, 4 h) into serum-starved HeLa cells, we carried out WB analysis to measure the upregulation of IRS1 phosphorylation (Figure 5d); elevated expression level of phospho-IRS1 was detected in MABS197-treated cells as well as those treated with insulin + Na3VO4. This result is in accordance with a study that showed treatments of cells with PTP1B inhibitors, with or without insulin, markedly enhanced IR and IRS1 phosphorylation.67 Previously, it was reported that, upon treatment with EGF (a growth factor), fibroblasts lacking PTP1B exhibited an increase and sustained phosphorylation of EGFR.67 As shown in Figure 5e, we also observed significant upregulation of EGFR phosphorylation (but not total EGFR expression) in MABS197-treated A431 cells, but not in control cells (treated with control IgG, compound 3, or Na3VO4). These combined results thus indicated direct endogenous PTP1B inhibition in both HeLa and A431 cells by cytosolically delivered MABS197. Our results also confirmed the antibody-bound biodaptor CpA1, by binding to the Fc domain and not the Fab region, did not alter endogenous antigen binding as expected. It should be noted that MABS197 was directly used as provided from the vendor with no purification. Thus, in the future, our “mix-and-go” antibody delivery approach may be conveniently used to investigate other intracellular antigens that are normally considered “undruggable” by small-molecule inhibitors, e.g., nonreceptors, transcription factors, and others.68

Antibody-Mediated Degradation of Endogenous Proteins. Targeted protein degradation using proteolysis targeting chimeras, or PROTACs, has in recent years become one of the most promising therapeutic tools in drug discovery.69 This method, however, has only been applied
PROTAC designs require target proteins to be druggable by small molecules, the right match between degraders and target proteins, as well as optimal linker lengths between the E3 ligase recognition motif and ligand. While most reported antibody-based protein inhibition strategies require the antibody to bind to an epitope within the target that is capable of blocking protein activity/function, and at the same time can stoichiometrically compete with endogenous ligands, a method called "trim-away" recently developed by Clift et al. makes use of an antibody-mediated protein degradation strategy, which elegantly leverages the advantage of antibodies in their unique ability to bind to virtually any protein target with high affinity and specificity. In principle, this protein degradation strategy is therefore universally applicable to targeting any protein-of-interest (POI) in mammalian cells. In the "trim-away" approach, microinjection or electroporation was needed for intracellular antibody delivery. Upon successful cell entry and recognition of POI, the resulting antibody-POI complex was subsequently recognized by TRIM21 (an E3 ubiquitin ligase), leading to TRIM21-mediated ubiquitination and degradation of the POI by the proteasome (Figure 6a). With our TRIM-21-derived cell-permeant bioadaptor (CpT, also named Ni-NTPCPD-(His)6TRIM21), we wanted to know if a less invasive/disruptive method for intracellular delivery of antibodies could be realized.

The commercially available (His)6-TRIM21 was first mixed with IgG by using our "mix-and-go" approach followed by DLS and TEM measurements of the resulting complex (Figure 6b); successful formation of a stable (His)6-TRIM21/IgG complex was observed in the form of nanoparticles (~120 nm in size), which was similar to earlier PrtA/IgG complexes. Next, by mixing the fluorescently labeled IgGFITC with CpT to generate CpT-IgGFITC, the resulting complex was subsequently incubated with HeLa cells under earlier optimized conditions, and successful cytosolic cargo delivery was confirmed by WB analysis and in-gel fluorescence scanning of the resulting cell lysates (Figure 6c,d); (His)6-TRIM21 was detected in cells treated with either CpT alone or the CpT-IgGFITC complex (lanes 2 and 3 in Figure 6c), whereas IgGFITC was detected in cells treated with CpT-IgGFITC complex (lane 3 in Figure 6d).

We next investigated whether our cytosolically delivered antibodies could take part in the antibody-mediated degradation of endogenous protein targets with the "trim-away" strategy. α-Synuclein is a protein known to be strongly associated with Parkinson’s disease (PD), which cannot be cured currently. Studies have shown that aberrant forms of α-synuclein are neurotoxic species and can readily aggregate to form insoluble fibrils under pathological conditions in PD and related neurodegenerative diseases. Antibodies or their fragments capable of binding to α-synuclein have been shown to reduce the extent of α-synuclein dimerization/oligomerization and thus minimize its aggregation. successfully to a small number of endogenous protein targets.

Figure 6. Antibody-mediated degradation of endogenous α-synuclein. (a) Scheme showing targeted protein degradation with CpT-Ab complex, by combining our "mix-and-go" antibody delivery with "trim-away". (b) (Left) TEM image of TRIM21-IgG. Scale bar = 100 nm. (Right) Summary of size distribution of TRIM21-IgG and controls. Data are presented as mean ± s.d. (n = 3). (c, d) WB analysis of lysates from HeLa cells incubated with CpT or CpT-IgGFITC (50 nM, 4 h). Total lysates were immunoblotted with the corresponding antibodies in c, d or detected by in-gel fluorescence scanning in d. (e) WB analysis of lysates from SH-SYSY cells electroporated with indicated protein/antibody combinations (working concentration of Absyn 555 nM). Whole-cell lysates were harvested 16 h after electroporation. (f) WB analysis of lysates from SY-SH5Y cells upon "mix-and-go" delivery of anti-α-synuclein antibody (50 nM). Whole-cell lysates were harvested 18 h postdelivery. GAPDH/β-actin were run as loading controls in c–f. (*) Signals from anti-(His)6-tag antibody.
Targeting α-synuclein, by either inhibiting its aggregation or decreasing its endogenous expression in pathogenic neuronal cells, therefore constitutes a promising therapeutic strategy for PD. To investigate whether the expression level of endogenous α-synuclein in SH-SYSY neuronal cells could be reduced by “trim-away”, we first co-delivered recombinant (His)$_6$-TRIM21 together with an anti-α-synuclein antibody (named Ab$^{99m}$) to the cells by electroporation as previously reported (Figure 6c);$^{14}$ successful reduction of endogenous α-synuclein (∼70% degradation as determined by WB in Figure 6e) caused by proteasome-mediated degradation was clearly observed (lane 4), which was both TRIM21- and antibody-dependent, as wild-type SH-SYSY cells did not express a detectable level of endogenous TRIM21, and omission of either reagent in the experiment led to a complete inhibition of target degradation (lanes 2 and 3, respectively). In an independent experiment, we delivered both (His)$_6$-TRIM21 and Ab$^{99m}$ to SH-SYSY cells with the “mix-and-go” method by simply incubating cells with CpT-Ab$^{99m}$, followed by WB analysis of the resulting cell lysates 18 h post-delivery (Figure 6f); similar Ab$^{99m}$-dependent degradation of endogenous α-synuclein was observed (∼50% degradation as determined by WB in Figure 6f), as no target degradation in experiments with either omission of Ab$^{99m}$ or control IgG was detected (compare lane 4 with lanes 2 and 3). These results thus confirmed that our antibody delivery approach with the TRIM21-derived biodaptor (CpT) could indeed be combined with “trim-away” to achieve effective proteasome-based target degradation in unmodified mammalian cells, while avoiding the need of microinjection or electroporation. The overall delivery efficiency for the “mix-and-go” approach was slightly lower than that of electroporation (i.e., as judged by (His)$_6$-TRIM21 uptake in Supplementary Figure 21). Under the current experimental conditions, while electroporation could induce ∼70% degradation of α-synuclein, CpT-Ab$^{99m}$ caused ∼50% degradation with our “mix-and-go” approach. This indicates that the overall intracellular delivery of the functional antibody in both approaches was comparable. With a wide variety of off-the-shelf antibodies available that can bind to many different proteins, potential therapeutic applications of such “mix-and-go” + “trim-away” experiments are therefore highly appealing and may be used to complement existing small-molecule drug discovery programs or expand currently druggable space for many diseases.

**DISCUSSION**

In order to take full advantage of the high affinity/selectivity endowed by most antibodies in their antigen recognition for expanded biomedical applications, effective cytosolic delivery of native functional antibodies to engage intracellular targets needs to be first realized. In the current study, we have successfully equipped IgG-binding proteins (protein A and TRIM21) with cell-penetrating poly(disulfide)s (CPDs), providing the corresponding cell-permeant biodaptors (CpA1, CpA2, and CpT), which could be readily appended to off-the-shelf, commercially available antibodies by a simple “mix-and-go” protocol, thus enabling efficient cytosolic antibody delivery with immediate bioavailability. Compared to other known antibody delivery strategies, e.g., the L17E and lipid-based approaches, our newly developed strategy allowed convenient antibody uptake in different mammalian cells with greater efficiencies at a significantly lower operational concentration and with minimal cytotoxicity.$^{10,15}$ We also compared our “mix-and-go” strategy with electroporation, the “gold standard” for cytosolic delivery, and we found that punctate fluorescent signals of delivered antibody were not due to endosome trapping, but rather nanoparticle formation. Both “mix-and-go” strategy and electroporation offered excellent delivery efficiency (fluorescent-positive cells more than 95%), and electroporation could deliver twice the amount of antibody than a “mix-and-go” approach (judging from fold-increase in mean fluorescence intensity). While electroporation offered a slightly better delivery efficiency, its use is limited because it is low-throughput, disruptive, and poorly translatable to live organisms. By using live-cell bioimaging and quantitative flow cytometry, we have shown successful cytosolic delivery of various commercial antibodies, without additional manipulations and with easy cargo exchange. Direct intracellular target engagement of delivered antibody was demonstrated by using anti-NPC as an example, and localization on nuclear membrane was observed in live cells, in a fashion consistent with electroporation.

By employing this “mix-and-go” strategy, we first showed that a cytosolically delivered, fluorescently labeled antibody remained functional in the cell cytosol and was capable of live-cell imaging of endogenous protein glutathionylation (PSSG). Since there was no in-built fluorescence “Turn-ON” mechanism in this antibody-based biosensor, the excessive unbound antibody needed to be washed away by using a live-cell permeabilization protocol. While the use of a low-concentration detergent (0.015% Triton X-100) for membrane permeabilization effectively kept the cells alive, some unwanted losses of soluble cytosolic proteins (sometimes including desired antigens) from such a protocol might be inevitable.$^{75}$ This might have accounted for the moderate fluorescence signals detected in our live-cell PSSG experiments. Notwithstanding, we have shown such a “live-cell immunofluorescence” approach is indeed feasible and in the future may be used for live-cell bioimaging of other post-translational protein modification (PTM) events, which is currently possible by using genetically encoded biosensors but with very limited success. We are mindful, however, that the current live-cell imaging protocol needs to be extensively optimized before it can be widely used for real biological studies. A possible improvement includes the strategic introduction of a suitable quencher/fluorophore pair within the antibody-based biosensor, thus equipping it with a fluorescence “Turn-ON” property that might enable direct “no-wash” imaging in live mammalian cells.

In our second and third biological studies, antibody-based activation of endogenous caspase-3 activity and inhibition of endogenous PTP1B activity were achieved upon cytosolic delivery of the respective antibodies by using the “mix-and-go” method. The serendipitous discovery that a caspase-3-specific antibody was capable of enzyme activation possibly indicates a more widespread presence of other antibodies (similar to small molecules) as potential enzyme activators, as well as their future biological applications, but this will require careful design and screening of antibodies capable of binding to different epitopes within the same antigen.$^{74}$ Our success of endogenous PTP1B inhibition by using a cytosolically delivered antibody known to specifically bind to the catalytic domain of PTP1B further highlights the enormous potential of antibody-based therapy. With the existence of a large collection of commercially available, off-the-shelf, and well-validated antibodies that are widely used in basic research, our newly
developed bioadaptors for convenient and effective cytosolic delivery of these antibodies may allow interrogation of many intracellular protein targets which are currently undruggable by small-molecule inhibitors.

Finally, by using a CPD-modified cell-permeant TRIM21 bioadaptor, and combining the “mix-and-go” method with “trim-away”, we showed a cytosolically delivered anti-α-synuclein antibody could be used for antibody-mediated endogenous protein degradation, and it caused a substantial decrease in the endogenous expression level of α-synuclein, a promising therapeutic target for Parkinson’s disease. Our delivery approach thus effectively delivered a native functional antibody against the protein-of-interest (POI) into mammalian cells without the need for microinjection and electroporation, which are low-throughput and disruptive, and often require specialized instruments. With our cell-permeant TRIM21 bioadaptor, protein degradation could in principle be used on any TRIM21-binding antibodies to achieve proteasome-mediated knockdown of their antigens and may be applicable for large-scale and routine pharmaceutical applications.6 We noted, however, that the target degradation efficiency by using the current protocol was not higher than that with the standard “trim-away” method, suggesting a need for further improvement. Notwithstanding, unlike current PROTACs which use small molecule-target recognition, the possibility of using antibody-target recognition to achieve effective endogenous protein degradation highlights the great potential of this “mix-and-go” method, not only in antibody-based therapy, but also for interrogation of many disease-related signaling pathways currently inaccessible by chemical knockdowns with small molecule inhibitors.

In conclusion, our newly developed “mix-and-go” strategy was able to achieve cytosolic delivery of commercially available off-the-shelf antibodies without any intervention from chemists. The strategy was shown to be versatile, practical, and highly efficient, and afforded antibodies with immediate and good bioavailability upon intracellular delivery. By successfully demonstrating this approach in a number of biological applications, including antibody-based live-cell imaging of endogenous protein glutathionylation to detect oxidative cell stress, antibody-based activation of endogenous caspase-3 and inhibition of endogenous PTP1B activity, and finally TRIM21-mediated endogenous protein degradation for potential targeted therapy, we show this strategy will have broad utilities in chemical biology and future drug discovery.

**ASSOCIATED CONTENT**

* Supporting Information
  The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscentsci.0c01379.

  Detailed experimental procedures and characterization data. Supplementary Scheme 1 Supplementary Tables 1−2 Supplementary Figures 1−21 (PDF)

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**Notes**

The authors declare no competing financial interest.

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