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Photocontrollable Analyte-Responsive Fluorescent Probes: A Photocaged Copper-Responsive Fluorescence Turn-On Probe

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Abstract: Analyte-responsive fluorescent probes are valuable chemical tools for dissecting complex living systems. However, the major shortcoming of fluorescent probes is that once they enter the cells, control over them is basically lost. It is critical to regulate fluorescent probes in a spatial and temporal manner, as functions of biomolecules are spatiotemporal. On the other hand, light can be manipulated in time and in the application site, so the photocaging technique allows researchers to control the biomolecules of interest in a temporal and spatial fashion. Herein, we propose for the first time the combination of the merits of sensing and photocaging technologies, which may afford the caging version of analyte-responsive fluorescent probes, referred to as photocontrollable analyte-responsive fluorescent probes (PCAFPs). These "smart" fluorescent probes apparently have the intrinsic advantage of spatiotemporal control when compared to traditional fluorescent probes, as the "sensing activity" of PCAFPs is photocontrollable. This should enable biologists to interrogate complex biological systems in a spatial and temporal manner with an innovative chemical tool. In this work, for proof of concept, we report the rational design, synthesis, photocontrollable sensing in solution and in living cells, and mechanistic studies of a molecular prototype of PCAFP for copper as the first paradigm of this new class of smart fluorescent probes. We believe that PCAFPs represent a substantial breakthrough in the sensing and photocaging fields, and that the general concept of PCAFPs should be broadly applicable for a wide variety of biologically relevant species.

Keywords: cage compounds · fluorescent probes · photoactivation · photochemistry · photolysis

Introduction

Biological systems are highly complicated, as they comprise countless interwoven interactions of biomolecules under precise temporal and spatial control. To explore the biochemistry of a living system, it is essential to have suitable chemical tools. One type of useful chemical tool is the fluorescent probe. In this contribution, "fluorescent probes", including fluorescent chemosensors and chemodosimeters, are defined as synthetic small molecules that react specifically with analytes to induce a marked change in fluorescence properties (Scheme 1a).[1] Since the seminal work by Tsien's group about Ca^{2+} probes,[2] a wide variety of analyte-responsive fluorescent probes for various targets has been developed. As fluorescent probes exhibit the advantages of high sensitivity and simplicity for implementation, they are valuable in bioimaging applications for dissecting complex living systems.[3] However, the major shortcoming of analyte-responsive fluorescent probes is that once they enter cells, control over them is basically lost.[4] It is critical to regulate fluorescent probes in a spatial and temporal manner, as functions of biomolecules are spatiotemporal.

On the other hand, photocaging technology has been widely employed in studies of molecular and cellular dynamics.[4,5] Masking the key functional components of biomolecules with photolabile protecting groups may afford caged compounds, which are essentially biologically inert. However, upon photolysis, the photolabile groups are deprotected to release the native active biomolecules (Scheme 1b). The pioneering work by Hoffman and coworkers on caged ATP has stimulated development in the photocaging field.[6] So far, diverse biologically relevant molecules including metal ions, neurotransmitters, nucleotides, peptides, enzymes, nucleic acids, and drugs have been caged.[4–7] As light can be manipulated in time and in the ap-

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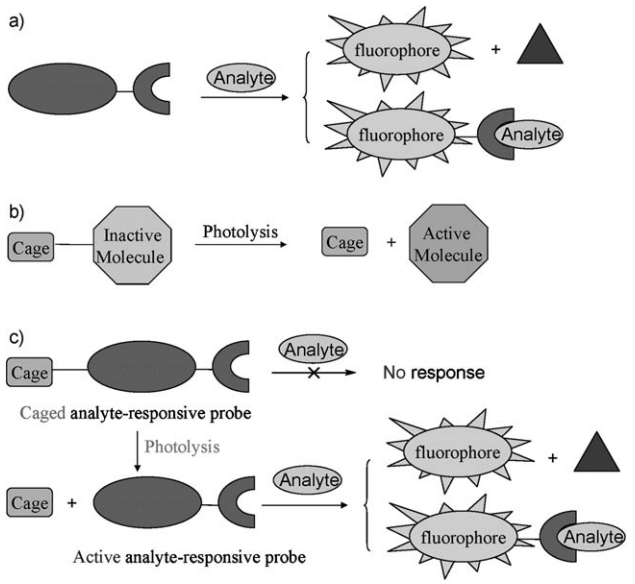


Scheme 1. a) Schematic diagram showing the principle of the traditional analyte-responsive fluorescent turn-on probes (e.g., chemosensors and chemodosimeters). b) Schematic diagram illustrating the principle of the conventional caged compounds. c) Schematic diagram depicting the working hypothesis of our proposed PCAFPs.

plication site,[4–8] it is then possible to regulate these biologically relevant molecules with high temporal and spatial resolution using photocaging technology. Thus, the photocaging method allows researchers to control the biomolecules of interest in a temporal and spatial fashion.

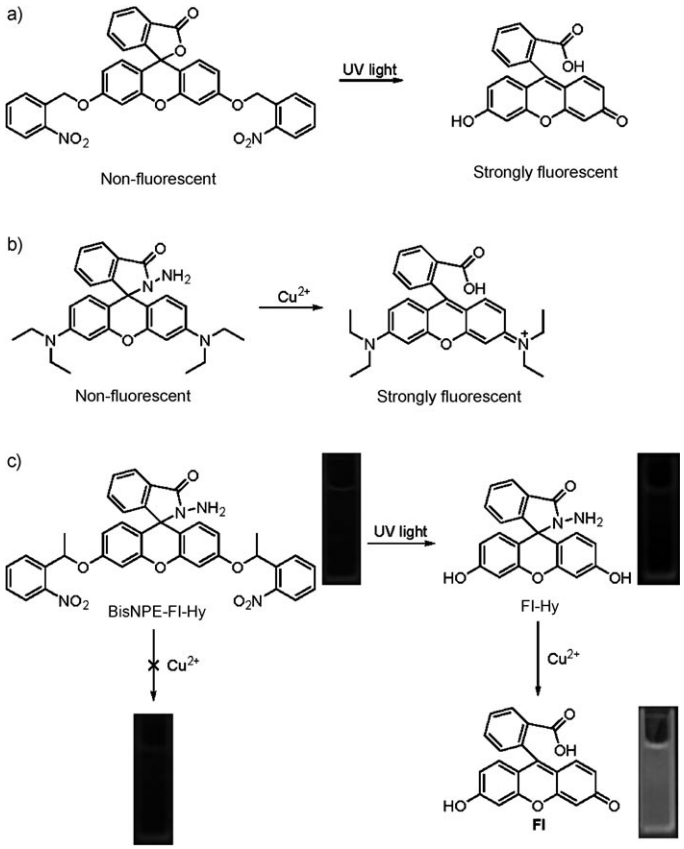
Herein, we propose that combining the advantages of the analyte-responsive fluorescent probes and the photocaging technology may afford a novel type of "smart" fluorescent probes, which we refer to as photocontrollable analyte-responsive fluorescent probes (PCAFPs). In PCAFPs the sensing activity to the analyte of interest is caged, so PCAFPs do not exhibit a sensing response to the analyte in the dark (Scheme 1c). However, upon exposure to light, caged PCAFPs are uncaged, and are converted to functionally active analyte-responsive fluorescent probes. Thus, the sensing activity is recovered. When compared to traditional analyte-responsive fluorescent probes, PCAFPs apparently have the intrinsic advantage of spatiotemporal control, as the sensing activity of the PCAFPs is photocontrollable. This may provide biologists with a novel, powerful, chemical tool for investigating complex living systems in a spatial and temporal manner. It is important to note that the new concept of PCAFPs is conceptually distinct from the previous so-called caged fluorescent probes.[9] Smart PCAFPs are the caging version of fluorescent chemosensors and chemodosimeters, which, upon photolysis, can specifically interact with a target analyte to elicit a fluorescence response. In contrast, the previous so-called caged fluorescent probes are simply the caging version of dyes, and do not respond to the analytes of interest after photolysis, as they lack a sensing unit. The aim of this paper is to introduce the concept of PCAFPs. For proof of concept, we present the rational design, synthesis, photocontrollable sensing in solution and in living cells, and mechanistic studies of a molecular prototype of a copper PCAFP.

Results and Discussion

Rational design and synthesis of photocontrollable analyte-responsive fluorescent probes (PCAFPs): In the development of valid PCAFPs, some design criteria should be considered: 1) the probes should be stable in aqueous solution at physiological pH; 2) they should be inert to the analyte of interest in the dark; 3) they should not exhibit a fluorescence response after photolysis in the absence of the analyte of interest; 4) they should display a significant fluorescence turn-on or ratiometric response after photolysis in the presence of the analyte; 5) the wavelengths of the uncaging light should be above 300 nm to minimize UV-induced cell damage.

To examine the feasibility of the construction of smart PCAFPs based on the sensing and caging technologies, we were interested in creating a prototypic PCAFP for Cu^{2+} as a step toward the regulation of sensing activity by light. With the above design criteria in mind, we judiciously designed the probe bisNPE-Fl-Hy as the first paradigm of a PCAFP for Cu^{2+} (Scheme 2c), inspired by a rhodamine hydrazide based fluorescent copper probe (Scheme 2a) and a nitrobenzyl-caged fluorescein (Scheme 2b). It is known that copper may induce the hydrolysis of the non-fluorescent spirolactam form of rhodamine hydrazide into the fluorescent ring-opened carboxylic form of rhodamine,[10] and that the non-fluorescent nitrobenzyl-caged fluorescein derivative could be converted into the fluorescent fluorescein dye upon photolysis.[9a] Thus, we envisaged that integrating the sensing platform of the rhodamine hydrazide based copper fluorescent probe with the caging strategy of the fluorescein dye could afford a first-generation prototypic copper PCAFP (compound bisNPE-Fl-Hy in Scheme 2c). We anticipated that bisNPE-Fl-Hy would be essentially non-fluorescent and resistant to Cu^{2+}-induced hydrolysis in the dark, as both the hydroxyl groups of the fluorescein dye are alkylated, which may inhibit the ring-opening reaction (see the spectral and mass spectrometry studies shown below). However, upon photolysis, the photolabile nitrobenzyl groups are removed to uncage the non-fluorescent fluorescein hydrazide (Fl-Hy), which may be further hydrolyzed by Cu^{2+} to provide highly fluorescent fluorescein (Fl).

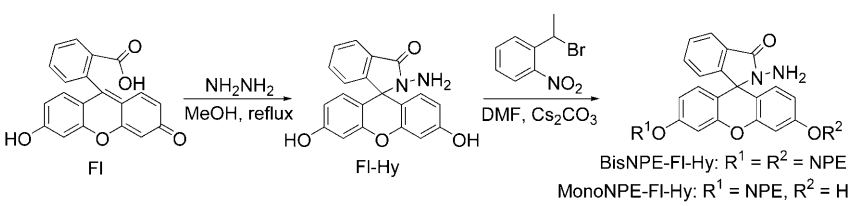
Notably, bisNPE-Fl-Hy is fundamentally different from the recently reported "caged copper"[7j] or "light-activated copper" probes.[7p] In their approach, the probes initially bind to Cu^{2+} to form a complex, which is then subjected to UV light photolysis to release Cu^{2+}[7j] or Cu^{2+} and fluorophore.[7p] In other words, in this case, the interaction of the probes with Cu^{2+} occurs before photolysis, and therefore is not controllable by light. In sharp contrast, in our PCAFP strategy, the probes do not interact with Cu^{2+} before photolysis. Only after photolysis is the caged probe bisNPE-Fl-Hy



Scheme 2. a) Sensing chemistry of a rhodamine hydrazide-based fluorescent copper probe. b) Uncaging chemistry of a conventional caged fluorescein. c) The design hypothesis of bisNPE-Fl-Hy, a first-generation prototypic PCAFP for copper.

released to give the functionally active copper-responsive probe Fl-Hy, which can then interact with Cu^{2+} to elicit a fluorescence turn-on response due to the copper-promoted hydrolysis. Thus, in the PCAFP strategy described herein, the sensing of copper can be regulated by light.

The synthesis of compound bisNPE-Fl-Hy is outlined in Scheme 3. Briefly, condensation of fluorescein with hydrazine afforded the key intermediate, fluorescein hydrazide (Fl-Hy) in high yield.[11] Subsequent alkylation of Fl-Hy with an excess amount of 1-(1-bromoethyl)-2-nitrobenzene under basic conditions gave the desired caged compound bisNPE-Fl-Hy in excellent yield. The compound monoNPE-Fl-Hy containing only one photolabile nitrobenzyl group was also synthesized as a reference by reacting Fl-Hy with roughly 0.8 equivalents 1-(1-bromoethyl)-2-nitrobenzene under basic conditions. The structures of bisNPE-Fl-Hy and monoNPE-Fl-Hy were fully characterized by ^{1}H and ^{13}C NMR spectroscopy, and mass spectrometry.



Scheme 3. Synthesis of caged compound bisNPE-Fl-Hy and the analogue monoNPE-Fl-Hy.

Fluorescence response of the caged probes to UV light exposure: After acquiring the caged compound bisNPE-Fl-Hy, we evaluated its fluorescence response to UV light exposure in the absence or presence of a fixed concentration of Cu^{2+}. As the utility of a caged species is dependent on its hydrolytic stability at physiological pH in the absence or presence of Cu^{2+} in the dark, we initially examined the fluorescence of the caged compound bisNPE-Fl-Hy in the absence or presence of Cu^{2+} before photolysis. As shown in Figure 1,

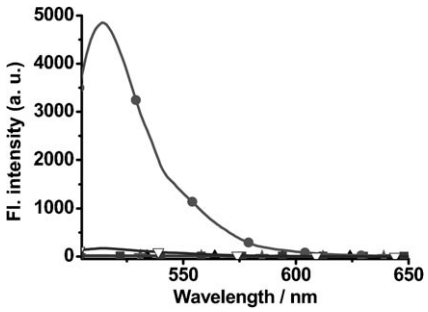


Figure 1. The fluorescence spectra of compound bisNPE-Fl-Hy (5 μM) in the absence or presence of Cu^{2+} in 25 mM HEPES (pH 7.4) containing 20% CH_3CN as a co-solvent, kept in the dark for various periods: bisNPE-Fl-Hy (5 μM) in the absence of Cu^{2+} for 0.5 h (▲); bisNPE-Fl-Hy (5 μM) + Cu^{2+} (10 equiv) for 0.5 h (■), bisNPE-Fl-Hy (5 μM) + Cu^{2+} (10 equiv) for 8 days (★). For comparison, the fluorescence spectra of bisNPE-Fl-Hy (5 μM) in the absence of Cu^{2+} after UV light exposure for 120 s (▽) and bisNPE-Fl-Hy (5 μM) + Cu^{2+} (10 equiv) after UV light exposure for 120 s (●) are also shown. Excitation at 492 nm.

bisNPE-Fl-Hy is essentially non-fluorescent ($\Phi_f < 0.0002$, see Supporting Information) due to the spirolactam structure. Thus, the caged probe has a very low fluorescence background. In addition, even in the presence of Cu^{2+} in the dark, it is still almost non-fluorescent, indicating that the caged compound is very stable and does not hydrolyze to Fl in the presence of Cu^{2+} in the dark, in good agreement with the mass spectrometry analysis (Figure S2 in the Supporting Information). This is critical for the regulation of the sensing activity of bisNPE-Fl-Hy toward copper by light.

We then proceeded to investigate the emission spectra of bisNPE-Fl-Hy upon exposure to UV light (350 nm) in the presence of Cu^{2+}. A large fluorescence enhancement at 516 nm ($\Phi_f = 0.81$, see Supporting Information) was observed in the presence of Cu^{2+} after exposure to UV light (Figure 2). It should be noted that a huge fluorescence enhancement (up to 350-fold) around 516 nm was induced

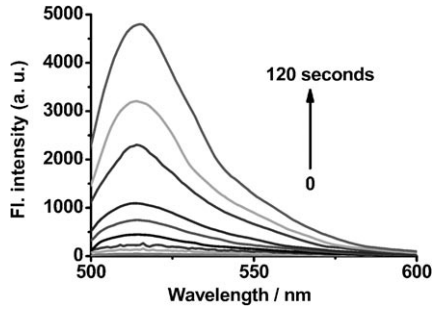


Figure 2. Fluorescence emission spectra of bisNPE-Fl-Hy (5 μM) in the presence of Cu^{2+} (10 equiv) before and after UV illumination for various periods (0—120 s). Excitation at 492 nm.

after photolysis. Such a dramatic fluorescence enhancement after uncaging is highly favorable for cell imaging applications. However, no significant fluorescence was observed after photolysis in the absence of Cu^{2+} (Figure 1). This evidently indicates that the fluorescence turn-on response was elicited by Cu^{2+} after photolysis. As a great fluorescence enhancement around 516 nm was achieved after 120 s of photolysis, this UV light exposure time was selected to examine further the sensitivity and selectivity of the caged probe bisNPE-Fl-Hy toward Cu^{2+}.

The compound monoNPE-Fl-Hy (Scheme 3) is the analogue of bisNPE-Fl-Hy with only a single nitrophenyl group, and it could also exhibit strong fluorescence in the presence of Cu^{2+} upon photolysis (Figure 3). However, monoNPE-Fl-

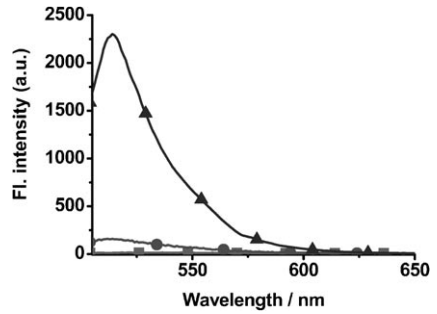


Figure 3. The fluorescence spectra of compound monoNPE-Fl-Hy (5 μM) in the absence or presence of Cu^{2+} in 25 mM HEPES (pH 7.4) containing 20% CH_3CN as a co-solvent, kept in the dark for various periods: monoNPE-Fl-Hy (5 μM) in the absence of Cu^{2+} for 0.5 h (■); monoNPE-Fl-Hy (5 μM) + Cu^{2+} (10 equiv) for 0.5 h (●). For comparison, the fluorescence spectrum of monoNPE-Fl-Hy (5 μM) + Cu^{2+} (10 equiv) after UV light exposure for 30 s (▲) is also shown. Excitation at 492 nm.

Hy already displayed marked fluorescence in the presence of Cu^{2+} even before photolysis ($\Phi_f = 0.082$, see Supporting Information). This indicates that, unlike bisNPE-Fl-Hy, monoNPE-Fl-Hy can be hydrolyzed by Cu^{2+} in the dark to afford monoNPE-Fl, in accordance with the results of the mass spectrometry studies for monoNPE-Fl-Hy in the absence or presence of Cu^{2+} in the dark or upon UV exposure (Figures S3 and S4). Thus, monoNPE-Fl-Hy is not ideal as a PCAFP for copper according to the above criteria set for PCAFPs. We decided to focus on the caged probe bisNPE-Fl-Hy for further studies of sensing sensitivity and selectivity to copper.

Fluorescence response of bisNPE-Fl-Hy to Cu^{2+}: After identifying the optimal photolysis time, we further examined the sensing response of the caged probe bisNPE-Fl-Hy to various concentrations of Cu^{2+} at the fixed UV illumination time (120 s) by fluorescence spectroscopy. The free probe is essentially non-fluorescent, but upon photolysis, addition of an increasing concentration of Cu^{2+} to the solution of bisNPE-Fl-Hy induced a gradual increase in the emission around 516 nm (Figure 4). Furthermore, the introduction of

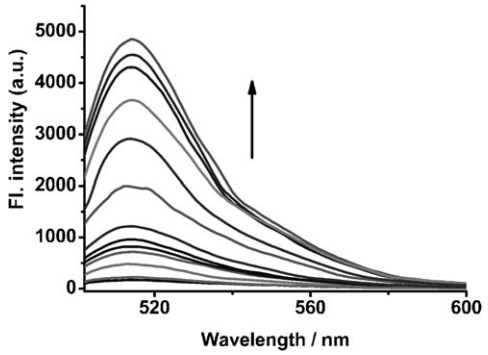


Figure 4. The emission spectra of bisNPE-Fl-Hy (5 μM) after UV illumination for 120 s in the presence of various concentrations of Cu^{2+} (0–10 equiv). Excitation at 492 nm.

Cu^{2+} turned the visual emission color of the solution of the probe bisNPE-Fl-Hy from dark to bright green after photolysis (Scheme 2), further reinforcing the fluorescence turn-on response. The probe bisNPE-Fl-Hy exhibited a linear fluorescence response toward Cu^{2+} ranging from 2.5×10^{-7} to 1.0×10^{-5} M (Figure S5 in the Supporting Information), with a detection limit of 1.9×10^{-7} M under the experimental conditions (Figure S6, in the Supporting Information).[12] Moreover, the studies of the effect of pH on the fluorescence response (Figure S7 in the Supporting Information) indicate that bisNPE-Fl-Hy could be employed to detect Cu^{2+} upon photolysis at physiological pH.

Studies of uncaging and sensing chemistry of BisNPE-Fl-Hy: To gain an insight into the photolysis and sensing processes associated with the caged probe bisNPE-Fl-Hy, the solutions of compound bisNPE-Fl-Hy before and after different durations of UV light exposure in the absence or presence of Cu^{2+} were subjected to mass spectrometry and emission and excitation spectroscopy studies. Prior to release, in the presence of Cu^{2+}, bisNPE-Fl-Hy only showed a major peak at m/z 645.0 ($[bisNPE\text{-}Fl\text{-}Hy+H]^+$); peaks corresponding to caged fluorescein or Fl were not present in the spectrum (Figure S8a in the Supporting Information), indicating that the caged probe bisNPE-Fl-Hy was very stable

and was not hydrolyzed to caged fluorescein or Fl by copper in the dark. This is in accord with the finding of the above emission spectroscopic studies that bisNPE-Fl-Hy is non-fluorescent in the presence of Cu^{2+} in the dark. However, with exposure to UV light in the absence of Cu^{2+}, two major new peaks appeared at *m*/*z* 347.0 ($[Fl\text{-}Hy+H]^+$) and 496.0 ($[monoNPE\text{-}Fl\text{-}Hy+H]^+$) (Figure S8 b–d, in the Supporting Information). Furthermore, with the increasing photolysis time, the peak corresponding to Fl-Hy increased, whereas that corresponding to monoNPE-Fl-Hy decreased, suggesting that the partially photolyzed product monoNPE-Fl-Hy was eventually converted to the completely photolyzed product Fl-Hy. However, upon photolysis in the presence of Cu^{2+}, a major new peak at *m*/*z* 315.0 ($[Fl+H-H_2O]^+$) (Figure S8 e in the Supporting Information) was noted. The same peak was also observed after the photolysis of bisNPE-Fl-Hy in the absence of Cu^{2+}, followed by addition of Cu^{2+} (Figure S8 f in the Supporting Information). In addition, the emission and excitation spectra of bisNPE-Fl-Hy after exhaustive photolysis in the presence of Cu^{2+} were identical to those of the standard Fl when excited or monitored at the appropriate wavelengths (Figure S9in the Supporting Information).

Thus, based on the analysis of mass spectrometry and emission and excitation spectroscopy, the uncaging and sensing chemistry of bisNPE-Fl-Hy is proposed as shown in Figure S10 in the Supporting Information. The light-mediated transformation of bisNPE-Fl-Hy to the partially photolyzed monoNPE-Fl-Hy, and then to the entirely photolyzed Fl-Hy, followed by the copper-induced hydrolysis, results in the formation of the final product Fl, which exhibits intense fluorescence. This proposal is consistent with the above design hypothesis of bisNPE-Fl-Hy (Scheme 2). An alternative pathway from monoNPE-Fl-Hy to the final product Fl may also be present. The existence of a minor peak at *m*/*z* 464.0 ($[monoNPE\text{-}Fl+H-H_2O]^+$) in the mass spectra of the mixture of bisNPE-Fl-Hy with Cu^{2+} upon photolysis (Figure S8 e,f in the Supporting Information) implies that the partially photolyzed product monoNPE-Fl-Hy can also be hydrolyzed by Cu^{2+} to afford monoNPE-Fl (Figure S10 in the Supporting Information), which may be photolyzed further to produce Fl, in good agreement with the aforementioned mass spectrometry analysis and the proposed uncaging and sensing chemistry of monoNPE-Fl-Hy (Figures S3 and S4 in the Supporting Information).

Selectivity studies of bisNPE-Fl-Hy: We proceeded to examine the selectivity of bisNPE-Fl-Hy to Cu^{2+} over other competing species upon photolysis. 10–200 equiv of various biologically relevant ions were added to solutions of bisNPE-Fl-Hy, then UV illumination was applied for 120 s. As shown in Figure 5, introduction of Cu^{2+} to bisNPE-Fl-Hy upon photolysis resulted in a significant enhancement of the fluorescence intensity at 516 nm. By contrast, no visible changes in the emission were noted upon addition of representative species such as Na^+, K^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, Co^{2+}, Fe^{3+}, Hg^{2+}, Mn^{2+}, SO_4^{2-}, Cl^-, NO_3^-, and F^-, indicating that

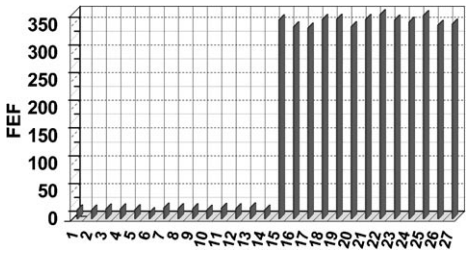


Figure 5. Fluorescence enhancement factor (FEF) of bisNPE-Fl-Hy (5 μM) for different competing analytes (200 equiv for Na^+, K^+, Ca^{2+}, Mg^{2+}, SO_4^{2-}, Cl^-, and NO_3^-, 10 equiv for Zn^{2+}, Cu^{2+}, Co^{2+}, Fe^{3+}, Hg^{2+}, Mn^{2+}, and F^-) after UV illumination for 120 s: 1) Free; 2) Na^+, 3) K^+, 4) Ca^{2+}, 5) Mg^{2+}, 6) Zn^{2+}, 7) Co^{2+}, 8) Fe^{3+}, 9) Hg^{2+}, 10) Mn^{2+}, 11) SO_4^{2-}, 12) Cl^-, 13) NO_3^-, 14) PO_4^{3-}, 15) Cu^{2+}, 16) Cu^{2+} + Na^+, 17) Cu^{2+} + K^+, 18) Cu^{2+} + Ca^{2+}, 19) Cu^{2+} + Mg^{2+}, 20) Cu^{2+} + Zn^{2+}, 21) Cu^{2+} + Co^{2+}, 22) Cu^{2+} + Hg^{2+}, 23) Cu^{2+} + Mn^{2+}, 24) Cu^{2+} + SO_4^{2-}, 25) Cu^{2+} + Cl^-, 26) Cu^{2+} + NO_3^-, and 27) Cu^{2+} + F^-. Excitation at 492 nm; emission at 516 nm.

the probe has a high selectivity for Cu^{2+}. This is further supported by the observation that other species have only negligible interference with the fluorescence response. Thus, bisNPE-Fl-Hy can sense Cu^{2+} selectively upon photolysis.

Photoregulated fluorescence sensing of Cu^{2+} in living cells: To investigate the photoregulated fluorescence sensing of Cu^{2+} in living cells, we treated HeLa cells with bisNPE-Fl-Hy and Cu^{2+}. The selected cells were irradiated with UV light through a fluorescence microscope.[9a,d] After photolysis for 30 s, the irradiated cells became brightly fluorescent (Figure 6 b). However, no marked fluorescence was ob-

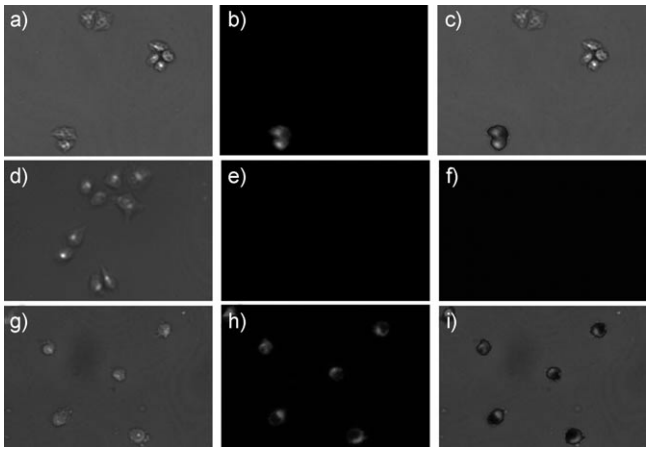


Figure 6. Local uncaging and imaging of bisNPE-Fl-Hy (15 μM) + Cu^{2+} (2 equiv) in living cells. a) DIC image of HeLa cells incubated with probe bisNPE-Fl-Hy + Cu^{2+}. b) Fluorescence image of the cells in the panel a) after 30 s illumination for the selected cells with blue light excitation. c) Overlay of images a) and b). d) DIC image of HeLa cells incubated with probe bisNPE-Fl-Hy in the absence of Cu^{2+}. e) Fluorescence image of the cells in panel d) after 30 s illumination as a negative control with blue light excitation. f) Fluorescence image of the cells only as a negative control with blue light excitation. g) DIC image of HeLa cells incubated with **Rh-Hy** + Cu^{2+}. h) Fluorescence image of the cells in panel g) with green light excitation. i) Overlay of images g) and h).

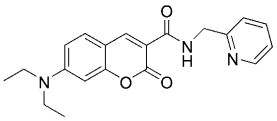
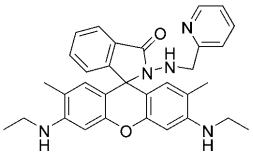
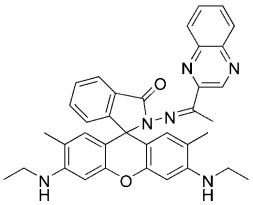
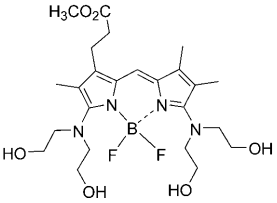
served in the cells that were not photolyzed. In a negative control experiment, HeLa cells were treated with bisNPE-Fl-Hy in the absence of Cu^{2+} upon UV exposure (Figure 6e), but no significant fluorescence was noted. These data clearly established that the bisNPE-Fl-Hy probe is cell-membrane permeable, and that this probe can be employed for local uncaging and sensing of copper in living cells. It is important to note that, to our best knowledge, this is the first proof-of-concept illustration that sensing of an analyte in living cells can be controlled by light. For comparison, when the cells were incubated with the traditional copper probe Rh-Hy and Cu^{2+}, the cells all essentially exhibited bright red fluorescence (Figure 6h),[13] suggesting no spatial control fluorescence sensing, which is consistent with the fact that the fluorescence response of the conventional probe Rh-Hy to Cu^{2+} is not amenable to light regulation. Thus, the newly constructed probe bisNPE-Fl-Hy exhibited an evident advantage over the traditional copper probe Rh-Hy in that the copper imaging and sensing in the living cells is photocontrollable.

Comparison with other Cu^{2+} fluorescent probes/reagents: A brief comparison of some representative Cu^{2+} fluorescence probes/reagents reported elsewhere[10,14] is given in Table 1. Most of the reagents exhibit a high selectivity and sensitivity to Cu^{2+}. The fluorescent probes exhibited either a fluorescence enhancement or quenching response to copper ions. Furthermore, some of them have been employed for Cu^{2+} imaging in living cells.[14d–g] However, the fluorescence response of the existing fluorescent probes is not photocontrollable. By contrast, the sensing ability of the new probe bisNPE-Fl-Hy can be regulated by light. Thus, the imaging and sensing of copper can be conducted in a spatiotemporal manner by using the new probe, as illustrated in the above studies.

Conclusion

We have described, for the first time, a new type of smart fluorescent probe, termed photocontrollable analyte-responsive fluorescent probes (PCAFPs). For proof of concept, a molecular prototype of the caged copper-responsive fluorescent probe bisNPE-Fl-Hy was rationally constructed. The bisNPE-Fl-Hy probe was essentially non-fluorescent and inert to Cu^{2+}-induced hydrolysis before light exposure.

Table 1. Comparison of some Cu^{2+} fluorescent probes/reagents.

Reagents	Analytical ranges (detection limit)	Selectivity	Comments	References
phen green FL/ phen green FL diacetate	no data available (near 100 nM)	modest	MOPS buffer pH 7, fluorescence quenching	[14a,b]
europium tetracycline hydrogen peroxide complex	0.2–0.9 μM (0.2 μM)	modest	MOPS buffer, pH 6.9, fluorescence quenching.	[14c]
Rh-Hy	no data available (10 nM)	good	HEPES buffer/CH_3CN (8:2, v/v), pH 7, fluorescence enhancement	[10]
	no data available (0.5 μM)	good	HEPES buffer/DMSO (9:1, v/v), pH 4–10, fluorescence quenching, living cell imaging	[14d]
	8.0×10^{-7}–1.0×10^{-5} M (3.0×10^{-7} M)	good	Tris-HCl buffered/C_2H_5OH (8:2, v/v) fluorescence enhancement, living cell imaging	[14e]
	5.0×10^{-7}–1.0×10^{-6} M (37 nM)	good	phosphate buffered saline, pH 6.2–7.8, fluorescence enhancement, living cell imaging	[14f]
	no data available	good	HEPES buffer/DMSO (9:1, v/v), pH 7, fluorescence quenching, living cell imaging	[14g]
bisNPE-Fl-Hy	2.5×10^{-7}–1.0×10^{-5} M (1.9×10^{-7} M)	good	HEPES buffer/CH_3CN (8:2, v/v), pH 6–10, fluorescence enhancement, living cell imaging	this work

However, upon photolysis, the photolabile nitrobenzyl groups were removed to release the active copper-responsive fluorescent probe, which could sense copper in solutions with a large (350-fold) fluorescence enhancement. In addition, we have demonstrated that the sensing of Cu^{2+} in living cells could also be photoregulated with a spatial resolution. Thus, the new, caged, bisNPE-Fl-Hy probe showed a clear advantage over the traditional copper probe Rh-Hy in that the copper sensing in living cells is light-controllable. Although bisNPE-Fl-Hy, the proof-of-principle paradigm of a PCAFP for Cu^{2+} illustrated herein, is regarded as a valuable step toward the ultimate goal to regulate sensing activity by light, we believe that the concept of PCAFPs represents a valuable breakthrough in the analyte-responsive fluorescent probe and photocaging fields, and that more sophisticated PCAFPs will no doubt become powerful chemical tools for exploring the spatiotemporal information of the biochemistry of life. Furthermore, because of the great advance in the sensing chemistry of analyte-responsive fluorescent probes and the well-developed uncaging chemistry, the general strategy of PCAFPs should be broadly applicable for a wide variety of biologically relevant targets. Our future efforts will focus on improving the sensitivity of the first-generation copper PCAFP, and developing various types of PCAFPs with diverse photolabile groups susceptible to various uncaging conditions (i.e., two-photon photolysis) as new chemical tools for biological exploration in a temporal and spatial fashion.

Experimental Section

Materials and instruments: Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. Solvents used were purified by standard methods prior to use. Twice-distilled water was used throughout all experiments. The melting points of compounds were measured on a Beijing Taike XT-4 microscopy melting point apparatus, and all melting points were uncorrected. Mass spectrometry was performed using an LCQ Advantage ion trap mass spectrometer from Thermo Finnigan or an Agilent 1100 HPLC/MSD spectrometer. NMR spectra were recorded on an INOVA-400 spectrometer, using TMS as an internal standard. Electronic absorption spectra were obtained on a SHIMADZU UV-2450 spectrometer. Photoluminescent spectra were recorded using a HITACHI F4500 fluorescence spectrophotometer with excitation slit widths of 2.5 nm and emission slit widths of 5 nm, and an excitation wavelength of 492 nm. All photochemical reactions were conducted in a Rayonet RPR-600 Reactor using 350 nm mercury lamps. Cell imaging was performed using a Nikon Eclipse TE2000U inverted microscope. TLC analysis was carried out on silica gel plates, and column chromatography was conducted over silica gel (mesh 200–300), both of which were obtained from the Qingdao Ocean Chemicals.

General procedures of photolysis, spectroscopic measurement, and mass spectrometry analysis: A solution of bisNPE-Fl-Hy (5 μM) in HEPES buffer (25 mM, pH 7.4), containing 20% CH_3CN as a co-solvent in the absence or presence of Cu^{2+} (0–10 equiv) or other ions (200 equiv for Na^+, K^+, Ca^{2+}, Mg^{2+}, SO_4^{2-}, Cl^-, and NO_3^-, 10 equiv for Zn^{2+}, Cu^{2+}, Co^{2+}, Fe^{3+}, Hg^{2+}, Mn^{2+}, and F^-) (1 mL) was photolyzed using a Rayonet RPR-600 Reactor (350 nm mercury lamps). After each illumination, the absorption and emission spectra were recorded after 30 min with excitation at 492 nm. An assay time of 30 min was chosen according to the kinetic profile of the Cu^{2+}-induced hydrolysis of Fl-Hy. As shown in Figure S1 in the Supporting Information, in the absence of Cu^{2+}, compound Fl-Hy (5 μM) exhibited no observable changes of emission intensities at 516 nm, indicating that Fl-Hy was not converted to Fl when Cu^{2+} ions were not present. In contrast, upon addition of Cu^{2+} at room temperature, a maximal fluorescence enhancement was noted after approximately 30 min. Thus, the assay time of 30 min was selected for the further examination of the sensitivity and selectivity of bisNPE-Fl-Hy toward Cu^{2+} after photolysis. For mass spectrometry analysis, the photolyzed samples were analyzed using an LCQ Advantage ion trap mass spectrometer.

Cell culture and fluorescence imaging: HeLa cells were grown in MEM (modified Eagle's medium) supplemented with 10% FBS (fetal bovine serum) in an atmosphere of 5% CO_2 and 95% air at 37°C. The cells were plated on 6-well plates and allowed to adhere for 24 h. The cells were washed with HEPES buffer immediately before the experiments, and then incubated with bisNPE-Fl-Hy (15 μM) and Cu^{2+} (6 equiv) for 30 min at 37°C in HEPES buffer containing 3% CH_3CN as a co-solvent. After washing three times with PBS, the selected cells were illuminated for 30 s with UV light through a fluorescence microscope.[9a,d] Subsequently, the cells were incubated for a further 30 min at 37°C, and the fluorescence images were acquired through a Nikon Eclipse TE2000U inverted fluorescence microscope equipped with a cooled CCD camera. The imaging of copper by Rh-Hy (15 μM) was conducted in a similar way, but without exposure to UV light.

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