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Endogenous Proteins Modulation in Live Cells with Small Molecules and Light

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Protein modulation by light illumination enables the investigation of biological function in high spatiotemporal precision. Compared to genetic methods, the small molecule approach is uniquely suited for modulating endogenous proteins. Endogenous protein modulation in live cells with small molecules and light has recently advanced on three distinctive frontiers: i) the infrared-light-induced or localized decaging of small molecules by photolysis, ii) the visible-light-induced photocatalytic releasing of small molecules, and iii) the small-molecule-ligand-directed caging for photomodulation of proteins. Together, these methods provide powerful chemical biology tool kits for spatiotemporal modulation of endogenous proteins with potential therapeutic applications. This Concept article aims to inspire organic chemists and chemical biologists to delve into this burgeoning endogenous protein modulation field for new biological discoveries.

Introduction

Modulating proteins in high spatiotemporal precision is critical to probing their biological functions accurately.[1] With its easily tunable wavelength, duration, and intensity, light irradiation provides a near-ideal tool for the modulation of proteins. The optogenetic method is a revolutionary light-modulation method for neuroscience and related life science research, which utilizes photosensitive proteins to probe biological functions (Figure 1a).[2] Among those optogenetic methods, the caged proteins strategy enables the site-specific modulation of protein functions by photodecaging light-sensitive unnatural amino acid residues.[3] While these optogenetic methods are highly influential, introducing photosensitive proteins requires genetic sequence incorporation into the genome, which is unsuitable for modulating endogenous proteins. The novel genetic engineering techniques (e.g., CRISPR/Cas9) may enable protein expression at the endogenous level, but they are unsuitable for hard-to-transfect cells and suffer from off-target effects.[4] In addition, genetic engineering inevitably disturbs the homeostasis of endogenous proteins.[5]

Various biological pathways fine-tune endogenous protein functions in native cells in their natural environments, which precise understanding is biologically relevant for therapeutic applications. To this end, the small molecules approach is attractive.[6] The decaging of neurotransmitters by photolysis has enabled neuroreceptor function investigations in high spatiotemporal precision (Figure 1b).[7] However, scientists have discovered two main limitations of the smallmolecule decaging approach. *i*) The commonly used UV light irradiation led to compromised biocompatibility and penetration;[8] *ii*) The free diffusion of decaged small molecules decreased the spatiotemporal resolution of the small-molecules-decaging methods.[9] Over the past few years, scientists have developed new strategies to overcome these limitations for endogenous proteins modulation in live cells, and we will discuss these

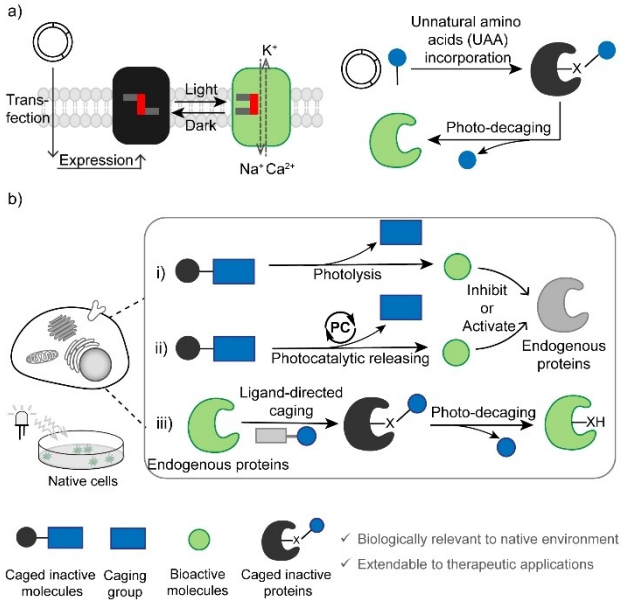

Figure 1. Endogenous proteins modulation in live cells with small molecules and light. a) The optogenetic methods for protein modulation in transgenic cells. b) The endogenous protein modulation with small molecules and light in native cells.

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strategies here. We hope this Concept article will inspire organic chemists and chemical biologists to delve into this burgeoning interdisciplinary field of endogenous protein modulation for new biological discoveries.

Small Molecules Decaging by Photolysis

Modulating endogenous proteins by small-molecule decaging was first achieved on caged cyclic adenosine monophosphate (cAMP) **1** and adenosine triphosphate (ATP) **2** (Figure 2a).[10] With the caged small-molecule approach, the cAMP-dependent proteins kinase and sodium-potassium pumps were modulated by UV light irradiation in high photolysis efficiency, respectively. Afterward, this caged small-molecule approach enabled the investigations of various dynamic biological processes with neurotransmitters and anticancer/antibacterial drugs.[11] However, the high cytotoxicity and low penetration of UV light irradiation limited their *in vivo* applications. The boron-dipyrromethenes (bodipy) and cyanines were later developed as the far-red and near-infrared (NIR) light cage groups to circumvent UV light use.[12] Klán and co-workers reported NIR-responsive bodipy cages, and the far-red light-induced bodipy cages were further developed by tuning the photochemical properties of bodipy derivatives (Figure 2b).[13] The photorelease of bodipy-caged dopamine **3** increased the beating frequency of human cardiomyocytes with far-red (652 nm) light irradiation.[14] The cyanine-caged 4-hydroxycyclofen drug **4** inhibited the MCF-7 cell viability by near-IR light irradiation.[15]

As spatially organized proteins have different physiological functions, the confinement of the small molecule release to subcellular compartments is advantageous.[16] To limit the free diffusion of decaged small molecules, the subcellular-targeted motifs were linked to cage molecules. Weinstein and co-workers reported the organelle-targeted moieties enabled the bodipy-caged *p*-nitroaniline to be released in mitochondria, endoplasmic reticulum (ER), and lysosomes selectively (Figure 3).[17] The mitochondrial-localizable triphenylphosphine was linked to

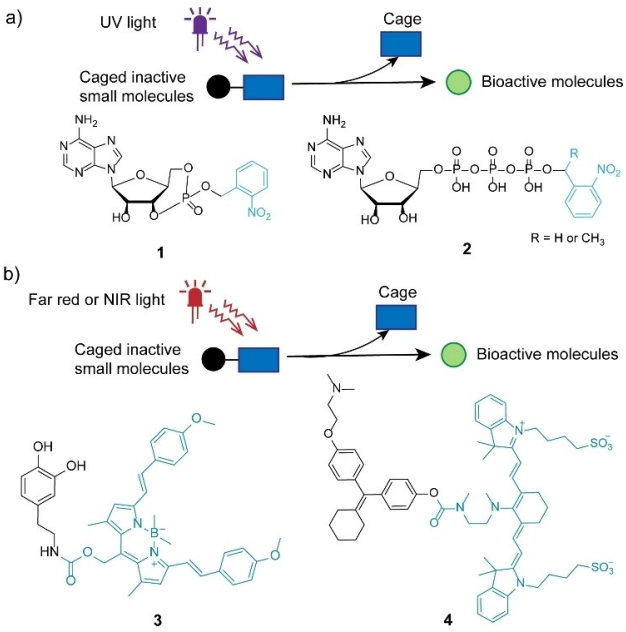

Figure 2. The small-molecule decaging by photolysis. a) Schematic diagram of small-molecule decaging by photolysis and early examples. b) Far-red or NIR light-induced small-molecule decaging.

DNP **5** to achieve mitochondria depolarization.[18] Sasmal and co-workers designed caged doxorubicin **6** with triphenylphosphine moiety to induce cell death by NIR (two-photon) light irradiation.[19] Singh and co-workers reported dihydrorhodamines with dual functions for photosensitization and mitochondria-localization.[20] The anticancer drug chlorambucil (cbl **9**) was linked to dihydrorhodamine to provide the inactive cbl **7**, which was oxidized by reactive oxygen species (ROS) in cancer cells to rhodamine-caged cbl **8** with mitochondrial localization. Under the green-light irradiation, the mitochondria-localized **8** released cbl **9** in cancer cells, which had improved spatial precision in the mitochondria.



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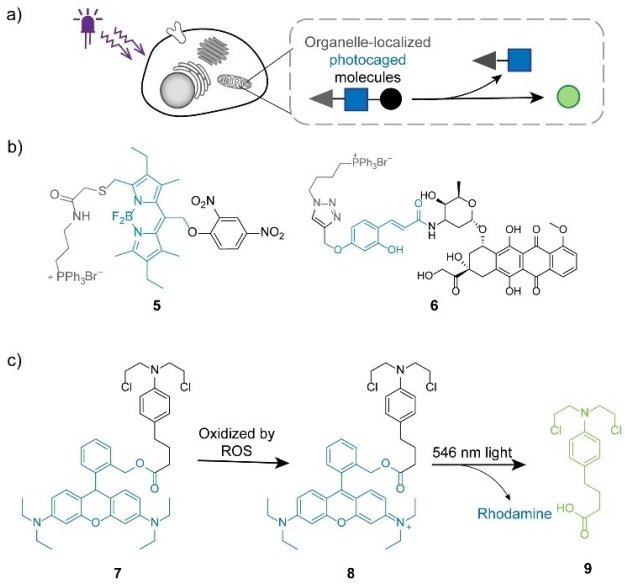

Figure 3. The organelle-localized small-molecule decaging. a) Schematic diagram of photolyzed decaging on organelle-localized small molecules. b) Mitochondria-localized small molecules with triphenylphosphines. c) The latent mitochondria-localized small molecules with dihydrorhodamines, which were oxidized by ROS to rhodamine with mitochondrial localization.

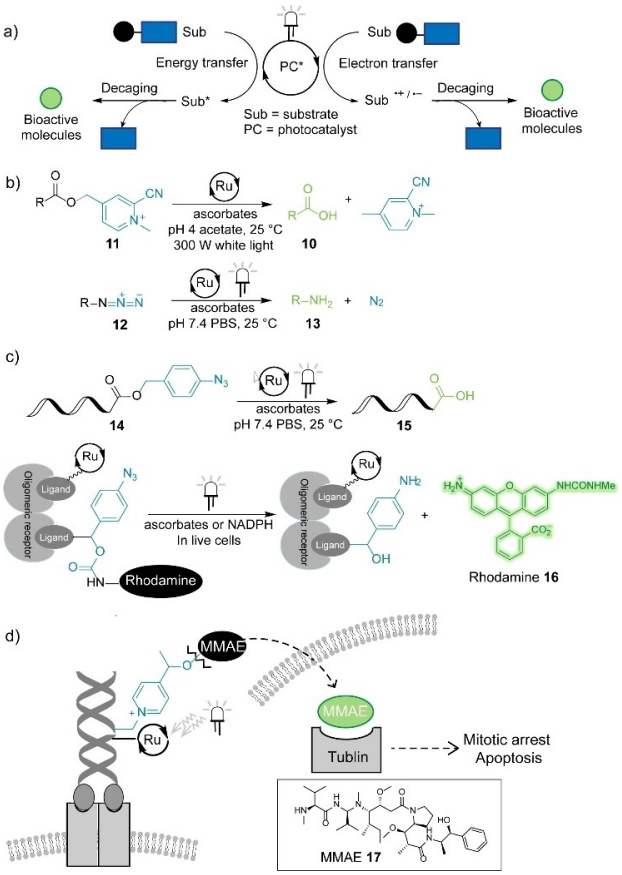

Figure 4. The photocatalytic releasing of small molecules by bond-cleaving reactions. a) Schematic depicting photocatalytic releasing of small molecules. b) Photocatalytic decaging by pyridinium salts and azides reduction. c) Azide reduction for carboxylates releasing and the dimeric protein-templated reactions. d) DNA-templated decaging of MMAE to induce cell apoptosis.

Photocatalytic Releasing of Small Molecules

Compared to small-molecule decaging by photolysis, the small-molecule decaging by photocatalysis is less studied for biological applications, which have recently emerged as a blooming frontier.[21] In the photocatalytic decaging reactions, the photosensory domain and the to-be-released target molecule are decoupled as the photocatalyst and the caged molecule, so the caged molecule does not need to be photosensitive. Instead, the photocatalyst absorbs the light irradiation to reach the excited state, which initiates the electron transfer or energy transfer with the photo-insensitive caged molecule to afford the decaged active molecule (Figure 4a). The photocatalytic release of carboxylate **10** was first reported with pyridinium salt **11** as the caging group with the ruthenium photocatalysis.[22] However, as the reaction required to perform in acidic solutions and the reaction rate was slow, the 300 W white xenon irradiation in acidic pH 4 acetate buffer was used and limited the biological applications (Figure 4b).

Liu and co-workers disclosed in 2011 the photocatalytic azide reduction reaction with biocompatibility.[23] The ingenious DNA-encoded reaction discovery system revealed the photocatalytic azide **12** reduction to release amine **13** by visible light irradiation in neutral aqueous conditions. Compared with pyridinium salt **11**, azide **12** underwent rapid photocatalytic reduction using ascorbates as reductants. The reaction was chemoselective and compatible with various sensitive functional groups, including alkynes, alkenes, phenols, and aryl halides. The azide reduction reaction was applicable for amine decaging and extended to carboxylate **15** decaging by the 1,6-elimination after the azide **14** reduction (Figure 4c). Winssinger and co-workers later utilized this photocatalytic azide reduction in the nucleic acid-templated and protein-templated versions. The amine-containing rhodamine **16** was released in live cells and vertebrates to report miRNA and protein expressions.[24] The tubulin ligand monomethyl auristatin E (MMAE) **17** was also photoreleased in the dimeric protein-template version under the hypoxia to induce cell apoptosis (Figure 4d).[25]

The biocompatible bond-forming reactions are as powerful for the photocatalytic release of small molecules as bond-cleavage reactions, however their developments lag behind due to the bimolecular reaction characters.[21a] Chen and co-workers have systematically investigated the photocatalytic biocompatible bond-forming reactions under oxidative quenching[26] and reductive quenching conditions[27] (Figure 5a). The cyclic iodine(III) hydroxylbenziodoxole (BI-OH) was discovered as a biocompatible oxidative quencher under photocatalytic conditions (Figure 5b).[26] The deboronative alkynylation of **18** with BI-alkyne **19** in neutral aqueous conditions constructed alkyne **20**, which photocatalytic use of BI-OH was compatible with amino acids, nucleotides, sugars, proteins, nucleic acids, and cell lysates. The reductive quenching with ascorbates was also compatible with various biomolecules, and

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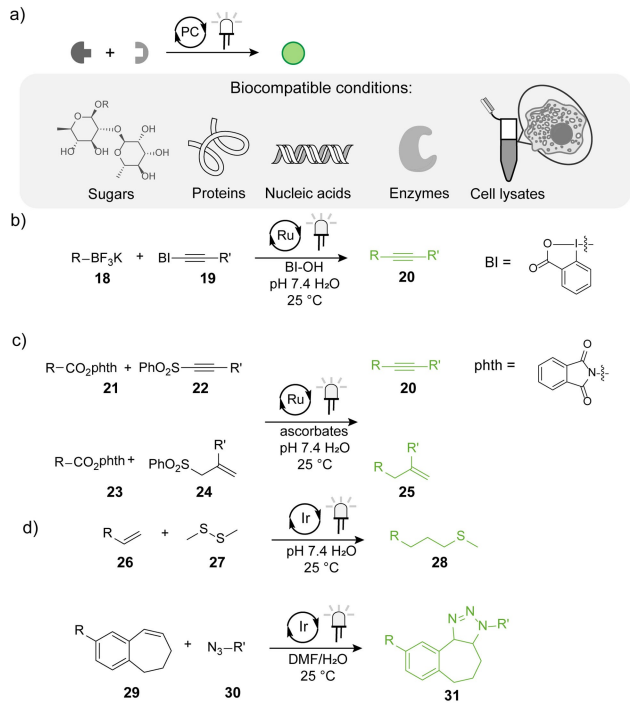

Figure 5. The systematic investigation of biocompatible photocatalytic bond-forming reactions. a) Schematic depicting biocompatible bond-forming reactions by photocatalysis. b) The biocompatible deboronative alkynylation via the oxidative quenching. c) The biocompatible decarboxylative alkynylation and allylation via the reductive quenching. d) The biocompatible bond-forming reactions via the energy-transfer pathway.

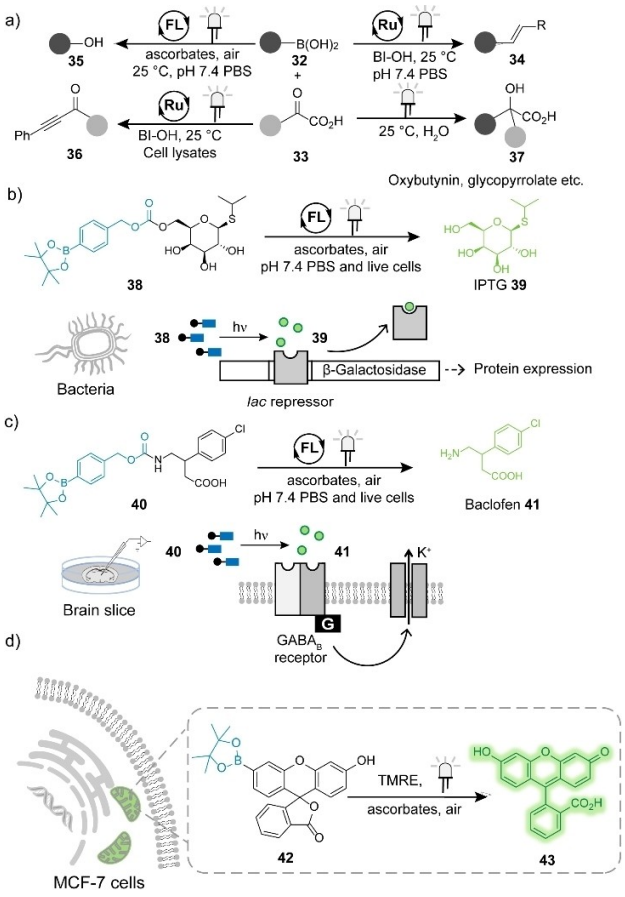

Figure 6. The photocatalytic reactions inside live cells, including neurons. a) Versatile biocompatible reactions of boronic acids and ketoacids. b) Photorelease of IPTG for the lac repressor modulation. c) Photomodulation of $GABA_B$ receptor in neurons from mice brain slices. d) Mitochondria-localized photorelease of small molecules.

it did not affect the enzymatic activity of a human carbonic anhydrase II (Figure 5c).[27a] The photocatalytic decarboxylative alkynylation of carboxylphthalimide **21** constructed alkyne **20** with alkynyl sulfone **22** effectively in neutral aqueous conditions. Under similar reductive conditions with ascorbates, the decarboxylative allylation of **23** with allyl sulfone **24** performed well at ultrafast rates and finished in seconds.[27b]

In addition to the single-electron-transfer pathway, the photocatalytic energy-transfer pathway also demonstrates biocompatibility. Glorius and co-workers reported the *anti*-Markovnikov hydrothiolation of alkene **26** via the energy-transfer activation of disulfide **27**, and the reaction was compatible with various biomolecules and cell lysates.[28] The cycloaddition reactions were previously applied for small-molecule release to modulate protein functions.[29] Weaver and co-workers developed the photocatalytic isomerization of unstrained cis-cycloalkene **29** to strained trans-cycloalkene via the energy transfer, which further reacted with the insulin-modified azide **30** by cycloadditions to form **31** in the mixed aqueous DMF solutions.[30]

Under biocompatible oxidative and reductive conditions, versatile photocatalytic bond-forming reactions were developed in neutral aqueous conditions (Figure 6a). Various bioactive molecules were constructed from boronic acid **32** and ketoacid **33** as biocompatible building blocks.[31–33] The BI-OH-enabled photocatalytic deboronative alkenylation synthesized the polyethylene glycol (PEG)-linked water-soluble alkene **34**.[31a] The organic dye fluorescein generated a low concentration of hydrogen peroxides in the air atmosphere, enabling deboronative hydroxylation to release phenol **35**.[32] The ketoacid **33** underwent decarboxylative alkynylation with BI-alkynes **19** to yield ynones, ynoates, and ynamides.[31b] The photorelease of a mGlu5 receptor inhibitor ynone **36** was achieved in cell lysates, which spatiotemporal release had the therapeutic potential for both peripheral and central nervous system disorders. The combination of boronic acid **32** and ketoacid **33** resulted in the donor-acceptor complex formation, which absorbed visible light to afford lactate **37** without photocatalyst or additional reagent.[33] This reaction could perform in neutral aqueous conditions for the homoserine conjugation by the radical additions.

The photocatalytic reactions inside live cells are desirable, but they face two main challenges: i) The commonly used photocatalysts are transition-metal-based, and heavy-metal components have potential cytotoxicity to limit biological applications.[34] ii) The oxygen and redox-active species are ominous in live cells, which react with photocatalytic radical intermediates to compromise the reaction.[35] Chen and co-workers reported the first photocatalytic decaging reaction in

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live cells, including neurons.[32] The reaction utilized organic dyes fluorescein or rhodamine as the biocompatible photocatalysts, which were usually used for intracellular tracing and tracking.[36] The photochemical pathways were tuned to favor electron transfer rather than energy transfer, so the endogenous signal molecules hydrogen peroxides were generated in situ. At the same time, the cytotoxic singlet oxygen was not formed. The excellent biocompatibility of organic dyes and fast kinetics of the deboronative hydroxylation were the key to the live-cell applications, in which the alcohol and amine functional groups may be decaged by the 1,6-elimination.[37] The boronic acid **38** released isopropyl β-D-thiogalactoside (IPTG) **39** by blue-light irradiation to modulate live bacterial protein expression level (Figure 6b).

Baclofen **40** is the selective agonist of the $GABA_B$ receptor, which shows strong therapeutic effects in many neurological and psychiatric conditions. However, its ubiquitous activation of $GABA_BR$ in neuronal and non-neuronal tissues results in unwanted, adverse muscle relaxation effects.[38] The photorelease of baclofen **40** from caged baclofen **41** in spatiotemporal precision enabled the targeted modulation of neuron membrane potential in mice brain slices (Figure 6c). As the photocatalytically generated hydrogen peroxides undergo readily metabolic degradation in live cells, they are short-lived and confine the boronate **42** decaging to the vicinity of photoexcited organic dyes (Figure 6d).[39] When the mitochondria-localized organic dye tetramethylrhodamine ethyl ester (TMRE) was used, the mitochondria-selective photorelease of small molecule **43** could be achieved. This subcellular-selective release of bioactive molecules holds great potential to modulate endogenous protein functions with spatiotemporal precision.

Covalent Conjugation of Small-Molecule-Ligands to Proteins for Photomodulation

While various methods have been developed to improve the targeted release of small molecules, the challenges arising from the diffusion of decaged small molecules cannot be completely addressed, which limit their spatiotemporal precision for protein photomodulation. To this end, the caged proteins strategy provides an attractive alternative. The unnatural amino acids (UAAs) incorporation method site-specifically introduces the caged amino acid residues to the proteins of interest. However, the genetic incorporation of orthogonal tRNAs and tRNA synthetases is required, and it is only applicable to exogenous proteins. The affinity labeling method has been developed for endogenous protein labeling and profiling, which covalently modifies target proteins *via* selective ligand-protein recognition in the crowded intracellular environment.[40]

The photoswitchable affinity ligands (PAL) strategy tethers proteins with small-molecule PAL by the selective ligand-protein interactions. Erlanger and co-workers developed the first PAL to switch the on-off activity of the acetylcholine receptor.[41] After the disulfide reduction, the photo-isomerizable azobenzene was anchored on the protein of interest by the cysteine alkylation. Kramer and co-workers utilized acrylamides to tether PAL **44** on the cysteine residues of potassium ion channels.[42] The exposure to 500 nm light kept the azobenzene in the *trans* isomer to inhibit the tethered potassium ion channels. In contrast, the 380 nm light irradiation switched the azobenzene to the *cis* isomer to unblock the tethered channel. They also used photoswitch **44** to reveal the function of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels in the initial axon segment (Figure 7a).[43]

However, the dependence on the disulfide bond reduction and the slow reaction kinetics of the method hampered its further applications. To this end, Gorostiza and co-workers reported a new PAL **45** composed of glutamate, azobenzene, and *N*-hydroxysuccinimide as the tethering group, conjugating lysine residues and avoiding the excess reductants (Figure 7b). With **45**, the kainate receptor channel (GluK1) was photoactivated under violet-light irradiation to yield the immediate photocurrents in the GluK1-overexpressing cells.[44] After optimization, this method was applied to ultrafast synapses of the auditory system in live mice.[45] However, this method modified all four lysine residues of GluK1, and it was unsuitable for enzymes whose modified amino acid residues affect their native activity. The limitation of these PAL methods calls for new site-specific protein photomodulation methods.

Hamachi and co-workers reported the ligand-directed labeling strategy with the proximity effect for protein photomodula-

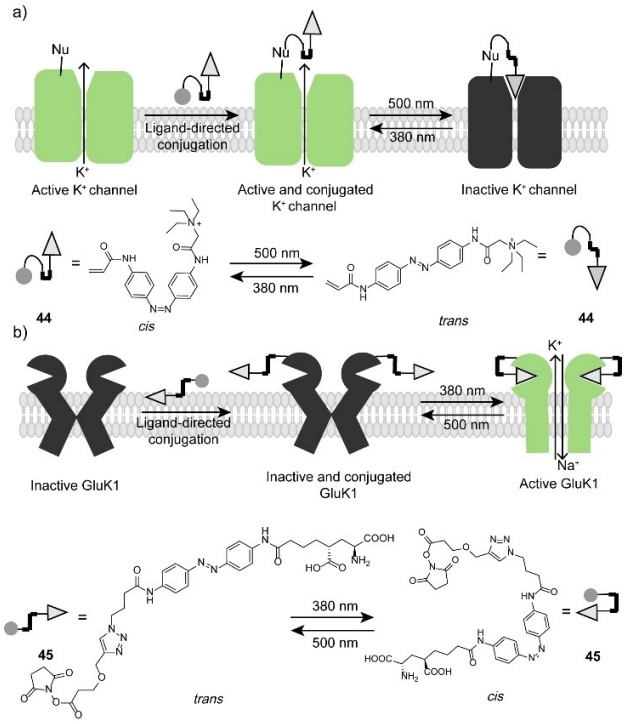

Figure 7. Photoswitchable affinity ligands for protein photomodulation. a) The acrylamides affinity ligands for potassium ion channels photomodulation. b) The *N*-hydroxysuccinimide affinity ligands for kainate receptor channel (GluK1) photomodulation.

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tion. The ligand-directed acyl imidazole (LDAI) chemistry was developed with the moderately reactive alkyloxyacyl imidazole **46** to transfer the acyl group to the nucleophilic amino acid residues (Figure 8a).[46] Based on LDAI, the small-molecule-ligand **47** selectively acylated the carbonic anhydrase I (CAI) to construct the caged CAI (Figure 8b).[47] The serine, lysine, and tyrosine residues of CAI were preferably labeled by **47**, and the caged CAI recovered activity by UV light irradiation. While the reaction performed well in test tubes and lysates, the high reactivity of acyl imidazoles was unsuitable for live-cell applications.[40]

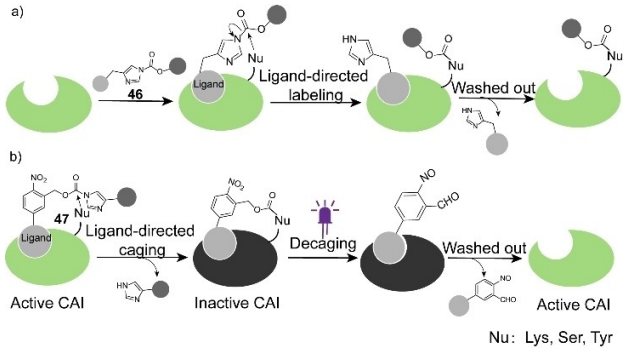

Figure 8. Ligand-directed proteins labeling by acyl imidazoles for the construction of caged proteins. a) The alkyloxyacyl imidazole for the ligand-directed labeling of proteins. b) The acyl imidazole for the ligand-directed caging of CAI nucleophilic residues.

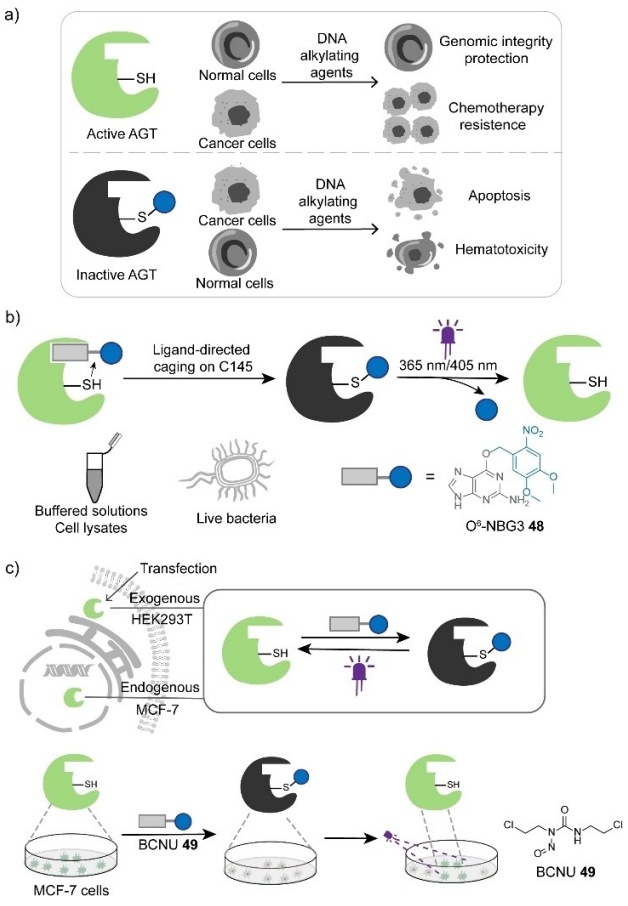

Figure 9. Small-molecule-ligand-directed caging and proteins decaging with light. a) The biological impacts of selective control of AGT activity in normal and cancer cells. b) The O^6-NBG3 ligand-directed caging of AGT. c) The AGT activity control for selective photomodulation of mammalian cells.

Chen and co-workers developed the first small-molecule-ligand-directed caging strategy to control endogenous DNA alkyltransferase activity inside live cells with light.[48] The O^6-alkylguanine-DNA alkyltransferases (AGT) selectively recognize DNA guanine O^6-methylation and transfer the methyl group to its cysteine-145 residue (Figure 9a).[49] The AGT activation protects normal cells' genomic integrity; however, it consumes the alkylating agents to defend cancer cells from chemotherapy.[50] In contrast, the AGT inhibition improves the chemotherapeutic drug efficacy as the anticancer adjuvant,[51] but it increases the normal cell mutation risks and results in hematotoxicity.[52] The O^6-benzylguanine derivative O^6-NBG3 **48** with the photoresponsive ortho-nitro substituents demonstrated superior AGT inhibition efficiency (Figure 9b). The catalytic active cysteine-145 residue was selectively blocked, and the spatially neighboring nucleophilic residues were unaffected. The O^6-NBG3 ligand-directed caging and decaging worked effectively in the buffered solutions and cell lysates, in which various bioactive components did not affect the reaction.

As the alkylated AGT is degraded by the facile ubiquitination in mammalian cells, the AGT photomanipulation in live cells poses more challenges.[53] When the O^6-NBG3 **48** was incubated in the human embryonic kidney cells (HEK 293T) transfected with AGT, the AGT activity was inhibited completely and may be recovered by light irradiation effectively. In the breast cancer MCF-7 cells without genetic manipulation, endogenous AGT activity could be inhibited by O^6-NBG3 **48** as well, and the light irradiation for 3 minutes recovered AGT activity (Figure 9c). The combined use of O^6-NBG3 **48** and light irradiation provides an attractive method to respond to the chemotherapy drug Bis-chloroethyl nitrosourea (BCNU) **49** treatment.[49] The light irradiation may effectively reverse the O^6-NBG3 **48**/BCNU **49** cocktail for cancer cell killing. The spatiotemporal control of the AGT function holds great potential for rescuing genomic integrity protection in normal cells while inducing the desirable apoptosis in cancer cells.

Summary and Outlook

In this Concept, we have discussed three distinctive frontiers of endogenous proteins modulation in live cells with small molecules and light (Figure 10a): i) the infrared-light-induced or localized decaging of small molecules by photolysis, ii) the visible-light photocatalytic release of small molecules, and iii) the small-molecule-ligand-directed caging of proteins for photomodulation. While these small-molecule methods are difficult to overcome the off-target effect, may be unsuitable for undruggable targets (e.g., protein-protein interactions), and have limitations to passing the blood-brain barriers,[54] they have the virtue of tunable oral bioavailability and cell permeability.[55] These methods together provide powerful

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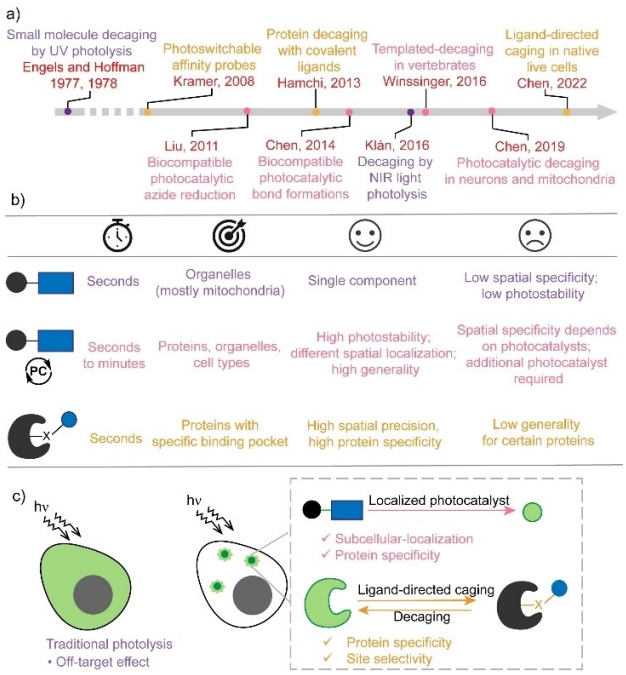

Figure 10. Strategies for endogenous proteins modulation with small molecules and light. a) Representative examples of endogenous proteins modulation with small molecules and light. b)The tabulated advantages and drawbacks of these spatiotemporal modulation methods. c) Schematic illustrations of different endogenous proteins modulation methods.

chemical biology tool kits for spatiotemporal modulation of endogenous proteins in live cells.

The spatiotemporal characters of these methods with pros and cons are summarized in Figure 10b, described below. Traditional photomodulation methods of endogenous proteins utilized small-molecule decaging by UV light photolysis. However, prolonged exposure to UV light has negative consequences on the biological system, and the tissue impenetrability of UV light is impractical for therapeutic applications. To this end, visible-light-cages and infrared-light-cages have been developed, but these caged molecules are synthetically sophisticated and unstable under ambient light conditions. Recently, various photocatalytic biocompatible reactions enabled the photo-release of photo-insensitive molecules. In these reactions, the photocatalysts not only perform electron-transfer or energy-transfer reactions with photo-insensitive molecules but also demonstrate versatile functions as fluorescence trackers and targeting motifs. As the photocatalyst is easily derivatized to target the cellular site of interest, the proximity decaging of small molecules,[32] the profiling of subcellular proteomics,[56] and the mapping of proteins interaction network[57] have been achieved by the localized photocatalysts.

To overcome the free diffusion of decaged small molecules, the covalent small-molecule-ligand strategy combines the fast kinetics from photochemical reactions and the site-specificity from protein-ligand recognition. The photoswitchable affinity ligands reversibly regulated the protein activity; however, the complete activity reversal by the photoisomerization was difficult in most cases. The construction of caged proteins provides an attractive alternative using the ligand-directed proteins labeling strategy; however, it usually resulted in unselective conjugation and was unsuitable for live-cell applications. AGT was the first endogenous protein to be site-selectively regulated by small molecules and light in live cells. The small-molecule-ligand-directed caging strategy is attractive to construct light-responsive proteins without genetic engineering, enabling site-specific turn-off and spatiotemporal turn-on of endogenous proteins' functions in live cells. This strategy, in principle, may easily extend to other protein classes with well-defined active sites and known ligands, such as kinases with well-developed covalent inhibitors, and Cas9 proteins with targeted DNA recognition sequences.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: endogenous proteins modulation · live cells · photocatalysis · photolysis · small molecules

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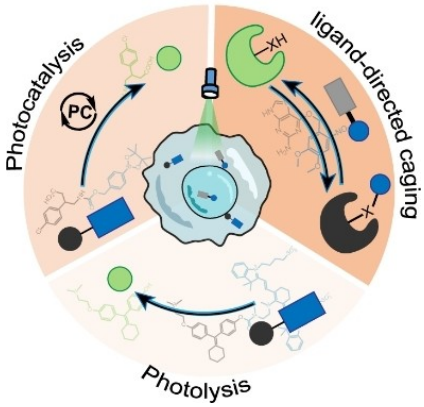
CONCEPT

Three distinctive small-molecule strategies for spatiotemporal modulation of endogenous proteins in live cells are discussed, including decaging of small molecules by photolysis, visible-light photocatalytic releasing of small molecules, and small-molecule-ligand-directed caging, which together provide powerful chemical biology tool kits with potential therapeutic applications.



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