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Resurfaced Fluorescent Protein as a Sensing Platform for Label-Free Detection of Copper(II) Ion and Acetylcholinesterase Activity

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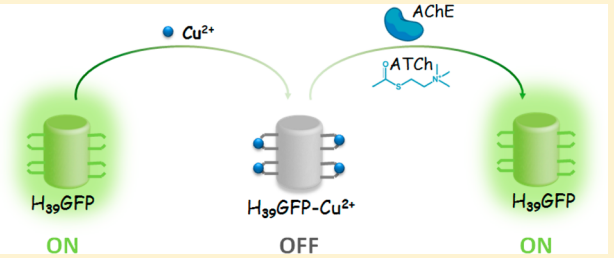
Supporting Information

ABSTRACT: Protein engineering by resurfacing is an efficient approach to provide new molecular toolkits for biotechnology and bioanalytical chemistry. H_{39}GFP is a new variant of green fluorescent protein (GFP) containing 39 histidine residues in the primary sequence that was developed by protein resurfacing. Herein, taking H_{39}GFP as the signal reporter, a label-free fluorometric sensor for Cu^{2+} sensing was developed based on the unique multivalent metal ion-binding property of H_{39}GFP and fluorescence quenching effect of Cu^{2+} by electron transfer. The high affinity of H_{39}GFP with Cu^{2+} (K_d, 16.2 nM) leads to rapid detection of Cu^{2+} in 5 min with a low detection limit (50 nM). Using acetylthiocholine (ATCh) as the substrate, this H_{39}GFP/Cu^{2+} complex-based sensor was further applied for the turn-on fluorescence detection of acetylcholinesterase (AChE) activity. The assay was based on the reaction between Cu^{2+} and thiocholine, the hydrolysis product of ATCh by AChE. The proposed sensor is highly sensitive (limit of detection (LOD) = 0.015 mU mL^{-1}) and is feasible for screening inhibitors of AChE. Furthermore, the practicability of this method was demonstrated by the detection of pesticide residue (carbaryl) in real food samples. Hence, the successful applications of H_{39}GFP in the detection of metal ion and enzyme activity present the prospect of resurfaced proteins as versatile biosensing platforms.



Protein resurfacing is an important technique in protein engineering, which consists of rationally designing a protein's surface while retaining the overall fold, core domain, and boundary regions of the intact protein.[1] Protein resurfacing is especially useful in altering protein−protein interaction,[2] modifying antibodies,[3] and endowing a protein with new functionalities.[4] For example, supercharged green fluorescent protein (ScGFP) with high surface net charge has been developed by resurfacing of GFP.[5] ScGFP shows exceptional solubility and stability against protein aggregation and potent ability to deliver biomacromolecules into cells,[5−7] exhibiting great promise for application in medicine, biotechnology, and even materials science.[8] Recently, H_{39}GFP, a new kind of ScGFP containing as many as 39 histidine residues in the primary sequence, was developed by further resurfacing of ScGFP.[9] Owing to their intrinsic fluorescence, robust stability, abundant surface functional groups, and cell-penetrating ability, it will be attractive to adopt the resurfaced GFPs as biosensors. However, the application of ScGFP in biosensing is scarce so far. Lately, our group has developed a versatile ScGFP-based biosensing platform for DNA detection and CpG methylation analysis.[10] Because of its multiple histidine residues, the newcomer of ScGFPs, H_{39}GFP, possesses several advantages, such as pH-mediated surface multicharge and metal ion coordination property, which is expected to be promising in the development of new biosensing methods.

Recently, the utilization of proteins to develop sensors has attracted much interest, and now several researchers have attempted to exploit fluorescent protein (FP)-based metal ion biosensors.[11−13] Generally, the metal-binding property is acquired by introducing natural (e.g., histidine)[14,15] or noncanonical amino acid residues (e.g., 3,4-dihydroxy-L-phenylalanine)[16] with chelating capability into FP via genetic engineering approaches. The binding site of metal ion is close to the chromophore of FP; thus, the complexation of metal ion results in the fluorescence quenching of FP. However, owing to single metal ion-binding site and correspondingly low binding affinity (K_d, 10^{-7}−10^{-5} M), the sensitivity of these FP-based sensors is still inefficient and needs to be improved. Multivalent binding has been proven to be an effective way to improve binding affinity and capability in antibody and drug design.[17,18] Hence, it is reasonable to expect that highly sensitive detection of metal ions would be achieved if H_{39}GFP was used as a biosensor probe, because so many histidine residues on the

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surface of H_{39}GFP provide multivalent binding sites for metal ions.

Copper is an essential trace element in the human body, and it plays an important role as an enzymatic cofactor.[19] Imbalance of copper homeostasis leads to many pathological sequences, including Alzheimer's, Menkes, and Wilson's diseases as well as tumor development and progression.[20] Copper is also widely used in many industrial processes and has become one of the major components of environmental heavy metal ions pollution.[21] Currently available methods, such as atomic absorption spectroscopy[22] and inductively coupled plasma mass spectroscopy,[23] are complicated and sample-destructive for determination of copper levels in both biological and environmental samples. Therefore, several FP-based sensors have been proposed for measuring the level of copper.[12−14,16] However, most of these biosensors only give satisfactory responses at high concentrations of Cu^{2+} (>20 μM), which is not sufficient in practice.

Acetylcholinesterase (AChE) is a critical enzyme in the central and peripheral nervous system, which has pivotal functions in Alzheimer's disease and nerve agent poisoning.[24] AChE inhibitors that penetrate the blood−brain barrier are useful in the symptomatic treatment of Alzheimer's disease.[25] In addition, pesticides including carbamates and organophosphorus compounds cause the irreversible inhibition of AChE activity, leading to acute toxicity on human health.[26] Therefore, many efforts have been made to probe the activity of AChE and to screen its potential inhibitors. Ellman's colorimetry is the technique mostly applied in the detection of AChE activity but is limited by its low sensitivity.[27] In recent years, nanomaterials, such as quantum dots,[28] gold nanoparticles,[29] and fluorescent conjugated polymers,[30] have also been employed for the detection of AChE activity. However, certain drawbacks still exist, such as multistep operations, sophisticated probe preparation, and long measuring time. Therefore, a novel label-free, simple, sensitive, and selective method for AChE activity and inhibitor monitoring is highly desirable.

Herein, we present a simple and novel method for metal ion assay using H_{39}GFP as a fluorescent probe. Benefiting from the multivalent metal ion-binding sites of H_{39}GFP and the fluorescence quenching effect of Cu^{2+}, this method provides a label-free, rapid, and highly sensitive detection of Cu^{2+}. Moreover, this H_{39}GFP-based Cu^{2+} assay is further applied to analyze AChE activity and AChE inhibitors by using acetylthiocholine (ATCh) as the substrate. In this assay, thiocholine (TCh), generated from the hydrolysis of ATCh by AChE, reacts with Cu^{2+} to form a Cu(I)−TCh complex. As a result, fluorescence of H_{39}GFP is restored and provides a facile switch-on assay for AChE activity detection. This work demonstrates that H_{39}GFP, a resurfaced GFP, can be used as a novel and versatile biosensing platform for the label-free detection of metal ion and enzyme activity, which will inspire the exploration of more widespread applications of engineered proteins in analytical chemistry.

EXPERIMENTAL SECTION

Materials. Acetylcholinesterase (AChE, from *Electrophorus electricus*), acetylthiocholine (ATCh), and tacrine were purchased from Sigma-Aldrich (Shanghai, China). Carbaryl was obtained from Agro-Environmental Protection Institude, Ministry of Agriculture (Tianjin, China). All other chemicals were of analytical grade. All solutions were prepared using ultrapure water (18.2 MΩ·cm) from Millipore (Milli-Q) system.

Cloning, Protein Expression, and Purification. The amino acid sequence of H_{39}GFP was referred to the reported literature.[9] The gene sequence encoding H_{39}GFP ($h_{39}gfp$) was reversely translated from the amino acid sequence of H_{39}GFP and optimized for *E. coli* codon usage. The full-length gene was synthesized and inserted in plasmid pUC19 by Takara (Dalian, China). Then $h_{39}gfp$ was transferred from pUC19-$h_{39}gfp$ to pET28a. The reconstructed plasmid pET28a-$h_{39}gfp$ was transformed into *E. coli* BL21 DE3 with electroporation.

Cells were cultured inLuria broth (LB) medium at 37 °C until OD_{600} (optical density at 600 nm) reached ∼0.6 and then were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 30 °C for 3 h for protein expression. After being harvested by centrifugation and resuspended in buffer (10 mM Tris pH 7.4 and 2 M NaCl), cells were lysed by sonication. Protein was purified by Ni-NTA (NTA = nitrilotriacetic acid) agarose chromatography (ÄKTA, GE), and then the buffer was exchanged into 10 mM Tris, 100 mM NaCl, and pH 7.4 by desalination chromatography (ÄKTA, GE). The purified H_{39}GFP was quantified by using the improved Bradford protein assay dye reagent kit with bovine serum albumin (BSA) as the standard and was stored at −20 °C.

Typical Experimental Process for Cu^{2+} Detection. A 10 μL solution of Cu^{2+} with different concentrations was mixed with 20 μL of 5× buffer (1× buffer: 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 7.0) and replenished to 90 μL by H_2O. Then 10 μL of H_{39}GFP (1 μM) was added to a final volume of 100 μL and incubated at room temperature for 5 min. Fluorescence spectra of each sample were recorded on an F-4500 fluorescence spectrophotometer (Hitachi, Japan). The excitation wavelength was 480 nm, and the emission wavelengths were in the range from 500 to 600 nm with both excitation and emission slits of 5 nm.

H_{39}GFP-dropped agarose hydrogels were prepared as follows: 100 mg of low-melting-point agarose was dissolved in 10 mL of water under microwave heating to produce 1% agarose, and then the solution was cooled to 50 °C by water bath. H_{39}GFP (133 μL, 15 μM) was mixed with 1% agarose to a final volume of 1 mL at 50 °C. Subsequently, an aliquot of 40 μL of the mixture was added into the mold (cap of 0.5 mL Eppendorf tube) and then was kept at 4 °C for 15 min to form the hydrogels. The H_{39}GFP-dropped agarose hydrogels were immersed in 1 mL of tap water samples containing varying concentrations of Cu^{2+} at room temperature for 30 min. The fluorescence was visually observed under UV light, and the images were taken by a Canon digital camera.

AChE Activity Assay. Four μL of 5× buffer, 1 μL of ATCh (10 mM), and 2 μL of AChE with various concentrations were mixed in a final volume of 20 μL and incubated at 37 °C. Thirty min later, 70 μL of 1× buffer containing 1 nmol Cu^{2+} and 10 μL of H_{39}GFP (1 μM) were added to a final volume of 100 μL and incubated at room temperature for 5 min. Fluorescence spectra of each sample were recorded on an F-4500 fluorescence spectrophotometer.

Inhibitor Screenings. For tacrine, AChE (0.5 mU) was incubated with different concentrations of tacrine in a final volume of 19 μL (1× buffer) at 37 °C for 5 min. Then 1 μL of ATCh (10 mM) was added and incubated at 37 °C for 30 min. After that, 70 μL of 1× buffer containing 1 nmol Cu^{2+} and 10 μL of H_{39}GFP (1 μM) were added to a final volume of 100 μL and incubated at room temperature for 5 min. Fluorescence

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Anal. Chem. XXXX, XXX, XXX−XXX

spectra of each sample were recorded on an F-4500 fluorescence spectrophotometer.

For carbaryl, AChE (0.2 mU) was incubated with different concentrations of carbaryl in a final volume of 19 μL (1× buffer) at 37 °C for 10 min. The following process was the same as that of tacrine.

Analysis of Carbaryl Residues in Food Samples. Apple, tomato, and cucumber were chosen as the sample matrixes to evaluate the carbaryl residue levels in the real application tests of this pesticide assay. A standard carbaryl solution (100 mg L^{-1}) was sprayed onto skins of the food samples. Twenty-five g of each sample were collected, chopped, and crushed to homogenate. Then 40 mL of acetonitrile was added, and the resulting mixture was filtered through a 0.22 μm membrane to remove the insoluble materials. The obtained filtrate was dried on a water bath. After that, methanol was added to a final volume of 5 mL (directly analyzed by high-performance liquid chromatography (HPLC)). One mL of the resulting solution was dried again, and H_2O was added to a final volume of 1 mL. Ten μL of the solution was incubated with AChE (0.2 mU) in a final volume of 19 μL (1× buffer) at 37 °C for 10 min. The following procedure was the same as described in the Inhibitor Screenings section.

RESULTS AND DISCUSSION

Fluorescence Quenching of H_{39}GFP by Cu^{2+}. H_{39}GFP was designed, cloned, expressed, and then purified from *E. coli*, according to the published H_{39}GFP sequence.[9] As shown in Figure 1A and Figure S-1, Supporting Information, there are 39

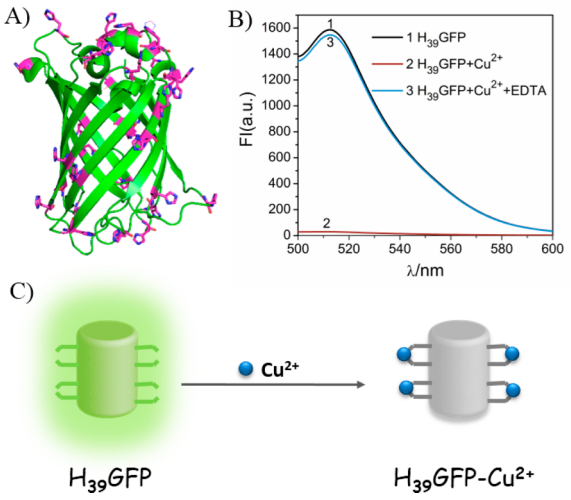

Figure 1. (A) Overall structure of H_{39}GFP. (B) Fluorescence spectra of H_{39}GFP (curve 1), H_{39}GFP−Cu^{2+} (curve 2), and H_{39}GFP/Cu^{2+}/EDTA (curve 3) in the buffer solution. [H_{39}GFP] = 100 nM, [Cu^{2+}] = 10 μM, and [EDTA] = 20 μM. (C) Principle of H_{39}GFP-based Cu^{2+} sensor.

histidine residues in the primary sequence of the H_{39}GFP molecule, and most of them are proximal to the surface of the H_{39}GFP molecule. It is known that histidine has a strong tendency to chelate Cu^{2+}, and Cu^{2+} is one of the best known fluorescence quenchers, which could induce the fluorescence quenching of organic fluorescent dyes and FPs through electron transfer.[31−33] Therefore, it is expected that mixing Cu^{2+} with H_{39}GFP will result in Cu^{2+} binding with the histidine residues on the surface of H_{39}GFP and subsequently quenching the fluorescence emission of H_{39}GFP by electron transfer. Figure 1B displays the fluorescence emission spectra of H_{39}GFP with or without the presence of Cu^{2+}. The addition of Cu^{2+} (10 μM) induced a significant decline in the fluorescence emission of H_{39}GFP (decreased ∼98%), which indicated that H_{39}GFP can be used as a sensor for Cu^{2+} detection. Furthermore, the addition of ethylenediaminetetraacetic acid (EDTA) (a stronger chelator for Cu^{2+}) could completely restore the fluorescence of H_{39}GFP, implying that the interaction between Cu^{2+} and H_{39}GFP is reversible.

To obtain a better sensing performance of H_{39}GFP for Cu^{2+}, some important issues were investigated. The fluorescence intensities at different incubation times were recorded and investigated as the dynamic monitoring of the interaction between Cu^{2+} and H_{39}GFP. As shown in Figure S-2, Supporting Information, the fluorescence of H_{39}GFP decreases quickly in the first 1 min (decreased ∼87%) and then gradually decreases with the incubation time increasing and tented to a saturation point at 5 min. This quick and high-efficiency fluorescence quench indicated that the proposed method was rapid and sensitive for Cu^{2+} detection. Additionally, the effect of pH was also examined. Fluorescence of H_{39}GFP and quenching efficiency kept stable as the pH of the buffer solution increased from pH 6.0 to pH 8.0 (Figure S-3, Supporting Information), showing that the proposed method can work well in the buffer with neutral pH. Thus, the following experiments were performed at pH 7.0 with an incubation time of 5 min.

Cu^{2+} Sensing Based on H_{39}GFP. Figure 1C shows the mechanism for Cu^{2+} detection based on H_{39}GFP. The proximal histidine residues on the surface of H_{39}GFP provide multiple binding sites for Cu^{2+}. After binding of Cu^{2+} on the surface, the fluorescence emission of H_{39}GFP is quenched by Cu^{2+}. On the basis of this principle, detection of Cu^{2+} in aqueous solution was studied under preoptimized conditions. When the concentration of Cu^{2+} increased from 0.05 to 15 μM, the fluorescence of H_{39}GFP decreased continually and was almost completely quenched by 10 μM Cu^{2+} (Figure 2A). According to the Cu^{2+} concentration-dependent quenching curve, the dissociation constant value (K_d) of 16.2 nM between H_{39}GFP and Cu^{2+} could be estimated by least-squares curve fitting analysis (details are shown in Supporting Information).[34] By comparison, the FP-based sensors with a single Cu^{2+}-binding site show lower fluorescence quench rates (<75%) and much lower affinity with Cu^{2+} (as shown in Table 1). From the Cu^{2+} concentration-dependent calibration curve, a good linear relationship (R^2 = 0.993) in the concentration ranging from 0.05 to 2.0 μM was obtained (inset in Figure 2B), and the lowest detection limit was 50 nM. The LOD of the proposed assay is much lower than those of many of the previous reported FP biosensors (Table 1),[11−13,16] which could be attributed to the multivalent binding sites and the corresponding higher affinity of H_{39}GFP toward Cu^{2+}.

To examine the specificity of the sensor, the fluorescence responses of H_{39}GFP toward various metal ions including Cu^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+}, Ag^{+}, Cd^{2+}, Pb^{2+}, Al^{3+}, Ni^{2+}, Ca^{2+}, Fe^{2+}, Fe^{3+}, and Hg^{2+} were studied under the same conditions; the final concentration of the ions was 10 μM. It can be seen that only Cu^{2+} and Hg^{2+} caused dramatic fluorescence quenching, and there were no obvious fluorescence changes for the other metal ions (Figure 3A). Comparatively, the fluorescence quenching induced by Hg^{2+} is much weaker as compared with that by Cu^{2+}, and the fluorescence response to Hg^{2+} can be masked upon the use of Cl^{-} as a coadditive. Moreover, the coexistence

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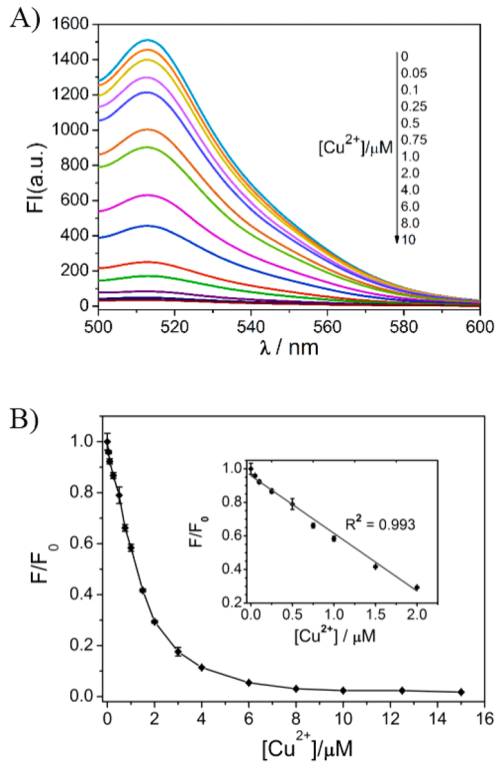

Figure 2. (A) Fluorescence spectra of H_{39}GFP (100 nM) in the presence of Cu^{2+} with varying concentrations. (B) Fluorescence responses of H_{39}GFP to Cu^{2+} with varying concentrations. F and F_0 are fluorescence intensities of H_{39}GFP in the presence and absence of Cu^{2+}, respectively. Inset shows the linear responses to Cu^{2+} ranging from 0.05 to 2 μM.

Table 1. Comparisons between H_{39}GFP-Based Sensor and Other FP-Based Biosensors in Cu^{2+} Detection

probe	K_d/μM	LOD/μM	QE[a]	ref
mEmerald-3H	0.2	0.1	~75%	11
His_6GFP	17	0.5	~15%	12
Rum13	10.9	0.1	~40%	13
GFPdopa	5.6	1.0	~35%	16
H_{39}GFP	0.0162	0.05	~98%	this work

[a]Fluorescence quenching rate in the presence of 10 μM Cu^{2+}.

of other metal ions showed no obvious interference for Cu^{2+} assays (Figure S-4, Supporting Information). Metal ions, such as Ni^{2+}, Zn^{2+}, and Co^{2+} could also chelate histidine residue and are widely used in immobilized metal affinity chromatography.[35] However, the binding affinity between histidine residue and Cu^{2+} is much stronger than with these metal ions,[36] and only Cu^{2+} shows remarkable fluorescence quenching effect. Thus, these ions (Ni^{2+}, Zn^{2+}, and Co^{2+}) do not cause interference to Cu^{2+} detection in the H_{39}GFP-based assay. Furthermore, to evaluate the practical application of this new assay, the response of H_{39}GFP to Cu^{2+} in urine (10%, v/v) was investigated, and it was found that fluorescence response was linearly associated the concentration of Cu^{2+} ranging from 0.1 to 10 μM (Figure S-5, Supporting Information). The concentrations of Cu^{2+} in tap water and urine samples were detected by applying a standard addition method, and satisfactory accuracy and precision were achieved (as shown in Table S-1, Supporting Information).

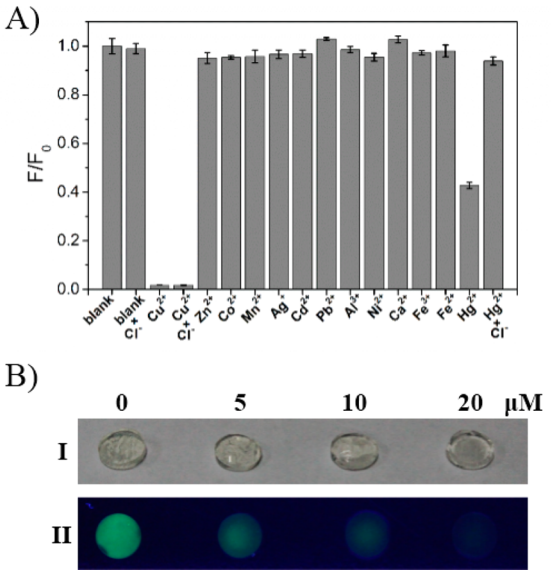

Figure 3. (A) Selectivity of the H_{39}GFP-based Cu^{2+} sensing system. The concentrations of Cu^{2+} and other metal ions were 10 μM, and the concentration of Cl^- was 50 mM. (B) Visual detection of Cu^{2+} (0, 5, 10, and 20 μM Cu^{2+}) using H_{39}GFP-containing agarose hydrogels under visible light (I) and UV light (II).

Visual detection by naked eyes is preferable for practical applications. It was reported that this H_{39}GFP can refold and recover its fluorescence when boiled and cooled at pH 4–5.[9] We found that H_{39}GFP kept 89% of its fluorescence after 9 months at −20 °C (data not shown). Such exceptional solubility and stability of H_{39}GFP gave us the chance to exploit the application of the sensor for visual detection of Cu^{2+}. Immobilizing H_{39}GFP into hydrogels is a reasonable solution because these materials have high loading capacity and low background. The large molecular weight (~27.4 kDa) of H_{39}GFP could restrict the diffusion and prevent the release of H_{39}GFP from the hydrogels, and the accumulative properties of functional hydrogels allow it to be used for visual detection of Cu^{2+}. To achieve this purpose, H_{39}GFP-dropped agarose hydrogels were prepared. Because of the superb stability of H_{39}GFP (e.g., the ability to refold after boiling[9]) and low melting temperature of low-melting-point agarose, the gelation process has no effect on the optical properties of H_{39}GFP, and the transparent agarose hydrogels emitted strong green fluorescence under UV irradiation, indicating that the H_{39}GFP-containing agarose hydrogels were successfully prepared. The fluorescence of hydrogels under UV lamp grew fainter with the increase of Cu^{2+} concentration and disappeared after the treatment with 20 μM Cu^{2+} (Figure 3B). The United States defined maximum contaminant level for Cu^{2+} in tap water is 20 μM; therefore, these H_{39}GFP-containing agarose hydrogels are suitable for instrument-free visual Cu^{2+} detection and water quality control under practical conditions.

AChE Activity Detection Based on H_{39}GFP. The function of H_{39}GFP as a biosensing platform was further expanded by exploring its application in enzymatic activity analysis. Taking advantage of the fact that the fluorescence of H_{39}GFP can be effectively quenched by Cu^{2+}, a novel fluorescent method for AChE activity assay was proposed. The sensing mechanism is shown in Figure 4A. Acetylthiocholine (ATCh), an analogue of acetylcholine (the natural substrate of AChE), can be hydrolyzed by AChE into

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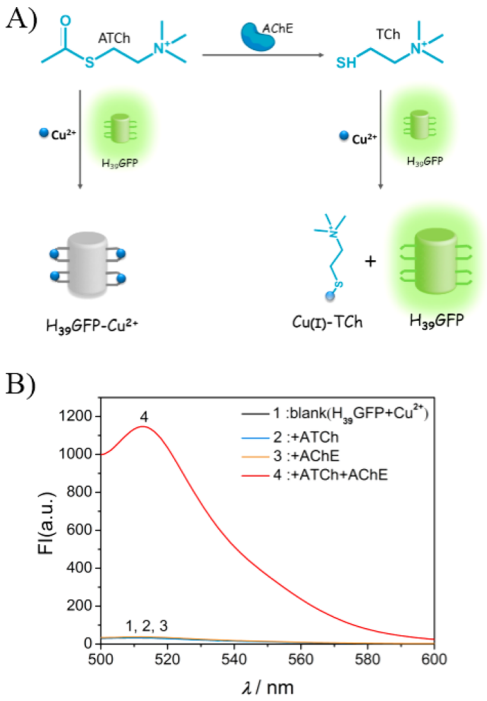

Figure 4. (A) Mechanism of the $H_{39}GFP/Cu^{2+}$-based fluorometric assay for AChE activity detection. (B) Fluorescence spectra of $H_{39}GFP/Cu^{2+}$ (curve 1), $H_{39}GFP/Cu^{2+}$ with ATCh (curve 2), $H_{39}GFP/Cu^{2+}$ with AChE (curve 3), and $H_{39}GFP/Cu^{2+}$ with both ATCh and AChE (curve 4). [ATCh] = 50 μM, [AChE] = 5 mU mL^{-1}.

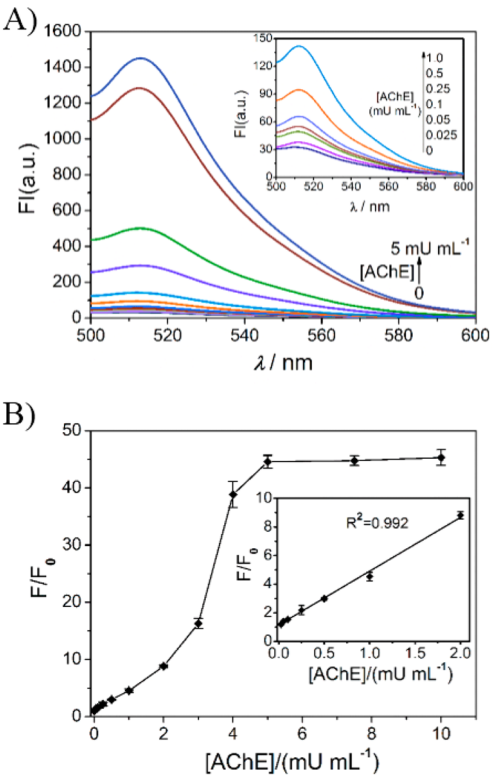

Figure 5. (A) Fluorescence spectra of $H_{39}GFP/Cu^{2+}$ at various concentrations of AChE. Inset A shows the fluorescence spectra of $H_{39}GFP/Cu^{2+}$ at low concentrations of AChE. (B) Relationship between the fluorescence intensity of $H_{39}GFP/Cu^{2+}$ and the AChE concentration. Inset B shows the linear responses to AChE ranging from 0.025 to 2 mU mL^{-1}. F_0 and F are the fluorescence intensities of $H_{39}GFP/Cu^{2+}$ in the absence and presence of AChE, respectively.

thiocholine (TCh). TCh, a kind of mercaptans, can react with Cu^{2+} to form the Cu(I)−TCh complex by the well-known reaction of copper with mercaptans.[37] The formation of Cu(I)−TCh complex prevents Cu^{2+} from binding $H_{39}GFP$. As a result, electron transfer between $H_{39}GFP$ and Cu^{2+} will not occur and $H_{39}GFP$ will emit intense fluorescence upon irradiation. However, in the presence of AChE inhibitors, the hydrolysis of ATCh is inhibited and TCh will not be generated, and Cu^{2+} will interact with $H_{39}GFP$ to form the $H_{39}GFP-Cu^{2+}$ complex, which leads to the quenching of $H_{39}GFP$'s fluorescence. Therefore, AChE activity and its inhibitor can be reflected by the fluorescence intensity change of $H_{39}GFP$.

Following the design, the feasibility of the proposed method was investigated. Figure 4B depicts typical fluorescence response of this AChE assay. After the incubation of AChE with its substrate ATCh, Cu^{2+} and $H_{39}GFP$ were added for detection. In comparison with the control sample ($H_{39}GFP/Cu^{2+}$), the AChE-catalyzed reaction caused a significant fluorescence enhancement of $H_{39}GFP$, which is ~38-fold as high as that of $H_{39}GFP/Cu^{2+}$. However, either AChE or ATCh caused negligible fluorescence responses relative to the control sample ($H_{39}GFP/Cu^{2+}$). Thus, the hydrolysis product of ATCh by AChE inhibited the binding of copper ion with $H_{39}GFP$, resulting in a remarkable restoration of the fluorescence and providing a "turn-on" detection of AChE. The optimal values for the concentration of ATCh and hydrolysis times of AChE were found to be 100 μM and 30 min, respectively (Figure S-6, Supporting Information). Under the optimized conditions, the dynamic response range of AChE in buffer solution was recorded through fluorescence changes of $H_{39}GFP$. When the concentration of AChE increased from 0 to 10 mU mL^{-1}, the fluorescence intensity of $H_{39}GFP$ was continually increasing (Figure 5A), implying a gradual hydrolysis of ATCh. The plot of fluorescence response versus the concentration of AChE is shown in Figure 5B with a plateau point at 5 mU mL^{-1} of AChE. A good linear range (R^2 = 0.992) from 0.025 to 2 mU mL^{-1} was obtained as the inset of Figure 5B shows. The assay allowed the detection of AChE as low as 0.015 mU mL^{-1} (S/N = 3), which is much lower than many previously reported AChE assays (detailed comparison is shown in Table S-2, Supporting Information).[29,38−41] To evaluate the specificity of the proposed fluorometric assay toward AChE, other enzymes, including horse radish peroxidase (HRP), carboxypeptidase Y (CPY), and protein kinase A (PKA), were investigated as control samples. Although the concentrations of these non-specific enzymes were much higher than that of AChE, none of them can induce remarkable fluorescence response in the assay as AChE did (Figure S-7, Supporting Information). This result clearly demonstrated that this assay based on $H_{39}GFP/Cu^{2+}$ could be selective for the targeted detection of AChE activity.

AChE Inhibitors Screening. To further demonstrate the potential application of this method in the inhibition assay, the experiments were performed in the presence of the AChE inhibitors. Tacrine, a well-known AChE inhibitor, which has been used for the treatment of Alzheimer's disease, was selected. The inhibition efficiency (IE) was determined by the following equation:

$$\mathrm{IE}\ (\%) = 100 \times (F - F_i)/(F - F_0) \quad (1)$$

where F_0 is the fluorescence intensity of the initial $H_{39}GFP/Cu^{2+}$ complex. F is the fluorescence intensity with AChE, and F_i

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Anal. Chem. XXXX, XXX, XXX−XXX

is that with both AChE and inhibitor. On the basis of the plot of inhibition efficiency versus the concentration of tacrine (Figure S-8, Supporting Information), the corresponding IC_{50} value (the inhibitor concentration required for 50% inhibition of the enzyme activity) was estimated to be 3.5 nM, which is similar to those obtained by other AChE assays.[42]

Carbamate pesticides are also known as inhibitors of AChE. Carbamate pesticides exhibit toxicity on human health and distribute through their residues in agricultural products or in drinking water. Carbaryl was employed to evaluate the viability of this method to the detection of carbamate pesticides. The relationship between inhibition efficiency and the carbaryl concentration is shown in Figure 6A. The inhibition efficiency

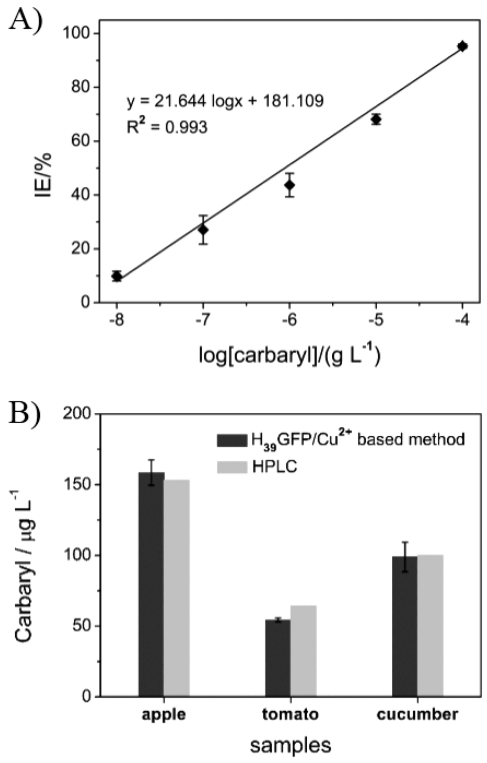

Figure 6. (A) Inhibition efficiency versus the logarithm of the carbaryl concentration. (B) Analytical results of carbaryl in apple, tomato, and cucumber by using $H_{39}GFP/Cu^{2+}$-based method (black) and by using HPLC (gray).

was linearly dependent on the logarithm of the carbaryl concentration ranging from 1×10^{-8} to 1×10^{-4} g L^{-1}, and the corresponding linear equation is IE (%) = 21.644 log[carbaryl] (g L^{-1}) + 181.109 (R^2 = 0.993). The limit of detection (defined as the concentration of inhibitor required to achieve 5% inhibition[43]) was 7.03×10^{-9} g L^{-1} (0.007 ppb), which is much lower than the maximum residue limits in the European Union (50 ppb) and United States (20 ppb) pesticides data set, as well as many previously reported assays (detailed comparison is shown in Table S-3, Supporting Information).[38,44−47]

The excellent sensitivity of the developed biosensor suggests that it might be directly applied for detecting pesticide residues in real samples. Therefore, the carbaryl residues in fruit and vegetable samples, such as apple, tomato, and cucumber, were detected by the proposed assay and high-performance liquid chromatography (HPLC). Figure 6B shows that the carbaryl concentration obtained by using this assay coincides well with those obtained by using HPLC, indicating that the proposed method can be successfully applied to detect pesticide residues in real samples.

CONCLUSION

In conclusion, we have developed a simple and label-free biosensor based on $H_{39}GFP$ for the detection of Cu^{2+}. The binding of Cu^{2+} induces quick and dramatic fluorescence quenching of $H_{39}GFP$, which makes this method rapid and facile, proved by its "mix-and-read" property and fast detection in 5 min, with excellent selectivity and high sensitivity (LOD = 50 nM). The $H_{39}GFP$-based sensor shows multifaceted advantages over previously reported FP-based copper ion sensors, such as higher binding affinity, better quenching efficiency, and lower detection limit, because of its multivalent binding of Cu^{2+}. Furthermore, $H_{39}GFP$-containing agarose hydrogels could be used for visual detection of Cu^{2+} in tap water. Moreover, $H_{39}GFP-Cu^{2+}$ interaction is further utilized to detect AChE activity based on the reaction of Cu^{2+} with the product of AChE-catalyzed ATCh hydrolysis. This homogeneous approach allows a low detection limit of AChE (0.015 mU mL^{-1}) and is feasible for not only AChE inhibitor screening but also for carbamate pesticides probing in agricultural products. The present study provides a new function of resurfaced FPs, as a versatile biosensing platform for the label-free detection of metal ion and enzyme activity. This work shows the prospect of the engineered proteins by resurfacing as a promising molecular toolkit in bioanalytical chemistry.

ASSOCIATED CONTENT

Supporting Information

Additional information including extensive figures as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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Anal. Chem. XXXX, XXX, XXX−XXX

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Anal. Chem. XXXX, XXX, XXX−XXX