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Synthetic Proteins behind the Plasma Barrier: Molecular Spies

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Cite This: *Acc. Chem. Res.* 2022, 55, 2055−2067

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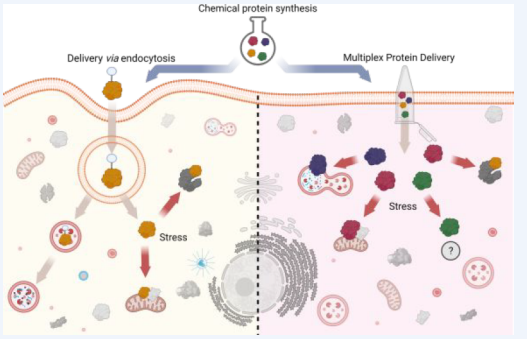
CONSPECTUS: There is a continuous demand to improve our understanding of fundamental processes that underlie human health and disease. Therefore, novel strategies that can assist in these efforts are required. For example, molecular biology and genetic approaches have revolutionized our understanding of protein-mediated processes by facilitating their direct visualization and analyses in living cells. Despite these developments, genetic manipulation has limitations in controlling events that occur after translation such as posttranslational modifications (PTMs), which are imperative regulatory elements. As a result, developing new methods to study PTMs in live cells is a major bottleneck in deciphering their exact roles in the myriad cellular processes.



Synthetic and semisynthetic proteins are prepared by combining solid phase peptide synthesis (SPPS) and chemoselective ligation approaches with synthetic or recombinant peptides. Employing protein synthesis allows chemists to incorporate natural and unnatural modifications with virtually unlimited number of functional groups into the protein's sequence, such as PTMs and their mimics. In addition, synthetic proteins can include additional elements such as fluorescent tags, reactive groups, caged units, and enrichment handles. Therefore, harnessing the power of chemical protein synthesis offers great opportunities to study fundamental biological processes.

Unfortunately, the low cell permeability of proteins limits their applications mainly to *in vitro* settings, excluding live cell studies. As a result, chemical biologists have been attempting to overcome these limitations by developing protein delivery methods that would enable the study of custom-made proteins in a biological context. Success with these strategies should enable accurate determination of protein localization, degradation, folding, interactions, and involvement in the assembly of membrane-less organelles formed by liquid−liquid phase separation inside cells. Importantly, protein delivery approaches are complementary to genetic manipulations, and combining these approaches should pave the way to new discoveries.

In this Account, we describe recent developments in protein delivery methods, with emphasis on those most compatible with synthetic proteins. We highlight experimental approaches and conceptual adaptations required to design and study synthetic proteins in live cells, with or without genetic manipulation. In addition, we highlight the strength and weakness of these approaches for both the delivery and the subsequent studies. We also describe our endeavors to deliver synthetic proteins to cells via cell penetrating peptides (CPPs) and multiplexed bead loading (MBL), as showcases of the applications of these methods to shed light on biological processes. Lastly, we contemplate other future applications of synthetic proteins to answer questions that are currently unapproachable.

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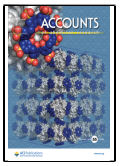
Received: April 17, 2022
Published: July 14, 2022



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Acc. Chem. Res. 2022, 55, 2055−2067

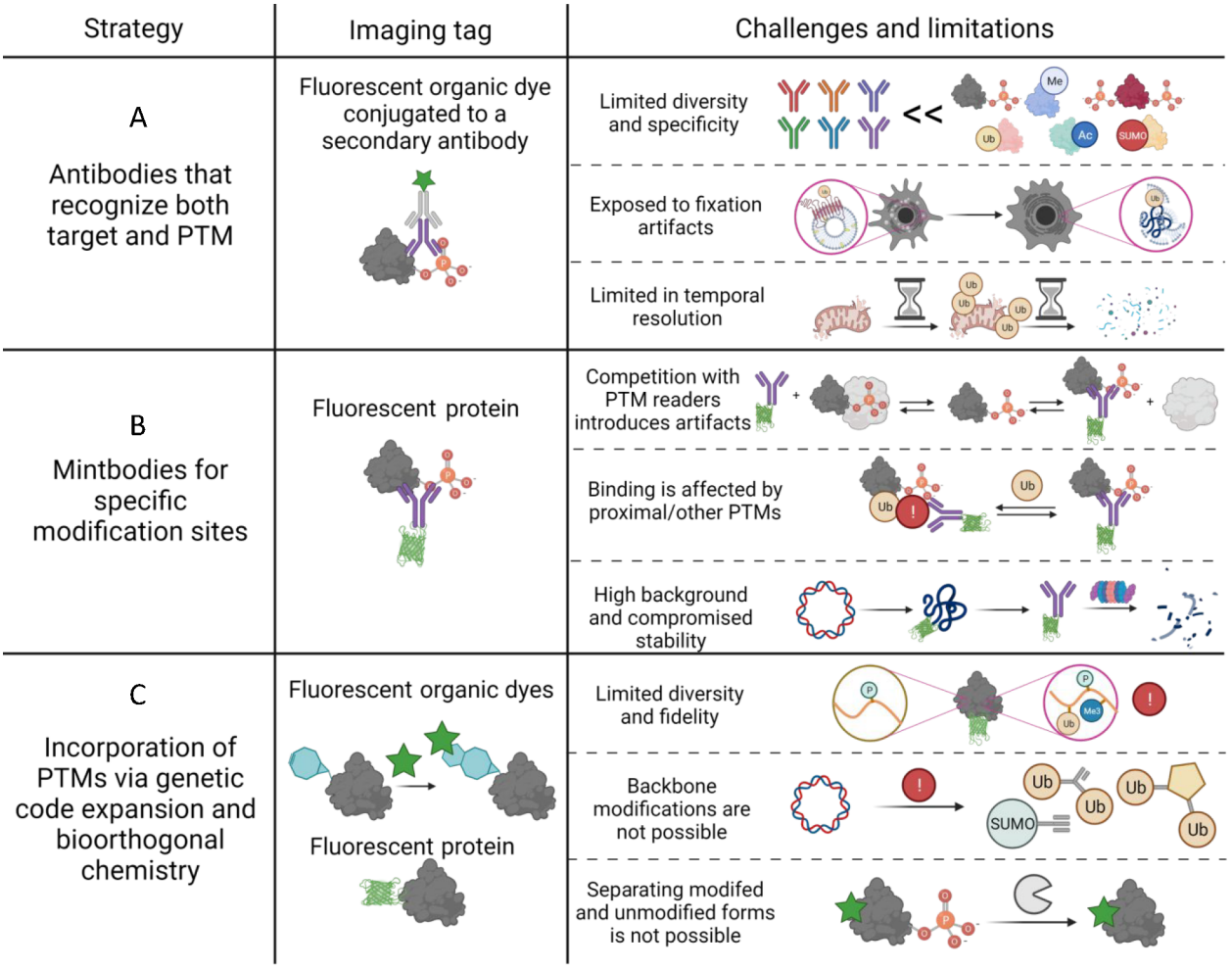

Figure 1. Challenges in studying PTMs using the current methods: (A) Antibodies are limited in their diversity and specificity with possible fixation artifacts and low temporal resolution. (B) Expressing mintbodies can alter the modified protein's biological function, and their imaging is affected by proximal PTM interference, probe degradation, and fluorescent background from unfolded and unbound probes. (C) GCE is limited to a handful of side-chain modifications and a limited number of sites and by possible processing by endogenous enzymes.

synthetic ubiquitin analogue bearing an unnatural phosphomimetic amino acid to probe its phosphorylation dependent cellular function.

- Mann, G.; Sadhu, P.; Brik, A. Multiplexed Delivery of Synthetic (Un)Conjugatable Ubiquitin and SUMO2 Enables Simultaneous Monitoring of Their Localization and Function in Live Cells. *ChemBioChem* **2022**, e202200122.[4] This study describes harnessing the power of multiplexed protein delivery to study several synthetic proteins in the same cells.

INTRODUCTION

Proteins: The "Work Horses" of Living Cells

Cells simultaneously perform numerous chemical reactions in order to maintain life. Each living cell is considered as a "chemical factory" surrounded by a plasma membrane (PM) that is impermeable to most macromolecular cargoes. Inside the cytosol, proteins are both the master regulators and workers for carrying out most chemical reactions in spatial and temporal resolution. Not surprisingly, proteins must be flexible and programmable molecules, yet understanding their function remains one of the greatest mysteries of our time.

Delivering macromolecular cargoes from an exogenous source to live cells is a powerful way to study their function in a biological context and modulate processes that are relevant for coping with different diseases.[5] Hijacking the cellular expression machinery to express a desired protein, is a powerful strategy that requires minimal PM perturbation.[6] As a result, molecular biology approaches to mutate and overexpress engineered proteins in living cells revolutionized our ability to study their functions. In particular, generating fluorescently tagged proteins by fusing them to green fluorescent protein (GFP)[7] or self-labeling protein tags (e.g., Halo-tag)[8] and incorporation of unnatural amino acids for biorthogonal labeling[9] significantly improved our ability to visualize and study proteins in various contexts. Nevertheless, recombinant approaches are not free of limitations. Overexpression of regulatory proteins can have significant impacts on their function.[6] Importantly, processes that occur at the protein molecular level such as turnover and posttranslational modifications (PTMs) cannot be controlled.[10]

Amplifying Protein Complexity via PTMs

Eukaryotic proteins are expressed from a limited number of genes (~25 000 in humans).[11] Despite this genetic "glass ceiling", proteins overcome this limitation through a PTM code to diversify and control their functions in various biological

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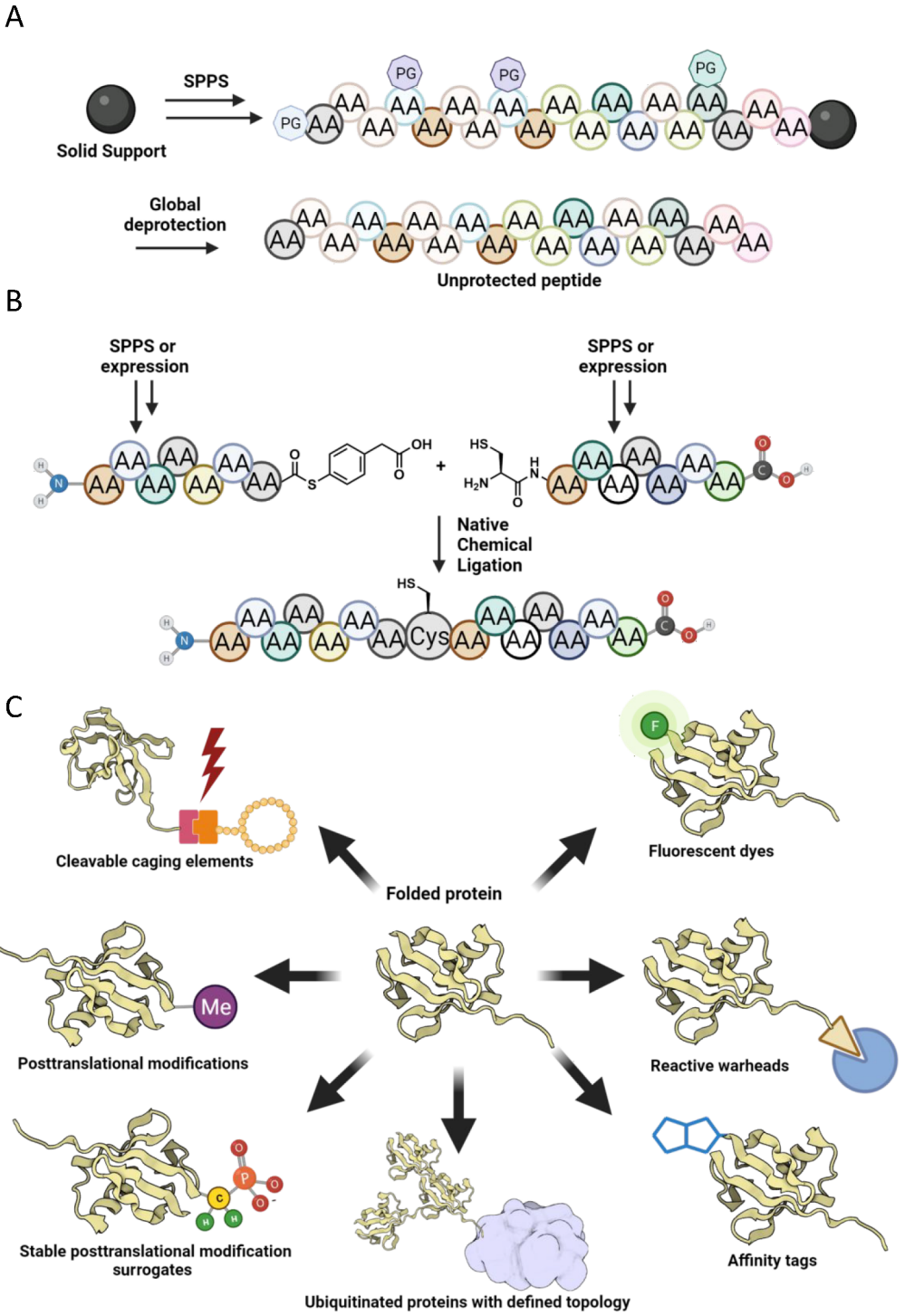

Figure 2. Protein synthesis as a powerful tool to produce unique proteins with various modifications. (A) Preparation of synthetic peptides by solid phase peptide synthesis (SPPS). (B) Combining peptide fragments by native chemical ligation, as an example of a chemoselective ligation method, to generate synthetic and semisynthetic proteins. (C) Examples of unique elements that can be directly incorporated into synthetic proteins via protein synthesis.

processes.[12] As a result, dysregulated PTMs correlate with various pathological states such as neurodegeneration and cancer.[12] Unfortunately, the large chemical space of PTMs, coupled with limitations in genetic engineering for their incorporation and removal in real-time, introduces substantial challenges in studying their cellular role.[13] Furthermore, imaging posttranslationally modified proteins is particularly challenging and requires site selective binding domains (e.g., antibodies) for their recognition.[10,13] Unfortunately, antibodies are both impermeable and unstable in the reducing environment of the cytosol, making them relevant mainly for fixed cells (Figure 1A).[14] Using alternative binders such as modification-specific intracellular antibody (mintbodies) is limited by slow development, cellular stability, proximal PTM interference, and their competition with PTM readers that can affect the PTM's function (Figure 1B).[13] Therefore, developing PTM binders to

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Acc. Chem. Res. 2022, 55, 2055−2067

the different possible sites with proven site-specific selectivity is hardly feasible.

Alternatively, molecular biology approaches including genetic code expansion (GCE) allow overexpressing and/or knocking down a particular cellular protein and its posttranslationally modified or tagged analogues.[15] Despite the knowledge gained from applying these methods, many questions remained unaddressable because of their various limitations. For example, many genetic approaches lack controllable stoichiometry and could lead to stochastic variations in expression levels.[6] Even when successful, these methods are limited in their ability to modify the protein backbone, in the diversity of modifications, in introducing multiple modifications, and in generating proteins with unique molecular compositions (e.g., ubiquitinated proteins and activity based probes) (Figure 1C).[15] As a result, controlling the atomic structure and function of proteins and monitoring their activity in real time has not yet been possible for most proteins.

Ubiquitin (Ub) and its related Ub-like modifiers are examples for complex PTMs that are involved in numerous cellular functions and are notoriously challenging to image in live cells.[16,17] These modifiers compete for the same modification sites[18] and exhibit cross-talk with additional PTMs.[19] For example, phosphorylation of Ub on mitochondrial proteins regulates the recycling of damaged mitochondria by phosphatase and tensin homolog (PTEN)-induced kinase 1 (PINK1)/parkin mediated mitophagy.[20] Following mitochondrial damage, PINK1 is stabilized on the outer membrane of damaged organelles and phosphorylates Ub at serine 65 (pUb).[21] Generating pUb conjugated to mitochondrial proteins is crucial for recruiting and activating the E3 Ub ligase parkin, which binds to Ub chains that contain pUb (poly-pUb).[22] Activated parkin collaborates with PINK1 to amplify the poly-pUb coat that recruits autophagy adaptors, such as NBP1 and P62, to induce autophagy of damaged organelles.[20]

To study pUb's involvement in mitophagy, several groups have utilized overexpression and Ub replacement strategies to mimic pUb by introducing S65E and S65D mutations that are structurally and functionally different than phosphorylated serine (pSer).[23] Using these mimics to study mitophagy is a compromise that can result in artifacts.[24] Importantly, this second layer of modification introduces additional challenges in imaging the substrates of these chains since site-specific antibodies that recognize both pUb and its substrates are unavailable. Such an example illustrates the complexity of PTM coding and performing studies to decipher their role in biological context. Therefore, developing additional methods to study modified proteins in cells is crucial for both fundamental research and therapeutic developments.[25]

SYNTHESIS OF UNIQUELY MODIFIED PROTEINS FOR BIOLOGICAL STUDIES

The preparation of proteins via total chemical protein synthesis and semisynthesis (Figure 2A,B) allow us to precisely modify proteins with control at the atomic level.[26] Importantly, protein synthesis has superiority in incorporating complex protein modifications such as those that are modified with Ub and Ub like modifiers.[27] Using protein synthesis, chemical biologists have prepared protein analogues that include, for example, natural and stable PTMs, affinity tags, reactive warheads, and fluorescent dyes for a variety of studies (Figure 2C).[26] Furthermore, protein synthesis allows introduction of caging elements to mask protein function for on demand activation to probe highly dynamic processes.[1] However, in order to fully exploit the potential of protein synthesis, we must develop methods for cellular delivery and on demand activation of these chemically synthesized and uniquely modified proteins. Unfortunately, in contrast to the uniformly charged nucleic acids that are delivered using transfection reagents, proteins are chemically diverse entities with unpredictable properties.[28] Therefore, proteins are more challenging to deliver and require a case-by-case study for finding the best approach.

In recent years, we have used and developed novel chemical approaches to prepare various uniquely modified proteins.[26] We have been particularly interested in studying proteins modified with Ub and Ub-like modifiers, such as the small ubiquitin like modifier (SUMO), to study how these modifications affect biological processes.[27,29] Until recently, we were mainly focusing on studying these unique constructs *in vitro*. Recent developments in delivery methods, from our laboratory and others, encouraged us to move forward with the delivery of synthetic proteins to live cells to study them in their native environment.

UTILIZING SYNTHETIC PROTEIN-BASED PROBES

Developing therapeutics that are both potent and free of side effects is the holy grail of modern medicine. This requires new strategies to probe disease related molecular events and provide a full picture of the disease's onset.[30] In this regard, synthetic protein probes could assist in filling the current gaps in our knowledge on the involvement of PTMs in various disease states. While, synthetic protein-based therapeutics are still far from reaching the clinic, protein-based probes function at a minimal concentration[8] and can be labeled for live cell imaging.[30,31] However, in order to image the delivered probes, the delivery process requires avoiding endosomal entrapment (i.e., the strong fluorescence signal resulting from cargoes trapped in endosomes and lysosomes) that can distort the analysis.[32]

TOOLBOX FOR PROTEIN DELIVERY

In this section, we highlight the delivery methods that are applied for synthetic and semisynthetic proteins. We comment on the strengths and weakness of each method for basic research or therapeutic applications. Despite their indisputable importance for protein delivery, we do not discuss methods to study delivery mechanisms or methods to validate the protein's cytosolic availability that were recently reviewed elsewhere.[5,33,34] We based our discussion on our personal experience in delivering synthetic proteins that are extremely precious and prepared in relatively small amounts. We do not discuss methods that might lead to differences in the encapsulation efficiency depending on the protein cargoes, such as liposomes,[35] despite their great relevance.

Pore-Forming Bacterial Toxins

Several strains of bacteria developed protein-based machineries to manipulate and kill host immune system cells to improve conditions for their growth. These machineries were hijacked for protein delivery by replacing their cytotoxic domains with a protein cargo. The anthrax lethal toxin (ALT) is the most studied of these delivery systems of proteins cargoes.[36] The cargo is first linked to the N-terminal domain of the lethal factor protein (LF_N) to induce the cargo's binding to the protective antigen complex that is associated with specific receptors on the target cell's PM. The cargo–LF_N, in a complex with the protective antigen, undergoes receptor-mediated endocytosis to

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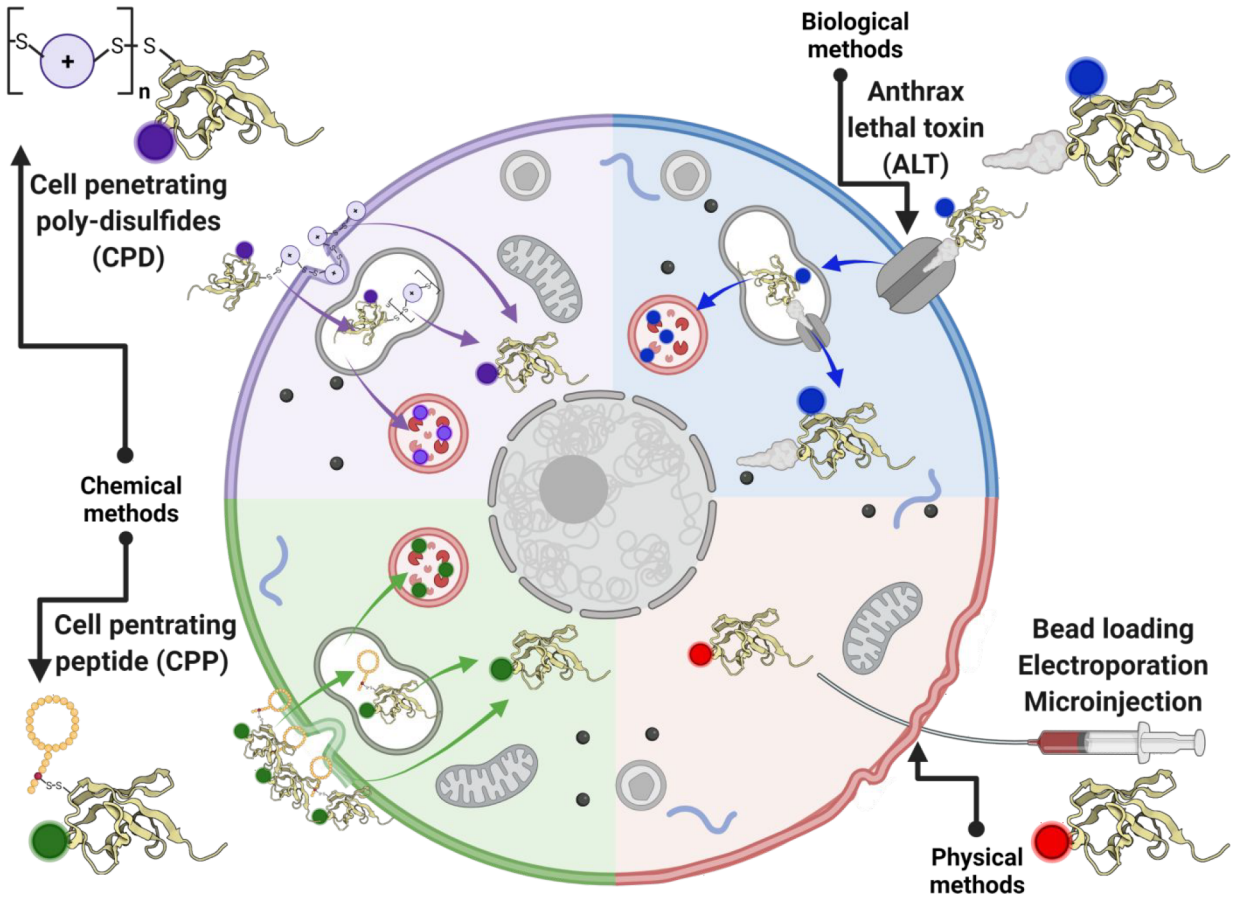

Figure 3. Summary of the most compatible delivery methods for synthetic proteins.

activate its translocation inside endosomes (Figure 3). In the last stage, the cargo unfolds to pass through the protective antigen's pore into the cytosol where it must refold.[36]

ALT was elegantly applied to deliver semisynthetic cargoes by ligating synthetic peptides to a recombinant LF_N with distinct advantages: (1) It is independent of the cargo's molecular weight. (2) It requires low concentrations to induce cargo delivery. (3) It has potential for cell type specific therapies. Nevertheless, ALT also has notable limitations: (1) The requirement to unfold and refold limits the delivery of cyclic peptides, proteins containing disulfides, and tightly folded domains by this approach. (2) The cargo's charge affects its translocation efficiency. (3) The need to ligate the cargo through an amide bond to the LF_N domain introduces further challenges in synthesis and cellular localization.

Using this approach, the Pentelute group delivered semi-synthetic peptides bearing unnatural amino acids with inversed chirality (i.e., D-amino acids). They discovered that one D-amino acid at the protein's N-terminus is sufficient to escape degradation by ubiquitinating enzymes operating through the N-end rule.[37]

Peptide and Protein Transduction Domains

Since the discovery of the first cell penetrating peptide (CPP), derived from the transactivator of transcription of HIV-1 (TAT),[38] covalently linking cargoes to protein transduction domains (PTDs) became one of the most direct approaches to deliver proteins.[39] Following this, many types of CPPs and cell penetrating poly(disulfide)s (CPDs)[40] were developed to further improve the permeability of cargoes. In most cases, PTDs are linked to a cargo using a disulfide bond to allow its release by glutathione (GSH) mediated reduction once inside the cytosol[41] (Figure 3). This prevents the PTD from affecting the cargo's localization, which is crucial for most applications.

Delivery by PTDs could occur through a combination of energy dependent and independent mechanisms.[33,42] The energy dependent delivery pathways proceed (mostly) via endocytosis, which requires the PTD−cargo complex to escape from endosomes to perform its function.[33] Although the escape is the most crucial stage, it is unpredictable with different cargo−PTD conjugates. As a result, the physical properties of a protein can make even the most effective PTD fail in the delivery process.[3] In most cases, even if a sufficient amount of cargo reaches the cytosol, endosomal escape is inefficient (e.g., 2% for TAT) with a significant amount of the cargo trapped in endocytic compartments.[43] This trapped cargo disrupts the analysis of both the delivery stage and localization, eventually resulting in lysosomal degradation of the trapped cargo.[33] At high concentrations, proteins could sometimes cross the PM through non-endocytic pathways; however, this is hard to predict and could be toxic to cells.[33,34]

By incorporating all the necessary elements for delivery and cargo in a single molecule, PTDs have clear advantages for delivering synthetic proteins. (1) The approach is relatively straightforward, which is a desirable factor for synthetic proteins that are mostly prepared on a very small scale. (2) Most PTDs are highly charged and improve the solubility of their cargo. (3) There are diverse approaches to link PTDs to proteins with different stabilities and cleavage conditions in cells. (4) PTDs have interesting potential for delivering therapeutic proteins. (5)

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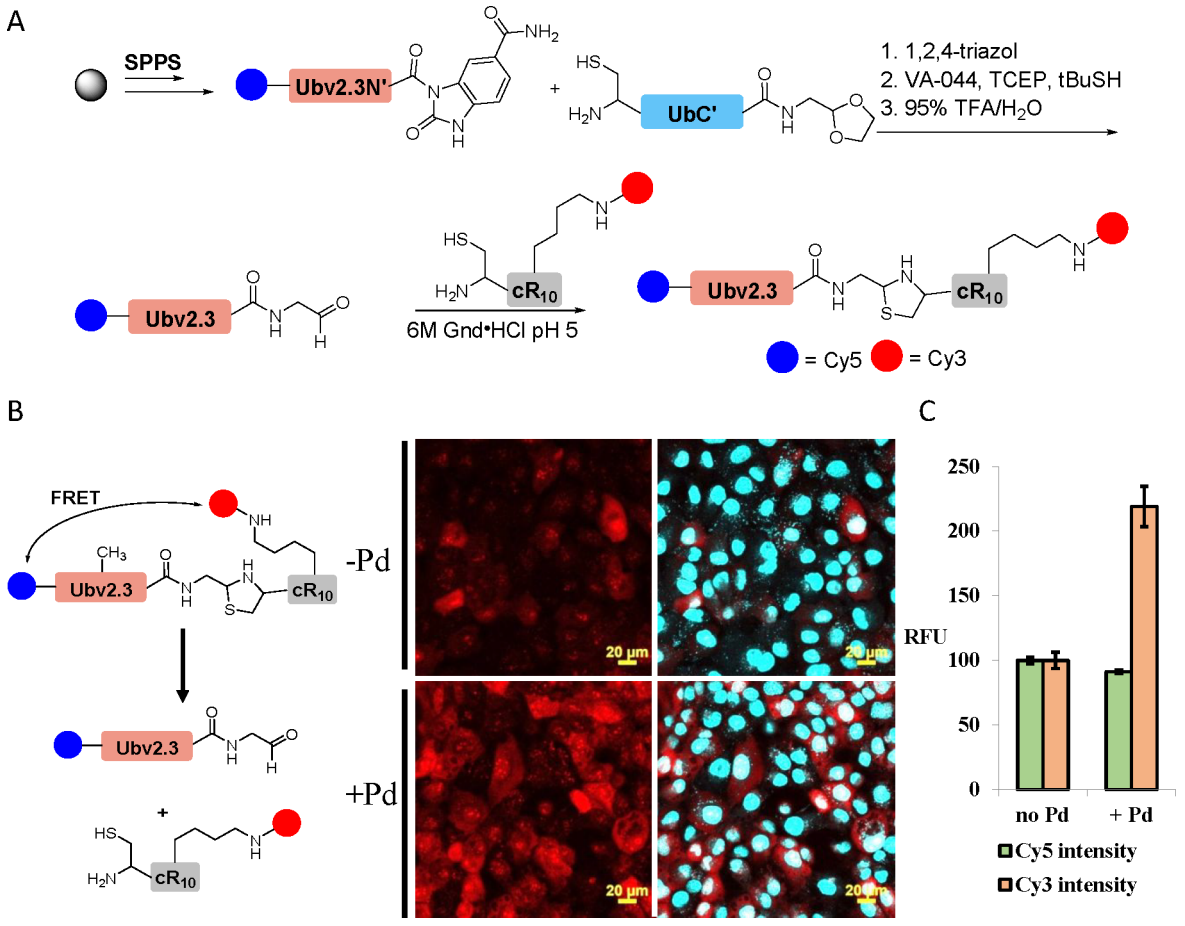

Figure 4. Palladium promoted activation of a synthetic protein in live cells. (A) Synthesis of Ubv2.3 caged at its C-terminus with thiazolidine linked cR10. (B) Confocal images of palladium promoted thiazolidine cleavage in DU145 prostate cancer cells containing caged Ubv2.3 with and without palladium treatment. Cy3 (red), Hoechst (Cyan). Scale bars 20 μm. (C) Quantification of the nuclear Cy3 and Cy5 intensities with and without palladium treatment in cells from panel C. Adapted with permission from ref 1. Copyright 2019 John Wiley and Sons.

Combining PTDs and additional approaches (e.g., endosome specific lytic peptides) can improve the endosomal escape efficiency. There are also limitations for this approach. (1) The dependence on the cargo's properties and variations in endosomal escape demands significant optimization for each case. (2) PTDs strongly adhere to the PM and in many cases to polymers used in the culture slides. (3) Even if performed correctly, the unexpected efficiency of endosomal entrapment does not guarantee delivery. (4) At high concentration, PTDs are toxic and induce cell death.

Using the CPP approach, Muir and co-workers performed chemical tagging of histone H2B with a fluorescent synthetic peptide in live cells. In this work, the authors used a disulfide linked TAT CPP to deliver a fluorescent peptide and attached it to H2B embedded in the cellular chromatin via protein trans-splicing.[44] The authors monitored the reaction by coupling the synthetic peptide's splicing to the separation of a dark quencher to increase the synthetic peptide's fluorescence through the reaction. Histone tails are highly modified and must be studied in their native biological context. Therefore, this approach could assist in studying PTM involvement in transcriptional regulation. In another work, Hackenberger and co-workers prepared semisynthetic nanobodies with "self-healing" fluorescent tags through proximity induced photostabilization. The authors used a cleavable CPP unit to simultaneously deliver two of these nanobodies for super-resolution microscopy of two endogenous proteins, which is challenging to achieve in live cells.[32]

Physical Methods and Transient Disruption of the Plasma Membrane

Using physical forces to transiently disrupt the PM allows protein diffusion into cells.[33] Despite these approaches apparently being accompanied by cell damage, several approaches achieve delivery without significant toxicity.[5] Moreover, the window between the PM's disruption and recovery is very short, making the time frame for these delivery strategies considerably shorter than other methods.[5] Importantly, this short delivery time does not enable endocytosis making these methods free of both cargo dependence and background signal from endosomes. As of today, the most common physical delivery methods are (1) microinjection, which generates a pore in the PM to deliver a small volume of protein solution directly to the cytosol;[5] (2) electroporation, which uses an electric shock to disrupt the PM and allow proteins to diffuse into the cytosol;[45] and (3) bead loading, which uses glass beads (~100 μm) to induce physical stress to cells and allow proteins to diffuse directly into their cytoplasm.[46] Among these methods, bead loading is the most robust, simple, and cheap method.[47]

Physical methods have significant advantages for basic research. (1) The properties of the protein cargo do not affect its delivery. (2) Simultaneous delivery of several cargoes to the

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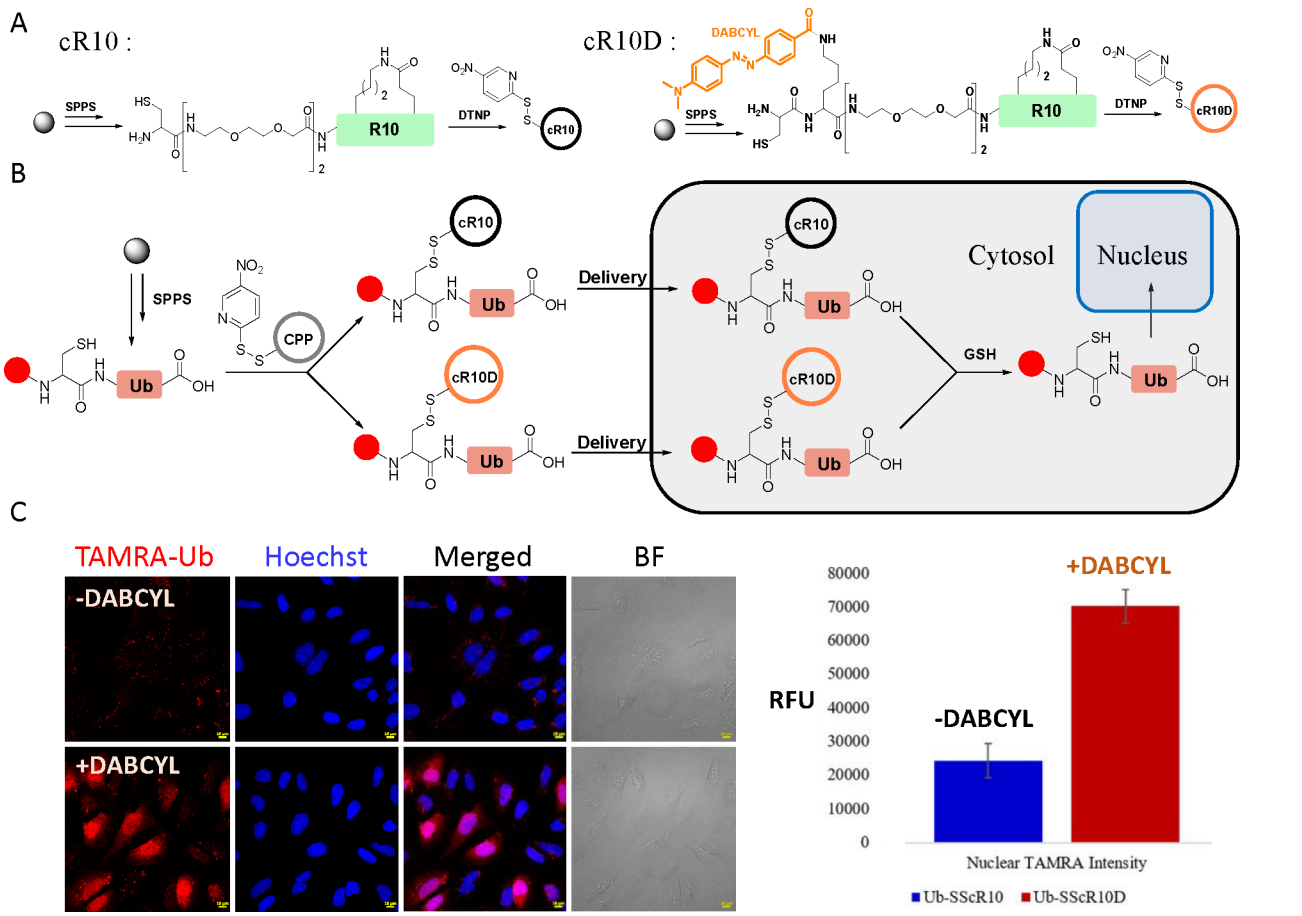

Figure 5. Improving cell delivery of synthetic proteins by cR10D. (A) Comparison between cR10 and cR10D with 2,2-dithiobis(5-nitropyridine) (DTNP). (B) Synthesis of Ub with disulfide linked cR10 and cR10D and the reduction by cytosolic GSH to release the Ub cargo inside cells. (C) Confocal images and quantification of nuclear fluorescence demonstrating improved Ub delivery with cR10D. TAMRA (red), Hoechst (Blue); scale bars 10 μm. Adapted with permission from ref 2. Copyright 2021 John Wiley and Sons.

same cells is possible. (3) None of these approaches requires covalent linkage to additional domains for delivery. (4) Direct cytosol transduction by these approaches is ideal for imaging applications. (5) These methods have low toxicity and neglectable effect on cell proliferation. On the other hand, the disadvantages of these approaches are (1) the low number of charged cells with the particular cargo, which limits their applications, (2) lack of therapeutic relevance, and (3) requiring specialized equipment for the delivery (e.g.; electroporation and microinjection).

Overall, these approaches are the most compatible for delivering synthetic proteins for research purposes. Using physical methods, chemical biologists successfully delivered synthetic proteins for protein engineering,[48,49] probing enzymatic activity,[50] introducing PTMs,[51,52] and imaging epigenetic PTMs.[53]

SELECTED EXAMPLES FOR THE DELIVERY OF SYNTHETIC PROTEINS TO STUDY CELLULAR PROCESSES

When planning to deliver synthetic proteins, the diversity of delivery methods can be overwhelming. After attempting several methods, we were successful with CCPs and bead loading approaches. In the following section, we highlight our recent studies where we synthesized uniquely modified proteins and used these approaches to deliver and study their involvement in cellular processes. From our experience, we conclude that protein delivery via CPPs is compatible for inducing cellular response; however the imaging quality is sacrificed due to endosomal entrapment. On the other hand, direct cytosolic delivery using physical methods is ideal for imaging the involvement of synthetic protein probes in cellular processes.

Palladium Promoted Activation of a Synthetic Protein in Live Cells

In recent years, several attempts have been made to expand the toolbox for both bond forming and bond cleavage reactions in live cells. As of today, most of these require GCE to express proteins containing a biorthogonal reactive handle on the side chain of an unnatural amino acid.[54] Despite its strength, only a handful of reactive elements are accessible by this approach, and many desired elements, which cannot be incorporated via GCE, remained unexplored. In other words, many unnatural elements in the protein's backbone, unnatural side chains with "bulky" groups (e.g., organic dye), and activity-based probes are not easily accessible, if at all. On the other hand, synthetic proteins are free of these limitations and allows essentially any desired modification, including mirror image proteins that are entirely unnatural.[55]

In this work, we used synthetic proteins to test the efficiency of our recently developed palladium promoted cleavage of proteins bearing a backbone thiazolidine linkage[56] for activating a caged protein in live cells.[1] Notably, thiazolidine cleavage

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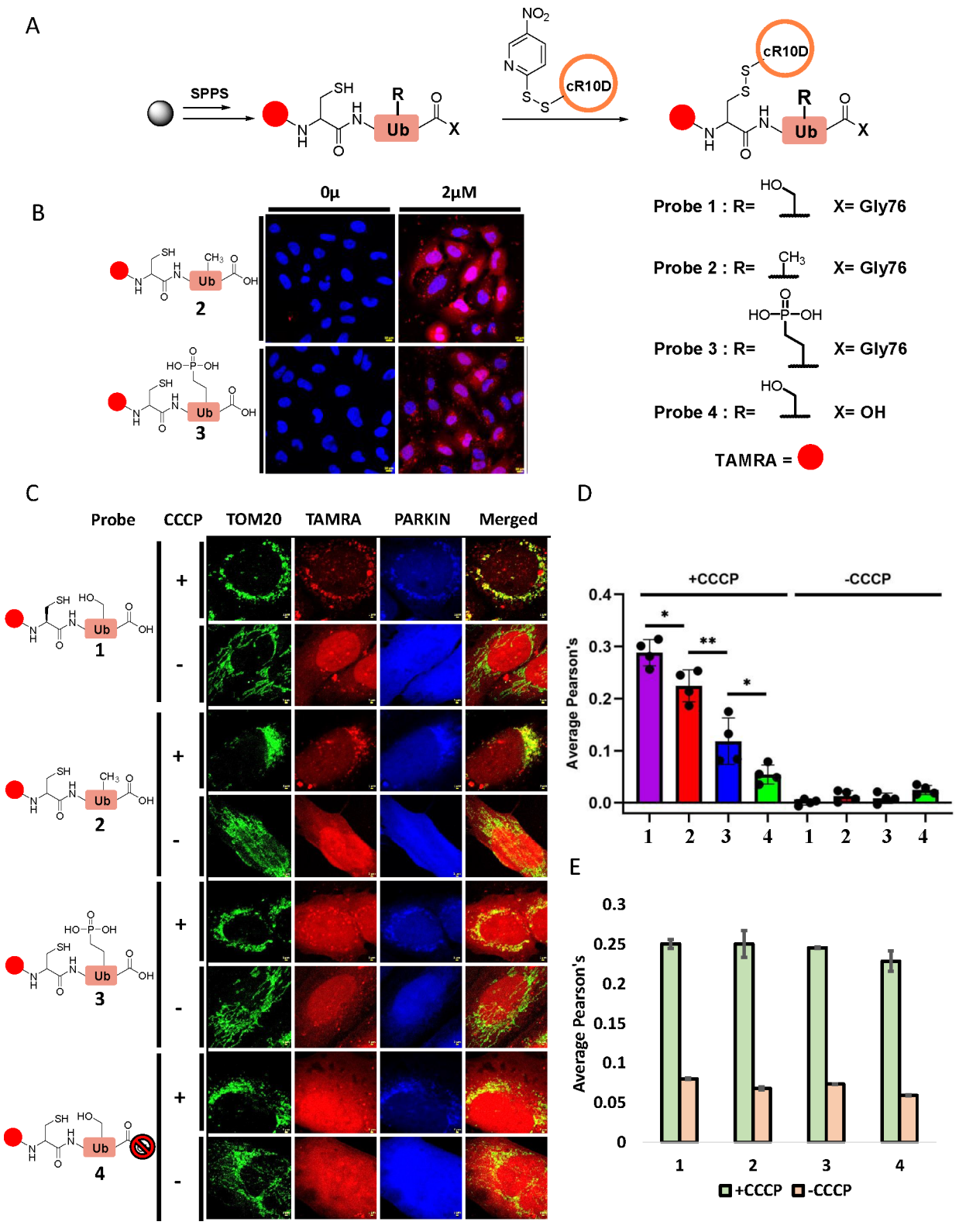

Figure 6. Studying parkin-mediated ubiquitination during mitophagy using synthetic proteins. (A) Synthesis of probes to study the localization of pUb during mitophagy. (B) Live cell LSCM images of U2OS cell with probes **2** and **3** conjugated to cR10D. TAMRA (red), Hoechst (blue); scale bars 10 μm. (C) Representative LSCM images of fixed parkin-expressing U2OS cells with and without CCCP and the synthetic probes **1**–**4**. TAMRA (red), TOM20 (green), parkin (blue); scale bars 1 μm. (D) Colocalization between synthetic probes and TOM20 using Pearson's coefficient (from the cells in panel C) shows that parkin prefers conjugation of unphosphorylated Ub. *$p < 0.05$, **$p < 0.005$. (E) Colocalization between parkin and TOM20 using Pearson's coefficient (from the cells in panel C) shows that parkin's recruitment to damaged mitochondria is not affected by the synthetic probes. Adapted with permission from ref 3, Copyright 2021 Royal Society of Chemistry.

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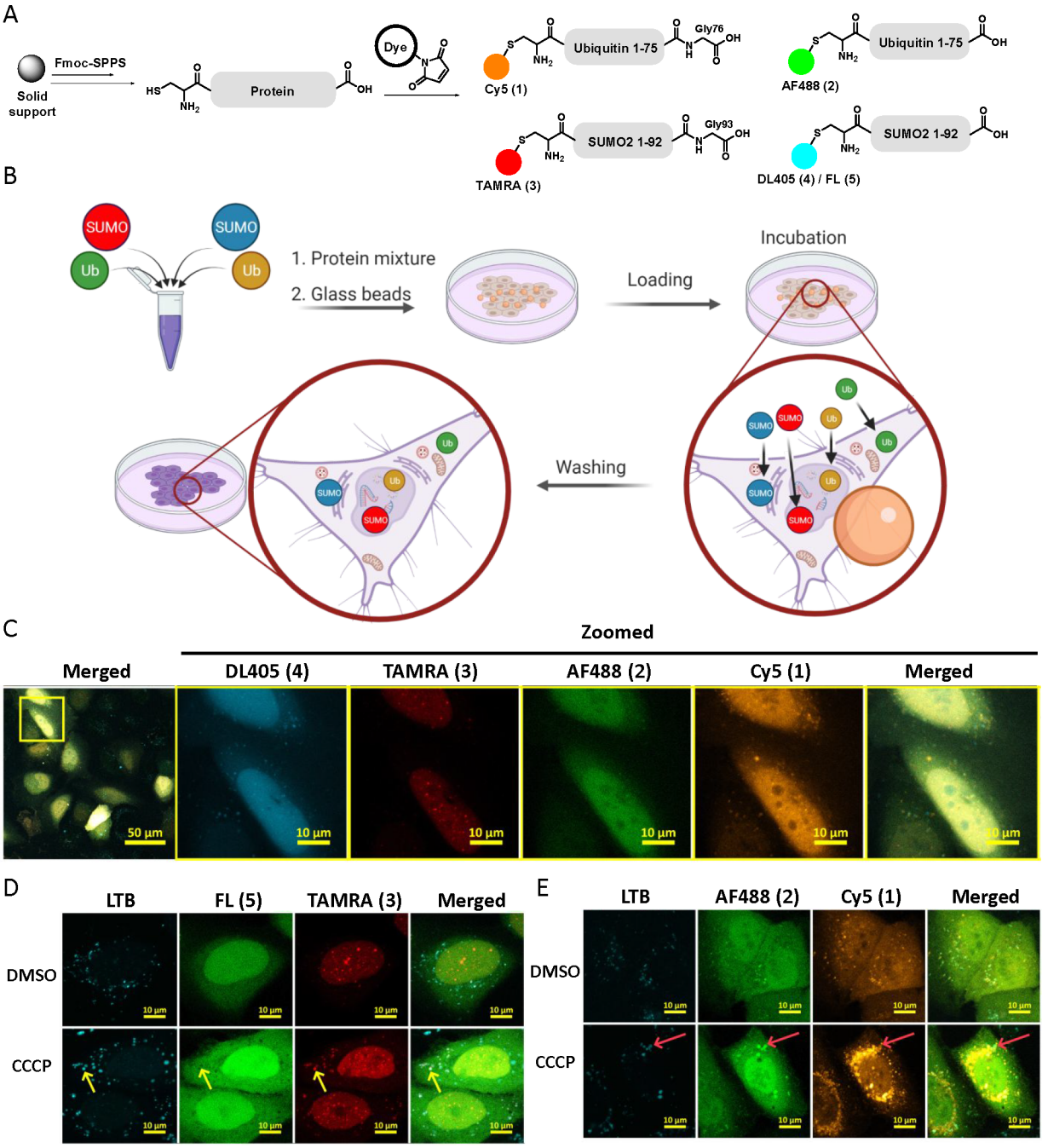

Figure 7. MBL of synthetic proteins to study ubiquitination and SUMOylation in live cells. (A) Synthesis of protein probes with fluorescent dyes: Cy5, TAMRA, fluorescein, Alexa Fluor 488 (AF488), and Dylight 405 (DL405). (B) Schematic illustration of protein delivery using MBL. (C) Confocal images of untransfected U2OS cells after MBL with four synthetic proteins: DL405−SUMO2ΔG93 (blue); TAMRA−SUMO2 (red); AF488−UbΔG76 (green); Cy5−Ub (gold). (D) Confocal images of parkin-expressing U2OS cells, with and without CCCP treatment, loaded with fluorescein−SUMO2ΔG93 (green) and TAMRA−SUMO2 (red) and stained with Lysotracker blue (LTB) (cyan). (E) Confocal images of parkin-expressing U2OS cells, with and without CCCP treatment, loaded with AF488−UbΔG76 (green) and Cy5−Ub (gold) and stained with LTB (cyan). Scale bars are 50 and 10 μm for full view and zoomed images, respectively. Adapted with permission from ref 4. Copyright 2022 John Wiley and Sons.

releases a protein with a C-terminal aldehyde, which is a known warhead to trap cysteine proteases. Particularly, Ub with a C-terminal aldehyde is known to selectively trap a specific type of deubiquitinating enzyme (DUB).[29] Using this feature, we aimed to perform on-demand activation of a synthetic protein inhibitor with selectivity for a prostate cancer related DUB, ubiquitin specific protease 2 (USP2). We therefore equipped a synthetic Ub variant, which is known to inhibit USP2 (Ubv2.3),[25] with an aldehyde warhead at the C-terminus to improve its potency (Figure 4A).

For the cellular delivery, we linked a CPP unit through a thiazolidine reactive group (Figure 4A). As CPP, we used the highly efficient cyclic deca-arginine (cR10) developed by Cardoso and co-workers[57] and further improved by the Hackenberger group (Figure 6A).[58] In addition, we used the endosomolytic peptide L17E as an additive to increase the endosomal escape of this cargo. This caged construct also

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included a proximity induced quenching through Förster resonance energy transfer (FRET) to monitor the thiazolidine cleavage in live cells (Figure 4B,C). Combining these elements in the same synthetic proteins, we demonstrated the on-demand selective inhibition of a cellular enzyme in live cells.

In this case, we chose the CPP method for the delivery to induce a biological effect and inhibit the endogenous USP2. To monitor the reaction without signal interference from the endosomally trapped cargo, we quantified the increase in the fluorescence intensity only inside the nucleus.

DABCYL Modified CPP Enhances Live Cell Delivery of Synthetic Proteins

At this stage, we were also interested to deliver proteins connected by a cleavable asymmetric disulfide to the CPP unit. Unfortunately, replacing the stable linkage to the CPP unit in the tetramethylrhodamine (TAMRA) labeled Ub resulted in significant endosomal entrapment (Figure 5). To improve the delivery of Ub and other synthetic cargoes, we examined the effect of 4-((4-(dimethylamino)phenyl)azo)benzoic acid (DABCYL), which is a hydrophobic dark quencher for both fluorescein isothiocyanate (FITC) and TAMRA. We observed that modifying the cR10 CPP with DABCYL (cR10D) significantly improved the delivery of synthetic proteins (Figure 5A,B,C).[2] Using cR10D, we were able to improve the delivery of Ub as a model cargo by over 3-fold compared to cR10 (Figure 5C). In addition, the improved cell permeability allowed us to deliver a DUB specific activity-based probe and SUMO paralogue 2 (SUMO2). It is still unclear how DABCYL affects the delivery mechanism, which is currently under investigation.

Synthesis and Delivery of Phosphorylated Ub to Examine Its Role in Mitophagy

After successfully delivering several synthetic proteins, we turned to exploit the power of protein synthesis and compare how a site-specific PTM affects the cellular function of synthetic proteins. For this goal, we chose to directly compare the phosphorylated and the native Ub involvement in PINK1–parkin mediated mitophagy.

In vitro comparison of parkin's substrate preference suggests that pUb activates parkin but is not a good substrate for conjugation by parkin.[59] However, this observation was never confirmed inside living cells, which are more complex than purified systems. Since phosphorylation is a highly dynamic PTM, we synthesized Ub with a stable pSer analogue at position 65 (SPSUb) to maintain the phosphorylation state without being affected by cellular phosphatases.[3] We then attempted the live cell delivery, with cR10, of SPSUb and its unphosphorylatable negative control, Ub with a S65A substitution (UbS65A). While UbS65A was permeable, the addition of two negative charges in SPSUb completely abolished its delivery using cR10. This strong difference in the delivery of two similar cargoes emphasizes the challenges in the CPP approach for studying modified proteins. Fortunately, replacing the CPP with cR10D facilitated the delivery of our relevant synthetic proteins with similar efficiency (Figure 6A). However, the need to fluorescently tag the mitochondria compelled us to fix the cells, following the damage response, to stain organelles using the known markers.

Once the delivery of SPSUb and its unphosphorylatable analogue UbS65A was optimized to a similar efficiency (Figure 6B), we also prepared Ub and its unconjugatable form with the C-terminal glycine 76 deletion (UbΔG76) as controls (Figure 6A).

Using laser scanning confocal microscopy (LSCM) and colocalization analysis, we were able to confirm that unphosphorylated Ub is a significantly better substrate for parkin after mitochondrial damage through treatment with carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (Figure 6C). By including UbΔG76 as a negative control, we were able to correlate that the mitochondrial recruitment of each probe depends on its conjugation (Figure 6D). Notably, our probes did not affect the mitophagy process, as evident by the identical parkin recruitment to damage sites (Figure 6E).

Our results support the current model, where parkin conjugates unphosphorylated Ub to damaged mitochondria and that the pUb is only formed in poly-Ub chains that are anchored to mitochondrial substrates. Phosphorylation of Ub, as a PTM of a PTM, is an interesting example of how cells utilize several layers of regulation to drive complex processes to their completion.[19] In this case, PINK1 and parkin are regulated by changes in their PTMs, stability, localization, and properties of their substrates (i.e., Ub that is conjugated to mitochondrial proteins) to drive the multistep mitophagy process to its completion.[60] To simplify our analysis of this process, we limited our examination to the conjugation of pUb by parkin during mitophagy. However, we are currently developing approaches for the cellular studies of modified Ub and ubiquitin-like modifiers that will address both the PTM and its substrate context.

MULTIPLEXED DELIVERY OF SYNTHETIC PROTEINS

Studying the role of a specific protein in the highly complex cellular environment, which contains heterogeneous populations (e.g., cell cycle, stress, etc.), requires reliable controls. This is particularly important when the biological question requires differentiating between similar targets that differ in a small chemical element (e.g., a PTM). Even Ub and Ub-like modifiers, which are complex PTMs are significantly smaller than fluorescent protein tags (e.g., GFP, RFP, mCherry) and the self-labeling protein tags (e.g., Halo-tag), which could affect the properties of tagged proteins.[45]

To directly image Ub and Ub-like modifiers without protein tags (Figure 7A), we envisioned that the bead loading delivery method and can simultaneously deliver several proteins into the same cells. Using this approach, termed multiplexed bead loading (MBL),[4] we delivered up to four different synthetic analogues of Ub and SUMO2 to the same living cells, each with a different fluorescent tag, to compare their localization in unstressed cells and mitophagy (Figure 7B). We probed the localization of these synthetic proteins, without altering the endogenous levels of Ub and SUMO2, by both LSCM (Figure 7C) and super-resolution microscopy. By comparing conjugatable and unconjugatable Ub and SUMO2 in the same cells, we revealed new involvement of SUMO2 (Figure 7D) and unconjugated Ub (Figure 7E) in lysosomes. In addition, the lysosomal localization of these proteins increased during late stages of mitophagy. Interestingly, while Ub recruitment was independent of conjugation, SUMO2 was only localized to lysosomes when Gly93 was present, suggesting that SUMO2 is conjugated at these sites. We also observed that the reported localization of SUMO2 to nuclear puncta (e.g., promyelocytic leukemia (PML) bodies) is strictly dependent on its conjugation.

Overall, this work emphasizes the power and simplicity of MBL to study synthetic proteins in same cells. This approach is

https://doi.org/10.1021/acs.accounts.2c00236
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ideal to compare modified proteins to study their interactions with both endogenous and ectopically expressed proteins. In this work, the number of delivered proteins was determined by the available fluorescent channels. However, MBL is not limited to the number of delivered proteins, if imaging is not necessary. We are currently working on applying this approach for proteome engineering using complex protein mixtures.

CONCLUSIONS

Mastering protein synthesis approaches grants direct access to unique proteins that are inaccessible by recombinant approaches.[27] Unleashing these synthetic proteins in the cellular environment is a powerful approach to study and manipulate biological processes. However, due to technical challenges in protein delivery, most synthetic proteins are still studied *in vitro*. We feel that the decades of developments in protein delivery brought us to a new direction in biological studies. Delivering unique proteins prepared by synthesis, semisynthesis, recombinant expression (with or without GCE), and late stage modification strategies into live cells can exploit the best of all worlds and improve our understanding of the fundamental roles of PTMs.

PTMs are one of the major ways that cells assign their "work horses" to their tasks. As a result, preserving the PTM's context, that is, in the presence of its substrates, writers, readers, and erasers, is an important consideration to fully decipher the PTM's message. Until today, most challenges in studying synthetic proteins in live cells were mainly attributed to the delivery process. With the new methods for synthetic protein delivery, one can focus on developing tailored approaches for their cellular studies. These must simultaneously provide information on the PTM's introduction, its substrate, and the biological process. For example, we found that SPSUb was not conjugated by the cellular machinery since the native pUb signal has to be introduced in the appropriate time and context, that is, preassembled ubiquitinated proteins at the mitochondrial damage sites.

Another exciting application is studying a PTM's involvement in regulating dynamic organization of proteins into membraneless organelles formed by liquid−liquid phase separation.[61] These organelles are involved in myriad cellular stress responses and show rapid dynamics in their assembly and disassembly. Therefore, we believe that synthetic proteins can find new applications to study these fundamental processes by introducing both defined PTMs and elements for their manipulations. Notably, the global changes in protein localization during phase separation processes is ideal for live cell imaging of synthetic protein probes.

In this Account, we highlighted the considerations for delivering synthetic proteins and provided examples of our recent contributions for direct live cell studies of PTMs in both stressed and healthy conditions.[1−4] Our work provides guidelines for planning future studies combining protein synthesis and molecular biology approaches to introduce modified proteins to investigate their role in a specific biological context. We mainly compared differences in localization for synthetic proteins using fluorescent microscopy. However, cutting-edge techniques such as rapid fluorescent lifetime imaging (rapid-FLIM) combined with FRET (FLIM-FRET) can separately image interaction events between synthetic, endogenous, and recombinant proteins, which is otherwise very challenging due to the dynamic and substoichiometric nature of PTMs.[62] In addition, Raman microscopy, protein NMR, microenvironment mapping, proteomics, and DNA points accumulation for imaging in nanoscale topography (DNA-PAINT)[63] can benefit from live cell delivery of synthetic proteins. Furthermore, delivering synthetic proteins can be beneficial in system biology in particular to the development of mirror-image artificial life[64−66] and mechanistic characterization of protein-based therapeutics.[25]

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Notes

The authors declare no competing financial interest.

Biographies

Guy Mann was born in Israel on October 22, 1986. Guy received his M.Sc. in organic chemistry from the Technion—Israel Institute of Technology. He is currently a Ph.D. student in Prof. Ashraf Brik's laboratory. His research interest is the chemical synthesis of posttranslationally modified proteins, their delivery to live cells, and developing approaches for their studies inside the cellular environment. Guy is interested in the live cell imaging of PTMs and protein labeling techniques.

Pradeep Sadhu was born in India on August 2, 1987. Pradeep received his M.Sc. in organic chemistry from Andhra University, India. He obtained his Ph.D. under Prof. Tharmalingam Punniyamurthy in 2017 from Indian Institute of Technology Guwahati (IIT-G), India. He is currently a postdoctoral fellow in Prof. Ashraf Brik's laboratory. His research focuses on the synthesis of challenging posttranslationally modified proteins, as well as the development of efficient methods for protein live cell delivery.

Ashraf Brik was born in Israel on June 29, 1973. Professor Brik obtained his Ph.D. in 2001 from the Schulich Faculty of Chemistry at Technion—Israel Institute of Technology. He did his postdoctoral studies with Prof. Chi-Huey Wong at the Scripps Research Institute and in 2004 was promoted to a Senior Research Associate. In 2007, he joined Ben-Gurion University of the Negev as an Assistant Professor and was promoted to Associate Professor in 2011 and Full Professor in 2012. In 2015, he moved to the Technion, where he currently holds the Jordan and Irene Tark Academic Chair. His research involves the development of novel chemistries and approaches for the synthesis and semisynthesis of posttranslationally modified proteins for biochemical, biophysical, and biological studies.

ACKNOWLEDGMENTS

A.B. holds The Jordan and Irene Tark Academic Chair. This project has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (grant agreement no. 831783).

https://doi.org/10.1021/acs.accounts.2c00236
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ABBREVIATIONS

PM	plasma membrane
SPPS	solid phase peptide synthesis
GCE	genetic code expansion
PTD	protein transduction domain
PTM	posttranslational modification
CPP	cell penetrating peptide
CPD	cell penetrating poly(disulfide)
MBL	multiplexed bead loading
ATL	anthrax lethal toxin
LF_N	lethal factor N-terminal domain
Ub	ubiquitin
SUMO	small ubiquitin like modifier
GFP	green fluorescent protein
TAT	transactivator of transcription of HIV-1
GSH	glutathione
cR10	cyclic deca-arginine
DABCYL	4-((4-(dimethylamino)phenyl)azo)benzoic acid
cR10D	DABCYL modified cyclic deca-arginine
DTNP	2,2-dithiobis(5-nitropyridine)
pUb	ubiquitin modified with phosphorylation at serine 65
SPSUb	ubiquitin with stable phosphoserine at position 65
UbS65A	ubiquitin with serine 65 mutated to alanine
DUB	deubiquitinating enzyme
FITC	fluorescein isothiocyanate
AF488	Alexa Fluor 488
TAMRA	tetramethylrhodamine
LTB	Lysotracker blue
LSCM	laser scanning confocal microscopy
FLIM	fluorescent lifetime imaging
FRET	Förster resonance energy transfer
CCCP	carbonyl cyanide 3-chlorophenylhydrazone

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https://doi.org/10.1021/acs.accounts.2c00236
Acc. Chem. Res. 2022, 55, 2055−2067