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Expanding the chemistry of fluorescent protein biosensors through genetic incorporation of unnatural amino acids

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Fluorescent proteins are essential tools in biological research, ranging from the study of individual biological components to the interrogation of complex cellular systems. Fluorescent protein derived biosensors are increasingly applied to the study of biological molecules and events in living cells. The present review focuses on a specific class of fluorescent protein biosensors in which a genetically installed unnatural amino acid (UAA*) acts as the sensing element. Upon direct interaction with the analyte of interest, the chemical and/or physical properties of UAA* are altered, which triggers fluorescence property changes of the biosensor and generates readouts. In comparison to mutagenesis approaches within the standard genetic code, introduction of UAA*s with a unique functionality and chemical reactivity could broaden the scope of analytes and improve the specificity of biosensors. Nonconventional functional groups in fluorescent proteins enable sensor designs that are not readily accessible using the common twenty amino acids. Recent reports of UAA*-containing fluorescent protein sensors serve as excellent examples for the utility of such sensor design. We envisage that the integration of the two powerful chemical biology tools, fluorescent protein sensors and genetic incorporation of UAA*s, will lead to novel biosensors that can expand and deepen current understanding of cellular processes.

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1. Introduction

Fluorescent proteins are useful probes for the study of biological molecules and processes.[1–6] Since the discovery and subsequent cloning of the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*,[7] many variants of fluorescent proteins have been isolated or engineered to give a color palette, which enables the monitoring of cellular processes in living systems under physiological conditions using fluorescence microscopy, fluorescence-activated cell sorting, and other related methodologies[1–4,8] Information extracted from these experiments leads to an unprecedented era of biological discovery. In recognition of the importance of fluorescent proteins in modern biological, biochemical, and biomedical research, the 2008 Nobel Prize in Chemistry was awarded to three researchers, Drs Osamu Shimomura, Martin Chalfie, and Roger Y. Tsien, for their key contributions to the discovery and development of fluorescent proteins.

In addition to many established applications in cellular biology, increasing numbers of examples focus on the design and the use of fluorescent proteins as biosensors to track biological molecules and events in living cells.[2,3] Genetically encoded fluorescent protein (FP) biosensors that provide real-time information on cellular activities with high spatiotemporal resolution are becoming indispensable tools in biological studies. In addition to superior physical properties of fluorescence signal detection, an FP biosensor can be easily introduced into the subject of interest in the form of DNA and be propagated as a part of the subject's genetic material. The FP biosensor itself is synthesized by the host's transcriptional and translational machinery and becomes an intrinsic part of the cellular system. Such genetically encoded biosensors cause limited perturbation to the intracellular environment. They are suitable for applications in sub-cellular compartments of individual cells, tissues, or even whole animals. Since the first publication of a green fluorescent protein (GFP) Ca^{2+} biosensor in 1997,[9] over 100 different genetically encoded FP biosensors have been developed to monitor intracellular pH, redox potential, enzyme activities, and concentrations of small molecules.[2,3]

The traditional design and construction of FP biosensors are based on the standard genetic code, which restricts the FP biosensor engineering to the common twenty amino acids and their relatively limited number of functional groups. An intriguing question is whether the introduction of non-canonical functional groups with novel physical, chemical, and biological properties through genetic incorporation of unnatural amino acids (UAA*s) into FPs could further diversify the proteins'

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structure and function and lead to novel designs of FP biosensors. The combination of FP engineering and UAA* mutagenesis methods will provide a new frontier for the design and construction of FP biosensors,[10–14] which is the main focus of this review. The intention of this review is to attract more attention towards this emerging research field and to provoke creative designs and innovative utilization of FP biosensors containing unnatural elements for biological studies in living systems.

2. Genetic incorporation of unnatural amino acids (UAA*s)

Genetic codes of all known organisms encode the same set of twenty amino acids with rare exceptions in selenocysteine and pyrrolysine. The side chains of these amino acids contain a limited number of functional groups, which restricts our ability to probe and manipulate protein structure and function. To overcome this barrier, a number of methods were developed to introduce UAA*s into proteins in living cells: (1) microinjection of *Xenopus* oocytes with a chemically misacylated Tetrahymena tRNA and a mutant mRNA with an amber nonsense codon resulted in the selective incorporation of UAA*s into proteins in response to amber codons;[15–17] (2) taking advantage of the substrate promiscuity of aminoacyl-tRNA synthetases, *E. coli* auxotrophy that cannot synthesize a particular amino acid was supplemented with an analog of this amino acid.[18] Cells were able to produce mutant proteins in which the unnatural analog replaced the specific natural amino acid at all positions.[19] This method can only be used to incorporate structurally similar analogs of a natural amino acid into proteins, such as fluorinated amino acid for ^{19}F NMR study[20] and methionine analogs, selenomethionine and telluromethionine, as heavy atoms for phase determination in X-ray crystallography.[21,22] The scope of this method can be expanded by relaxing the substrate specificity[23–25] or impairing proofreading functions of aminoacyl-tRNA synthetases;[26] (3) a general approach for the site-specific incorporation of UAA*s into proteins in living bacterial, yeast, and mammalian cells has recently been developed.[27–30] In this system, orthogonal tRNA–aminoacyl-tRNA synthetase pairs are generated to recognize a noncoding codon (also called a ''blank codon'', which does not encode a natural proteinogenic amino acid, such as the amber nonsense codon) and not to cross-react with any of the endogenous tRNAs and synthetases in the host strain. The orthogonal aminoacyl-tRNA synthetase is then modified to aminoacylate the cognate tRNA specifically with the desired UAA*. This method enabled high-efficiency incorporation of over seventy UAA*s with a variety of side chain structures and functions, including spectroscopic probes, metal chelators, posttranslational modification analogs, redox-active groups, and probes with unique chemical reactivities.[27–30]

3. Basic design principles of FP biosensors

Current FP biosensors are engineered by following several design principles (Fig. 1). In the first design (Fig. 1A), two compatible FP(s) are fused to a protein scaffold that acts as the sensing domain for the target recognition. Upon exposure to biological stimuli, the sensing domain undergoes a dramatic conformational change that leads to a change in the relative distance between the two fused FPs and results in a shift in the fluorescence resonance energy transfer (FRET) signal.[2,3,6] A well-known example is the ''cameleon'' class of Ca^{2+} sensors[31,32] in which the FP FRET pair is fused to a sensing domain containing calmodulin and a peptide that binds to calmodulin in the presence of Ca^{2+}, respectively.[53,54] The second design is based on bimolecular fluorescence complementation (BiFC; Fig. 1B).[6,33] The commonly known ''split'' FP approach entails the co-expression of two fusion proteins, while each contains half of the split FP and one part of the sensing domain. Association of the sensing domains in the presence of an analyte of interest brings the complementary fragments of the FP reporter within close proximity, promotes the reconstitution of the reporter FP into its native three-dimensional structure and the emission of the fluorescence signal. The ''split'' FP approach is commonly used to detect transient protein–protein interactions. Efforts are also devoted to building ''split'' FP biosensors to detect specific analytes. A classic example of such a split FP-biosensor is the split-pericam,[34] which responds to Ca^{2+} ions. The third general design is a class of single FP-based biosensors (Fig. 1C).[2,3,6] It is known that the surrounding molecular environment of an FP's chromophore defines its fluorescence signature. Here, an FP, usually a circularly permuted FP (cpFP), is fused to a protein scaffold(s) that acts as a sensing domain. Upon recognition of the target, the sensing domain causes a conformational change of the FP (or cpFP) and leads to a shift in the fluorescence intensity and/or the fluorescence hue. One representative example is a genetically encoded Ca^{2+} sensor, G-CaMP,[35] which has the M13 fragment of myosin light chain kinase and the calmodulin domain fused to the N- and C-terminus of a cpEGFP, respectively. The Ca^{2+}-induced interaction between calmodulin and M13 domains leads to conformational and fluorescence property changes of the cpEGFP.

Although current approaches to design FP biosensors are fairly straightforward, significant challenges remain in order to improve the specificity, the sensitivity, and the diversity of FP biosensors. Sensing elements of current FP biosensors are either naturally existing proteins, such as transcription factors, or engineered sensing moieties based on the chemistry of natural amino acids (*e.g.*, disulfide bond formation by cysteine residues). Such designs not only limit the specificity and the affinity of FP biosensors, but also restrict the types of analytes that can be efficiently detected. Furthermore, due to the signature multi-domain architecture, sensitivities of current FP biosensors are often dominated by their tertiary structures. The optimization of the spatial arrangement between the FP and the sensing element often relies on a trial and error approach with limited theoretical guidance.

We and others have been seeking to develop a novel group of FP biosensors through modifications of the natural chromophore of GFP variants with UAA*s. The amber suppression method is used to genetically incorporate UAA*s with unique

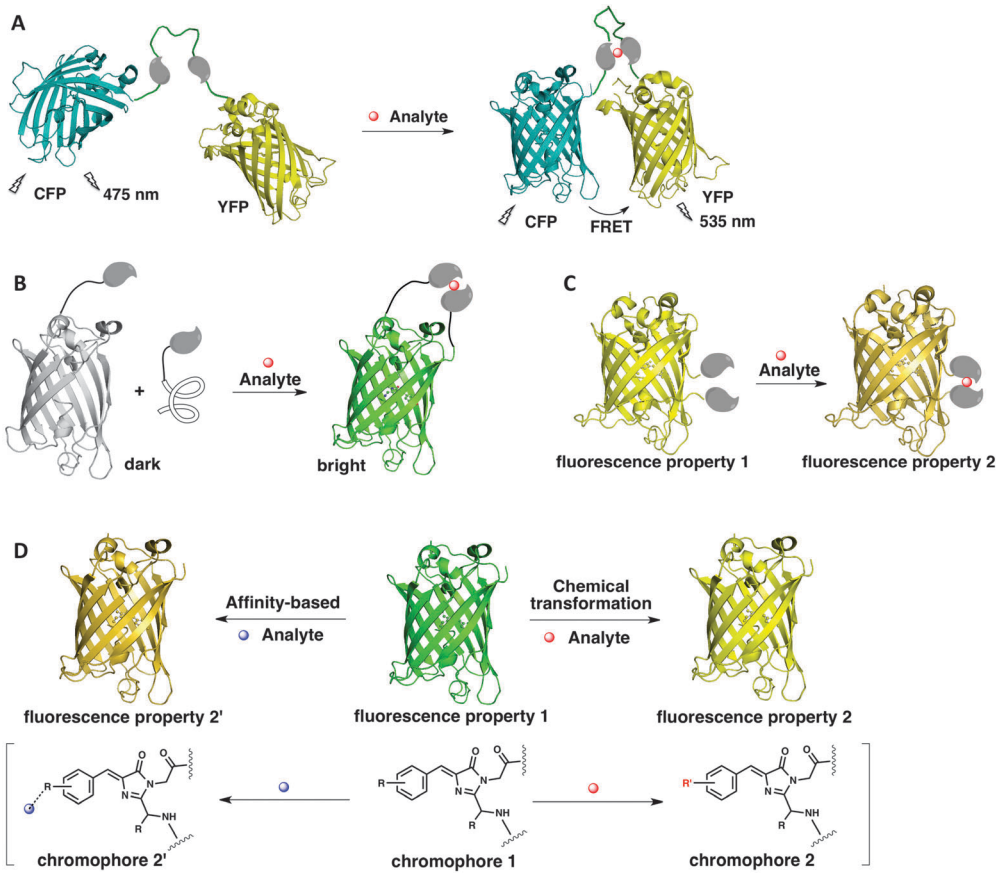

Fig. 1 Basic design principles of fluorescent protein (FP) biosensors. (A) The FRET-based FP biosensor; (B) the bimolecular fluorescence complementation (BiFC)-based FP biosensor; (C) the single FP-based biosensor; (D) the biosensor that is based on single FP containing UAA*s.

chemical and/or physical properties at defined positions of a chromophore. The chromophore of GFP is derived from a tripeptide sequence through a series of sequential chemical reactions, including the internal cyclization of Gly67 and Ser65, the dehydration reaction to form an imidizolin-5-one intermediate, and the final dehydrogenation reaction to complete the conjugation of the ring system.[5,36–43] The structure of an FP's chromophore is the major factor that defines its fluorescence signature. The novel UAA*-containing FP biosensor design aims to produce the maximum signal output by directly modifying chemical compositions of chromophores. Specifically, residue Tyr66 in the tripeptide sequence of GFP (or functionally equivalent residues of other FPs) is replaced with an UAA* bearing a side chain that either selectively reacts with or has high affinity towards the analyte of interest (Fig. 1D). A chemical transformation or a chelation event of the UAA*-containing chromophore leads to fluorescence property changes of the FP biosensor. Four desirable intrinsic properties are associated with this class of novel FP sensors: (1) high specificity; (2) large signal enhancement; (3) bioorthogonal to most cellular events; and (4) genetically encodable. The basic design principle also enables the direct transformation of many well-established aqueous phase chemical reactions into biological format and thus expands the repertoire of analytes that can be detected. This review will focus on the newly developed UAA*-containing FP

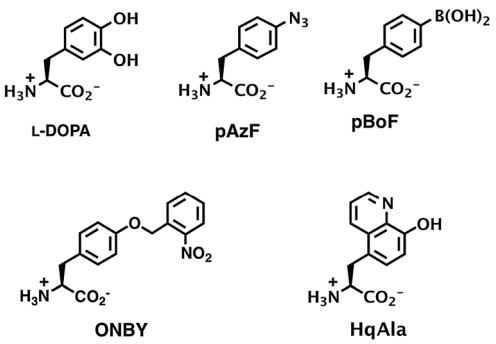

Fig. 2 UAA*s used in FP biosensors. Abbreviations: L-DOPA, 3,4-dihydroxy-L-phenylalanine; pAzF, *para*-azido-L-phenylalanine; ONBY, *o*-nitrobenzyl-O-tyrosine; pBoF, *para*-borono-L-phenylalanine; HqAla, 2-amino-3-(8-hydroxyquinolin-5-yl)propanoic acid.

biosensors for transition metals (Cu^{2+} and Zn^{2+}),[10,13] hydrogen peroxide (H_2O_2),[11] hydrogen sulfide (H_2S),[12] and light detection.[14] UAA*s (Fig. 2) that are successfully applied to the biosensor design include 3,4-dihydroxy-L-phenylalanine (L-DOPA; metal ion sensor),[10,45] 2-amino-3-(8-hydroxyquinolin-5-yl)propanoic acid (HqAla; metal ion sensor),[13] *para*-borono-L-phenylalanine (pBoF; H_2O_2 sensor),[11,44] *para*-azido-L-phenylalanine (pAzF; H_2S sensor),[12,45] and *o*-nitrobenzyl-O-tyrosine (ONBY, light-responsive FP).[14,46]

4. Transition metal sensors

Unique chemical properties of transition metal ions render them indispensable catalytic and structural components of many proteins that participate in a wide variety of cellular functions. Transition metal homeostasis is a delicate balance within a complicated network of uptake, storage, secretory, and regulatory pathways. Our knowledge of transition metal homeostasis is often inferred from the *in vitro* characterization of individual components, instead of direct monitoring of actual concentrations and changes within a single cell. Current most advanced transition metal FP biosensors are FRET-based Zn^{2+} sensors (eCALWYs) that have picomolar sensitivity.[47] FP biosensors for other transition metals, such as Cu^{2+}, Cu^{+}, Fe^{2+} and Mn^{2+}, are either at very early development stage or nonexistent. The major challenge in transition metal sensor design lies in the requirement of high selectivity among varieties of transition metal ions inside the cells and high sensitivity to analytes that often exist at low concentrations. This problem may be tackled by incorporating metal-binding amino acids, such as L-DOPA,[10,48] 8-hydroxyquinolin-L-alanine (HQA),[49] HqAla,[13] and bipyridyl-L-alanine (BpA),[50] into fluorescent proteins.

Single GFP-based *in vitro* Cu^{2+} sensors have been developed by the introduction of histidine and cysteine residues as copper-binding sites, which results in fluorescence quenching upon binding to Cu^{2+}.[51–54] To further improve the sensitivity and selectivity towards Cu^{2+}, Ayyadurai and co-workers[10] constructed a novel FP-based sensor by replacing all tyrosine residues in the GFP with metal-chelating L-DOPA.[10] The resulting GFPdopa mutant displayed good selectivity towards Cu^{2+} over other metal ions, including K^{+}, Mg^{2+}, Ca^{2+}, Na^{+}, Cd^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}. A 50% fluorescence quenching was observed at the Cu^{2+} concentration of 20 μM, and a complete quenching occurred at 100 μM of Cu^{2+}. Ayyadurai and co-workers proposed that the introduction of L-DOPA at positions Tyr66 (chromophore forming tyrosine residue) and Tyr92 might contribute the most to the observed response of GFPdopa to Cu^{2+}. Presumably, Cu^{2+} binds to the chromophore *via* interactions with DOPA66-His148 and DOPA92-chromophore chelation pairs (Fig. 3A). To demonstrate the utility of GFPdopa, Ayyadurai and co-workers constructed a sensor chip by fabricating GFPdopa patterns on the amine-coated glass surface using soft lithography. The fluorescence of the resulting sensor could be quenched with a linear behavior upon the addition of different concentrations of Cu^{2+}. On the other hand, the biosensor showed good reversibility when Cu^{2+} was removed by ethylenediaminetetraacetic acid (EDTA). Densitometry analysis revealed that 94% of the original fluorescence intensity of GFPdopa could be recovered with EDTA treatment. The copper dissociation constant of GFPdopa was shown to be 5.6 μM, which makes it relevant for Cu^{2+} detection under certain disease conditions[55–57] and in environmental applications.

The second example of a metal ion sensor came from a recent work of Liu and co-workers who site-specifically modified the chromophore of a circularly permutated sfGFP variant by replacing Tyr66 with a metal binding amino acid, HqAla

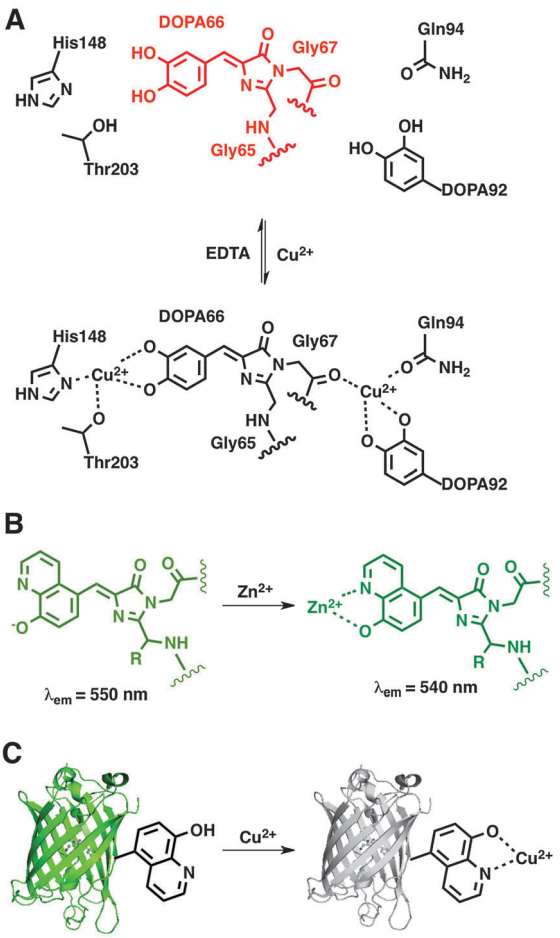

Fig. 3 Metal-binding amino acid-containing FP biosensors for metal ion detection. (A) The Cu^{2+} sensor based on L-DOPA; (B) the Zn^{2+} sensor based on HqAla; (C) the Cu^{2+} sensor based on HqAla.

(Fig. 2 and 3B).[13] The resulting FP biosensor, cpsfGFP-66-HqAla, showed fluorescence decrease in the presence of Cu^{2+}, Fe^{2+}, Co^{2+} and Ni^{2+}, and significant fluorescence increase (7.2-fold) with Zn^{2+}. It shows little response to other metal ions, including Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, and Mn^{2+} (all less than 1.3-fold change in fluorescence intensity).[13] In addition to changes in fluorescence intensity, the binding of Zn^{2+} also caused blue-shift in the absorption maximum (from 537 nm, the apo form, to 495 nm, the Zn^{2+}-bound form) and emission maximum (from 550 nm, the apo form, to 540 nm, the Zn^{2+}-bound form) of the sensor. Due to the importance of the Zn^{2+} ion in various biological processes, such as enzyme catalysis, cellular metabolism, gene expression, and neurotransmission, genetically encoded Zn^{2+} sensors with good sensitivity and selectivity are useful tools for *in vivo* monitoring. Unfortunately, the binding affinity of cpsfGFP-66-HqAla to Zn^{2+} is weak with an estimated K_D value of 50–100 μM. Since the concentration of the Zn^{2+} ion ranges from sub-nanomolar in mammalian cells[47,58–61] to about 300 μM in the mossy fiber terminals of the hippocampus,[62,63] this sensor is only applicable to measuring intracellular Zn^{2+} ion concentrations under limited physiological states.

Liu and co-workers also constructed and examined sfGFP-151-HqAla (Fig. 3C) as a metal ion sensor.[13] Since position 151 locates close to the chromophore of sfGFP, the binding of Cu^{2+} to HqAla was predicted to quench the GFP fluorescence through photoinduced electron transfer. In fact, a 65% quenching of fluorescence was observed in the presence of 1 μM of Cu^{2+}. The competitive metal capture analysis of sfGFP-151-HqAla showed an apparent K_D value of 0.1 fM towards Cu^{2+}.

The above proof-of-concept designs successfully demonstrate the feasibility of using metal binding UAA*s as the sensing element in FP metal biosensors. The initial success should promote further efforts into improving the selectivity and sensitivity of the above metal ion sensors through creative protein engineering and/or screening additional UAA*s with better selectivity and affinity to metal ions.

5. Hydrogen peroxide (H_2O_2) sensors

Hydrogen peroxide (H_2O_2) is an important second messenger that participates in the control of intracellular signaling cascades in response to external stimuli.[64–68] Methods that can be used to image intracellular concentration of H_2O_2 are highly desirable. A few small-molecule fluorescent probes have been designed based on the chemoselective reaction of arylboronates with H_2O_2.[69] These probes are highly specific for H_2O_2 and are bioorthogonal to most cellular processes. In order to generate intracellular H_2O_2 biosensors with good spatial resolution, a family of genetically encoded H_2O_2 sensors, Hyper,[70] Hyper-2,[71] and Hyper-3,[72] were constructed by inserting a circularly permuted yellow fluorescent protein (cpYFP) variant into the flexible region of the bacterial H_2O_2 transcription regulator (OxyR). Upon exposure to H_2O_2, the formation of a new disulfide bond in OxyR promotes the conformational change of the cpYFP and leads to enhanced fluorescence. A second class of a genetically encoded H_2O_2 sensor, roGFP2-Orp1,[73] is based on peroxidase-roGFP relays. Both Hyper and roGFP2-Orp1 families of sensors selectively respond to H_2O_2 but lead to less than 5-fold signal enhancements.

As an attempt to combine the sensitivity of arylboronate-based small-molecule fluorescent probes and the genetic encodability of FP biosensors, we have successfully constructed a H_2O_2 FP biosensor (UFP-Tyr66pBoPhe, Fig. 4A) by replacing the natural chromophore-forming Tyr66 residue of GFP with genetically incorporated pBoF (Fig. 2).[11] As shown in Fig. 4A, the arylboronate side chain of pBoF can be selectively oxidized by H_2O_2 in aqueous solution, which coverts the unnatural chromophore back to the wild-type chromophore of GFP. The signal output is derived from the fluorescence property difference between the unnatural and the wild-type chromophores. Therefore, the larger the quantum yield difference at specific wavelengths between the mutant and the wild-type GFP, the better the signal output of the biosensor. Interestingly, the GFP Tyr66pBoF mutant is not fluorescent, thus serving as an excellent starting point for the H_2O_2 sensor design. This observation may be attributed to the vacant 2p orbital of boron, which can readily accept electrons and makes the chromophore more electron deficient. Furthermore, based on the crystal structure of the GFP mutant,[11] the pBorono group rotates a small angle relative to the main plane of the chromophore due to its hydrogen bonding to the backbone nitrogen of His148

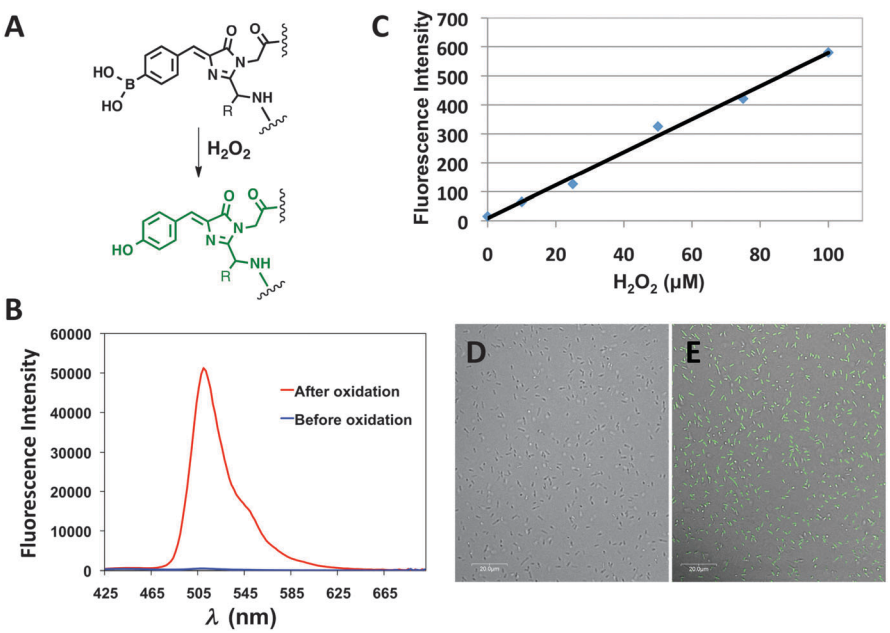

Fig. 4 pBoF-based FP biosensors for H_2O_2 detection. (A) Transformation of the chromophore containing arylborate in the presence of H_2O_2; (B) fluorescence of UFP-Tyr66pBoPhe before (blue) and after (red) H_2O_2 treatment; (C) H_2O_2 concentration-dependent fluorescence changes of UFP-Tyr66pBoPhe; (D) and (E), overlapped bright-field and fluorescence images of *E. coli*; (D) *E. coli* expressing UFP-Tyr66pBoPhe before H_2O_2 treatment; (E) *E. coli* expressing UFP-Tyr66pBoPhe after a 15-minute treatment with H_2O_2.

and to the hydroxyl side chain of Ser205. The bulky borono side chain also clearly pushes the His148 away from the chromophore. These structural changes could also contribute to the lack of any observed fluorescence.

After incubation with H_2O_2, the UFP-Tyr66pBoPhe biosensor emits strong fluorescence, which has the identical spectral property to that of the GFP_{UV} (λ = 508 nm) (Fig. 4B). The fluorescence intensity change correlated very well with the concentration of H_2O_2 in the micromolar range (Fig. 4C). *In vitro* characterization of the purified FP sensor showed response to H_2O_2 in the low micromolar range, which is above the normal physiological intracellular H_2O_2 concentration (0.01–0.7 μM),[67,74,75] but overlaps with the H_2O_2 concentration when cells are under oxidative stresses.[69,76] The detection limit of this sensor is comparable to the boronate-based small-molecule fluorescent probes.[69] In comparison to the best reported H_2O_2 FP sensors (Hyper, Hyper-2, and Hyper-3),[70–72] this new sensor is less sensitive but has an over 20-fold improvement in signal output. Next, we demonstrated the H_2O_2 sensor in living cells. As shown in Fig. 4E, addition of H_2O_2 into a bacterial culture expressing the UFP-Tyr66pBoPhe sensor led to the transformation of non-fluorescent cells into cells with bright green fluorescence. As a control, no fluorescence intensity change was detected with native host *E. coli* in the presence of H_2O_2 (Fig. 4D). Confocal fluorescence microscopy analysis also confirmed that the oxidation of UFP-Tyr66pBoPhe by H_2O_2 occurred inside *E. coli* cells (Fig. 4E).

Unlike the reversible disulfide bond formation reaction in Hyper[57,79,80] and roGFP2-Orp1,[73] the oxidation of an arylborate-based FP biosensor by H_2O_2 is irreversible. Therefore, the output signal of this type of biosensor is insensitive (bioorthogonal) to the rapidly recovered cellular redox state after a transient H_2O_2 burst. The intrinsic stability of arylborate-based FP biosensors could potentially simplify the imaging procedure in live cell studies. In addition, this type of sensor can be applied to monitoring the accumulative H_2O_2 signal in biological systems.

6. Hydrogen sulfide (H_2S) sensors

H_2S is an important gasotransmitter for the regulation of biological processes involving cardiovascular, neuronal, immune, endocrine, and gastrointestinal systems.[77–79] Given the high reactivity of H_2S in biological environments, traditional detection methods, *e.g.*, colorimetric/electrochemical assays, gas chromatography, and sulfide precipitation, could yield inconsistent results due to lengthy and/or destructive procedures.[80–83] Consequently, the reported biologically relevant concentrations of H_2S vary over a 10^5-fold range, from 100 pM to 300 μM.[81,84–88]

Recent reports described several small-molecule fluorescent probes for H_2S detection in living systems.[89–94] All these designs are based on H_2S-specific reactions. A reaction-based FP sensor for H_2S was also constructed by Chen and co-workers.[12] As shown in Fig. 5, the chromophore-forming Tyr67 residue of teal fluorescent protein (mTFP1) was replaced with pAzF. Chen and co-workers hypothesized that the reduction of azide into an amino group would change the fluorescence property of this mTFP1 mutant. Indeed, they observed slightly increased fluorescence after overnight incubation of mTFP1-Tyr67pAzF protein with NaHS (an H_2S precursor). The slow response may be a result of the restricted accessibility of H_2S to the chromophore of mTFP1 due to the protective beta-barrel structure of the protein.[12]

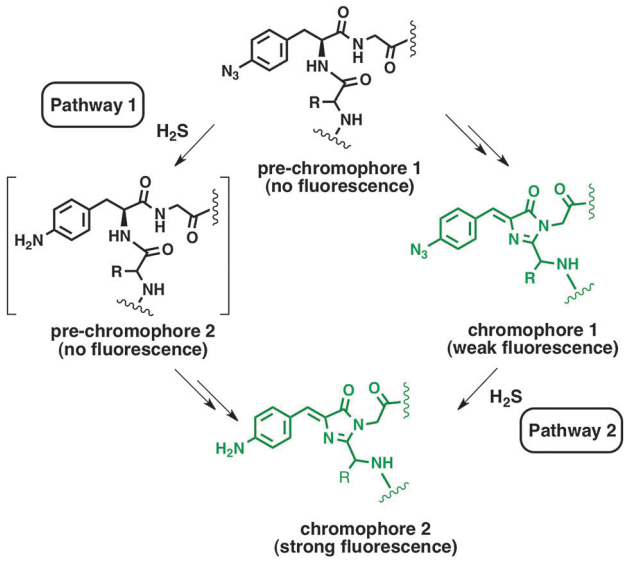

Fig. 5 pAzF-based FP biosensors for H_2S detection. Two hypothetical pathways that lead to the formation of chromophore 2 in the presence of H_2S are shown.

To improve the kinetics of the obtained FP biosensor, Chen and co-workers constructed a circularly permuted variant of GFP (cpGFP-Tyr66pAzF) mutant in which the Tyr66 residue was replaced with pAzF. Such modification led to about 3-fold fluorescence enhancement after a few minutes of incubation with 10 mM NaHS. The H_2S-treated cpGFP-Tyr66pAzF displayed the same excitation and emission profiles as those of an authentic cpGFP variant containing chromophore 2 (Fig. 5). To further confirm that the observed fluorescence change was a result of the reduction of the azide by H_2S, mass spectrometry analysis of the cpGFP-Tyr66pAzF before and after the H_2S treatment was conducted. A mixture of cpGFPs containing either premature (pre-chromophore 1; major peak) or mature (chromophore 1, minor peak) azide-containing chromophores was observed before H_2S treatment. On the other hand, a major peak that corresponds to cpGFP containing chromophore 2 was observed after H_2S treatment. These results support the authors' initial hypothesis that the azide functional group was reduced to an amino group by H_2S. Based on these observations, two hypothetical pathways that led to the formation of cpGFP containing chromophore 2 were suggested (Fig. 5). Pathway 1 that involves H_2S-induced maturation of a chromophore is the apparent major pathway. Chen and co-workers also demonstrated a linear relationship between the magnitude of fluorescence increase and the NaHS concentration when less than 50 μM of NaHS was used. When cpGFP-Tyr66pAzF was expressed in HeLa cells, the sensor responded to H_2S within minutes after the addition of 50 μM of NaHS. In comparison to

small-molecule H_2S sensors, the genetically encoded cpGFP-Tyr66pAzF should allow spatial resolution when it is fused to cell localization signals. Therefore, this unnatural fluorescent protein-based strategy for H_2S detection adds new tools to existing methodologies and should facilitate research on cellular production of H_2S.

7. Light-responsive fluorescent proteins

Photoactivatable FPs are a class of useful probes used in cell biology studies. This intriguing class of FPs undergoes irradiation-dependent photochemical reactions or isomerizations that alter spectral properties of the chromophores.[95] Therefore, the photoactivatable FPs can be applied to the imaging of sub-populations of cells and to experiments that require a higher resolution than the theoretical diffraction limit of photoactivated light microscopy.[96,97] This family of FPs include Kaede,[98] KikGR,[98] Dronpa,[99] and paGFP.[100] Since the photoactivatable behavior of these proteins relies on multiple sequence-specific mutations of parent GFP variants, the construction of photoactivatable FPs of other colors may require completely different mutations and/or strategies.

In order to develop a general applicable methodology for the construction of photoactivatable FPs, Groff and coworkers explored the genetic incorporation of a photocaged tyrosine residue (*o*-nitrobenzyl-O-tyrosine, ONBY; Fig. 2)[46] into the chromophore of a stabilized GFP variant (Fig. 6).[14] As shown in Fig. 6, the GFP66ONBY mutant is not fluorescent due to the photo-induced electron transfer (PET) effect. On the other hand, the removal of the *o*-nitrobenzyl group by irradiation at 365 nm restored the wild-type GFP fluorescence. The quantum yield (at 486 nm) of GFP66ONBY mutant increased over 100-fold upon photolysis. The observed fluorescence activation occurred on a timescale faster than 1 s, which is comparable to transition rates of other photoactivatable GFP variants.[100] Since the photoactivation mechanism of GFP66ONBY does not require the mutation of amino acids residues besides Tyr66, this strategy may be readily applicable to the generation of photoactivatable probes using other fluorescent proteins as the templates.

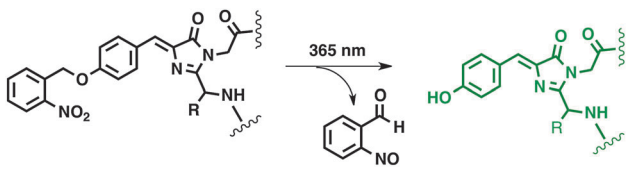

Fig. 6 ONBY-based photoactivatable FP. Photo-induced removal of the *o*-nitrobenzyl moiety converts a non-fluorescent chromophore into a wild-type GFP chromophore.

8. Conclusion and future outlook

In this review, we described UAA*-containing FPs as a new class of genetically-encoded and chemoselective biosensors. This emerging new strategy expands the foundation of protein sensor design beyond the 20 common amino acids that are specified by the standard genetic code. The inclusion of new functionalities in amino acid building blocks that are not found in nature will improve our current ability to construct selective FP biosensors. For example, the genetic incorporation of pAzF enabled the construction of the first FP-based H_2S sensor. All examples in this review except sfGFP-151-HqAla entail the substitution of the chromophore-forming tyrosine residue with a UAA*. Because of the almost universal mechanism of chromophore formation in FPs (ECFP requires tryptophan and EBFP requires histidine instead of tyrosine), the general design principle can be easily applied to other FPs and can potentially lead to facile creation of biosensors with different colors.

Albeit a powerful approach, the use of UAA*-containing FP biosensors for biological studies in living cells should be adopted with caution. The amber stop codon is the most used

Table 1 FP-based biosensors

Analyte	Sensor type	Sensor[a]	Selectivity	Sensitivity	Maximum fold of signal change[b]	Reversibility
Cu^{2+}	UAA*-based	GFPdopa[10]	Good (interfered by Fe^{3+})	K_D = 5.6 μM	>100	Yes
	UAA*-based	sfGFP-151-HqAla[13]	Did not examine	K_D = 0.1 fM	1.5–2	Yes
	Non-UAA*-based	HcRed[101]	Good	K_D = 3.6 μM	2–4	Yes
Zn^{2+}	UAA*-based	cpsfGFP-66-HqAla[13]	Very good	K_D = 50–100 μM	2–3	Yes
	Non-UAA*-based	eCALWYs[47]	Good (interfered by Pb^{2+}, Co^{2+}, Pb^{2+})	K_D = 2 pM	2.4	Yes
H_2O_2	UAA*-based	UFP-Tyr66pBoPhe[11]	Very good	Low μM	>100	No
	Non-UAA*-based	Hyper-1, 2, 3[70–72]	Very good	Medium nM	~2–3	Yes
H_2S	UAA*-based	cpGFP-Tyr66pAzF[12]	Good	Low μM	~7	No
	Non-UAA*-based	N/A	N/A	N/A	N/A	N/A
Light	UAA*-based	GFP66ONBY[14]	Very good	Good	>100	No
	Non-UAA*-based	Dronpa[99]	Very good	Very good	>100	Yes

[a] Due to the limited space of the table, only representative non-UAA*-based FP biosensors are listed. [b] No specific fold of signal change is given when the value is larger than 100.

triplet nonsense codon to encode UAA*s. Although studies showed it has no detectable cytotoxicity to bacterial and mammalian cell culture,[102–105] the heterologous expression of the suppression system will result in undesirable read-throughs that may cause nonlethal perturbations to cell physiology. Recent efforts in orthogonal ribosome[106,107] and quadruplet codon decoding approaches are expected to address this issue in cellular genetic code expansion.[108–112] Another component required for the expression of the UAA*-based biosensor is the UAA* molecule. Unlike small-molecule sensors, UAA* itself cannot produce signal readouts in the presence of an analyte. However, UAA*s often possess the functional groups that can react or bind to analytes. Therefore, complete removal of UAA*s post FP sensor expression is necessary in order to achieve the optimal sensor performance.

As shown in Table 1, reported UAA*-based FP biosensors generally have good selectivity and decent fold of signal change but lower than desirable sensitivity. Besides intrinsic limitations in the reactivity and/or binding affinity of the UAA*, all reported UAA*-based FP biosensors were recently developed and not optimized, while some of the regular FP biosensors were developed a decade ago and have been methodically optimized as commercial products. Further protein engineering needs to be performed in order to improve the sensitivity and the overall performance of UAA*-based FP biosensors. Another less than desirable aspect of most reported UAA*-based FP biosensors is that the sensor synthesis is largely limited to the *E. coli* host. Due to the important roles of small-molecule signaling in mammalian cells, efforts must be and are being made to enable mammalian expression of UAA*-based FP biosensors. One major breakthrough in this direction is the development of a method to genetically incorporate ONBY (Fig. 2 and 6) into proteins in mammalian cells.[104] Once the UAA*-based FP biosensors are compatible with the mammalian system, the next step would be to construct functionally equivalent FP biosensors of different hues, which will enable simultaneous detection of the same analyte in multiple subcellular compartments.

In addition to optimizing currently existing UAA*-containing FP biosensors, future efforts should also be devoted to the design and synthesis of new sensors in order to expand the scope of target molecules. This goal can be achieved by both incorporating UAA*s with novel functional groups and exploiting the diverse reactivity of already explored UAA*s. For example, the mechanism and design principles of many small-molecule biosensors, which are based on specific chemical reactions or molecular interactions between the sensor molecule and the analyte,[113] could be implemented into the construction of UAA*-based FP biosensors.

In summary, properties of reported UAA*-based FP biosensors need to be further optimized in order to be applied in real biological studies. Nevertheless, these sensors represent the first step towards the generation of highly sensitive and selective FP biosensors that may not be realized by following the standard genetic code. We expect the creative integration of modern protein engineering techniques with the expanded repertoire of amino acids building blocks to produce innovative designs and applications of this new class of FP biosensors in the near future.

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