Protein Biosensors

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Development of a Selective, Sensitive, and Reversible Biosensor by the Genetic Incorporation of a Metal-Binding Site into Green Fluorescent Protein**

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Copper is an important transition-metal ion in the human body. It plays a crucial role as a cofactor in oxidative scavenging mechanisms.[1] However, copper imbalance can have pathological consequences, such as Menkes, Alzheimer's, and Wilson's diseases as well as tumor development and progression.[2] Copper is also widely used as an antifouling agent in industrial processes and is one of the major components of environmental heavy-metal-ion pollution.[3] Currently available methods, such as atomic absorption spectroscopy, inductively coupled plasma mass spectrometry, and electrochemistry, are very complicated for the determination of the copper level in biological and environmental samples. As the samples may be destroyed during analysis, these methods are unsuitable for in situ copper detection.[4]

To date, a number of small-molecule-based sensors, chelator-based sensors, and enzymatic and electrochemical techniques have been developed for measuring the level of copper. The major limitation of these techniques is the modification of the binding elements with a reporter molecule to generate a signal.[3] On the other hand, the conversion of a protein into a sensor tool is currently attracting much interest, and now several researchers have attempted to use green fluorescent protein (GFP) as a copper biosensor.[5] In vitro biosensing systems have been developed by the introduction of copper-binding sites into GFP by various genetic-engineering and directed-evolution approaches, which resulted in fluorescence quenching in response to Cu^{2+}. In most of the cases, the metal-binding property was acquired and enriched by introducing histidine and cysteine residues into GFP.[6] However, the sensitivity and selectivity of GFP towards Cu^{2+} is still inefficient and needs to be improved. Similarly, the red fluorescent protein DsRed has also been reported as a metal biosensor.[7] The inherent metal-binding property of DsRed is potentially useful; however, DsRed has major drawbacks, such as its long maturation time, low expression yield, and poor folding efficiency.[8] Hence, the development of a simple and selective bioanalytical tool for monitoring Cu^{2+} in biological events is a great challenge in chemical biology.

The design and manipulation of target proteins with new and enhanced properties through the genetic incorporation of novel functionality derived from noncanonical amino acids (NCAAs) has become an important quest. Two experimental approaches have been used for the in vivo incorporation of NCAA into recombinant proteins: the reassignment of sense codons (genetic-code engineering) and nonsense suppression (site-specific incorporation).[9,10] In this study, we created a novel fluorescence-based copper biosensor by introducing the metal-chelating NCAA 3,4-dihydroxy-L-phenylalanine (L-DOPA) into GFP. It is well-known that transition-metal ions interact strongly with catecholamines. L-DOPA is an important catecholamine that coordinates with metal ions as a bidentate ligand through either the catecholate (O,O) or amino acid (O,N) part of the molecule.[11]

L-DOPA has been used in significant studies to explore protein–protein interactions following its genetic incorporation through the method of nonsense suppression.[12] Although such approaches are quite popular in academic communities, their practical usefulness is still limited for several reasons.[13] In this study, we created a protein-based biosensor by introducing L-DOPA into GFP through genetic-code engineering (Figure 1). Furthermore, we investigated possible interaction sites responsible for biosensing activity by the nonsense-suppression method. The residue-specific incorporation of L-DOPA was confirmed as described earlier.[14] As an initial test, we first evaluated the effects of metal ions such as K^+, Mg^{2+}, Ca^{2+}, Na^+, Mn^{2+}, and Cu^{2+} at high (1 mM; see Figure S1 in the Supporting Information) and low concentrations (0.1 mM) on GFP and GFPdopa (Figure 2a). The fluorescence of GFP and GFPdopa was slightly affected by the presence of K^+, Mg^{2+}, Ca^{2+}, and Na^+ metal ions at high and low concentrations. However, the addition of Cu^{2+} (0.1 mM) completely quenched the fluorescence of GFPdopa. In contrast, GFP retained more than 75% fluorescence after treatment with Cu^{2+} (0.1 mM). Among the tested metal ions,

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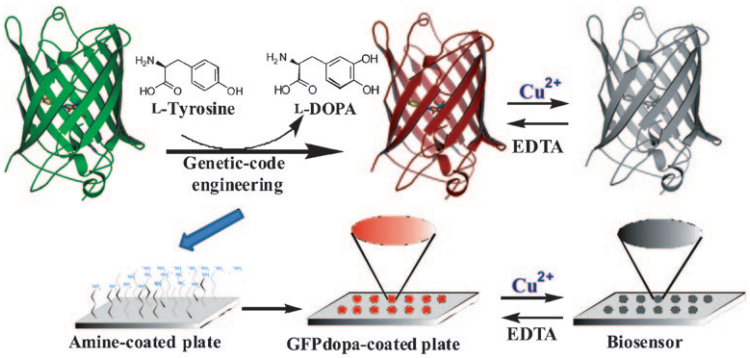

Figure 1. Proposed approach to the creation of a protein-based biosensor by genetic-code engineering.

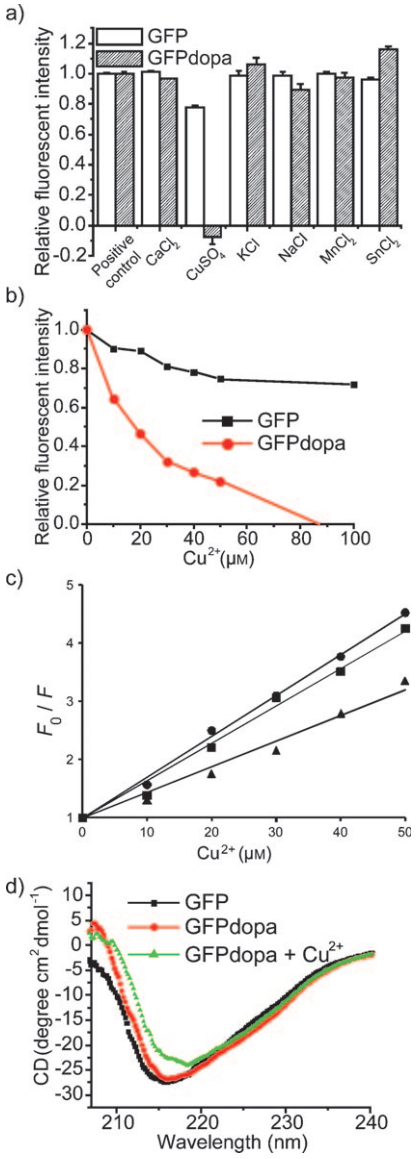

Figure 2. a) Effect of different metal ions on the fluorescence emission of GFP and GFPdopa. Each metal ion (0.1 mM) was incubated with GFP and with GFPdopa. b) Calibration curve generated for GFP and GFPdopa with different concentrations of Cu^{2+}. c) Stern–Volmer plots for GFPdopa in the presence of Cu^{2+} at three different temperatures: 25 °C (●), 30 °C (■), and 45 °C (▲). d) Circular dichroism profiles of GFP, GFPdopa, and GFPdopa in the presence of Cu^{2+} (0.1 mM).

only Cu^{2+} ions caused selective fluorescence quenching of GFPdopa. GFP and GFPdopa were also treated with the transition-metal ions Cd^{2+}, Co^{2+}, Fe^{3+}, Mn^{2+}, Ni^{4+}, and Zn^{2+}. Modest fluorescence quenching of both GFP and GFPdopa was observed for Fe^{3+}; however, the other ions had no effect. This result indicates that Fe^{3+}-induced fluorescence quenching may be due to an inherent property of GFP (see Figure S2 in the Supporting Information). Conversely, the binding of Cu^{2+} to GFPdopa did not lead to any wavelength shift in its fluorescence excitation and emission maxima (see Figure S3 in the Supporting Information).

A calibration curve was generated for GFP and GFPdopa by plotting the amount of fluorescence quenching against various concentrations of Cu^{2+} (Figure 2b). Cu^{2+} at a concentration of 20 μM showed 50% quenching of GFPdopa fluorescence. However, complete fluorescence quenching occurred when GFPdopa was treated with Cu^{2+} at a concentration of 100 μM. To calculate the dissociation constant of GFPdopa, we used Equation (1).[15]

$$\frac{\Delta F}{\Delta F_{max}} = \frac{\left(K_d + [Cu] + [P] \pm \sqrt{(K_d + [Cu] + [P])^2 - 4[Cu][P]}\right)}{2[P]} \quad (1)$$

In this equation, ΔF is the change in measured fluorescence, ΔF_{max} is the maximum fluorescence change, [P] is the total protein concentration, K_d is the dissociation constant of the Cu^{2+}-binding site, and [Cu] is the total concentration of Cu^{2+}. The copper dissociation constant of GFPdopa was identified as (5.6 ± 0.3) μM, which indicates the high affinity of GFPdopa for Cu^{2+}. The copper dissociation constant of GFPdopa was similar to that of DsRed and much lower than that of DsRed mutants Rmu13, drFP583, and gRF.[3,16] To confirm the mechanism of quenching, we generated Stern–Volmer plots by measuring the fluorescence of GFPdopa at various temperatures (Figure 2c). The quenching constant of GFPdopa decreased with an increase in temperature and vice versa. This relationship indicates that the quenching mechanism is static owing to the formation of a ground-state complex between Cu^{2+} and GFPdopa.

To further validate the static-quenching mechanism, we performed UV absorption scans of GFPdopa in the presence and absence of Cu^{2+}.[17] As a result of static quenching, the absorption spectrum of GFPdopa was not affected by the presence of Cu^{2+} (see Figure S4 in the Supporting Information). Furthermore, more than 70% of the fluorescence of GFPdopa was recovered within 5 min upon equilibration with ethylenediaminetetraacetic acid (EDTA; see Figure S5 in the Supporting Information). These results suggest that under equilibrium conditions, the continuous and reproducible monitoring of exchangeable Cu^{2+} is possible with GFPdopa. Furthermore, circular dichroism confirmed that the overall secondary structure of GFPdopa remained unchanged after treatment with Cu^{2+} (Figure 2d). This result suggests that the observed fluorescence quenching of GFPdopa is not due to structural or conformational changes in the protein.

On the basis of our results, we speculate that Cu^{2+} specifically binds to the catechol moiety of L-DOPA incorporated in GFPdopa, which might donate an electron to Cu^{2+} and possibly forms a nonfluorescent ground-state complex. It is well-known that Cu^{2+} ions coordinate preferentially with aminocarboxylate and catechol groups; they are typically found coordinated to L-DOPA side chains.[11] These groups might favor the binding of Cu^{2+} to GFPdopa and lead to fluorescence quenching. To determine the possible copper-binding sites in GFPdopa, we generated a 3D model structure of our GFP variant through homology modeling.[18] On the basis of the energy-minimized model, we surmise that the two residues His148 and Tyr92 may play a critical role in biosensing. The Tyr92 residue interacts with the chromophore with the help of a water molecule, and His148 interacts directly with the chromophore residue. The replacement of L-DOPA in these two pairs (to establish a DOPA66–His148 interaction and a DOPA92–chromophore interaction) might favor the binding of Cu^{2+} with the chromophore (Figure 3a,b).

To evaluate the mechanism, we introduced an amber mutation at the Tyr66 ($GFP_{Y66dopa}$) and Tyr92 ($GFP_{Y92dopa}$) positions and then incorporated L-DOPA site specifically by using an engineered *Methanococcus jannaschii* tRNATyr/tyrosyl-tRNA synthetase pair.[12] Similarly, a point mutation to Phe was introduced at Tyr92 ($GFP_{dopaY92F}$), and the remaining Tyr residues were globally replaced with L-DOPA through genetic-code engineering (see Table S1 and Figure S6 in the Supporting Information). We also introduced a double amber mutation at the Tyr66 and Tyr92 positions ($GFP_{Y66Y92dopa}$); however, owing to the low efficiency of the orthogonal tRNA/synthetase system, the desired protein containing L-DOPA at the Tyr66 and Tyr92 positions was not obtained. LC–MS/MS analysis confirmed the site-specific incorporation of L-DOPA in the mutant proteins (see Table S2 in the Supporting Information). The $GFP_{Y66dopa}$ mutant showed Cu^{2+}-sensing activity that was higher than that of the parent GFP and the $GFP_{dopaY92F}$ mutant, and lower than that of GFPdopa (Figure 3c). This response indicated that DOPA66 might not be the only chromophore residue involved in the Cu^{2+}-sensing activity. In contrast, the mutant $GFP_{Y92dopa}$ showed slightly higher Cu^{2+}-sensing activity than $GFP_{Y66dopa}$ (Figure 3c). Interestingly, the $GFP_{dopaY92F}$ mutant showed lower Cu^{2+}-sensing activity than both $GFP_{Y92dopa}$ and $GFP_{Y66dopa}$. This result indicates that the binding of Cu^{2+} is slightly favored by the presence of DOPA at position 92. Our results suggest that the synergistic effects of L-DOPA at multiple positions in GFP, especially at positions 66 and 92, are essential for the efficient Cu^{2+}-sensing activity.

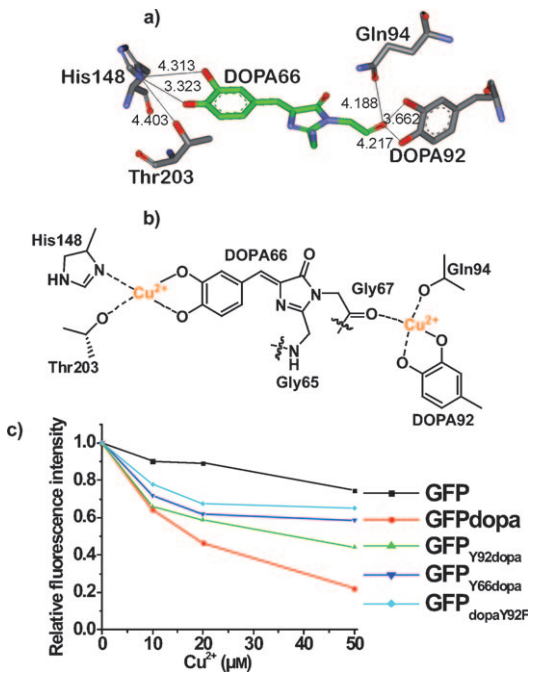

Figure 3. a) Chromophore-interaction sites in GFPdopa. b) Proposed biosensing interaction of GFPdopa with Cu^{2+}. c) Fluorescence intensity of GFPdopa and mutant variants in the presence of Cu^{2+} at different concentrations.

Finally, we aimed to integrate GFPdopa with a microfabricated device for the design of real biosensors. However, a crucial step in the design of a protein biosensor is the immobilization of the sensor protein onto a device without altering its structural and functional properties. Therefore, DsRed and GFP were encapsulated within a polyacrylamide matrix and a sol–gel, respectively.[5,19] Each immobilization technique has its own advantages and disadvantages. Recently, we showed that proteins containing L-DOPA can be selectively oxidized and covalently cross-linked with amine-containing polysaccharides.[14] The extraordinary features of GFPdopa led us to develop an application-oriented protein-based sensor. The immobilization of GFPdopa on an amine-coated material required *o*-quinone, which could be generated selectively by the oxidation of DOPA with $NaIO_4$.

We anticipated that the formation of *o*-quinone might interrupt the Cu^{2+}-sensing activity of GFPdopa. Therefore, we generated a calibration curve for $NaIO_4$-treated GFPdopa with respect to different concentrations of Cu^{2+}. Fluorescence was quenched according to the concentration of Cu^{2+} and was immediately recovered upon treatment with EDTA (see Figure S7 in the Supporting Information). The dissociation constant of $NaIO_4$-treated GFPdopa was identified as (4.3 ± 1.1) μM, which is similar to that found for GFPdopa in the absence of $NaIO_4$ (see Figure S8 in the Supporting Information). This result indicates that GFPdopa retained its Cu^{2+}-sensing activity even after treatment with $NaIO_4$. We hypothesize that the biosensing property of GFPdopa might be maintained in two ways: 1) During $NaIO_4$ treatment, the surface-exposed DOPA residues (DOPA74, DOPA143, DOPA151, DOPA182, DOPA200, and DOPA237) might be converted into *o*-quinone. In contrast, the DOPA residues buried internally (DOPA66 and DOPA92) might not be converted into *o*-quinone because of lack of accessibility to $NaIO_4$ and so may exist as catechol moieties without any modifications. The interaction of these residues with Cu^{2+} would lead to the fluorescence quenching. 2) Both the surface-exposed DOPA and internally buried DOPA might be converted into *o*-quinone, and the direct interaction of the resulting *o*-quinone groups with Cu^{2+} might lead to fluorescence quenching. However, additional studies will be

required for a complete understanding of Cu^{2+}-interaction sites in GFPdopa. We are currently pursuing such studies.

To investigate the feasibility of the proposed biosensor, we used soft lithography for the fabrication of GFPdopa patterns on the amine-coated glass surface.[20] The micropatterned proteins were observed under a fluorescence microscope. The pattern size was almost identical in each case, which confirms that the fabrication of GFPdopa on the amine slide is highly reliable and robust. Importantly, the patterned protein showed strong fluorescence signals, which indicated that GFPdopa retained its original functionality on the glass surface. To evaluate the performance of the protein biosensor, we incubated the GFPdopa slide with Cu^{2+} (100 μM) for 5 min and observed the difference in fluorescence. The fluorescence signal was completely quenched upon the addition of Cu^{2+} (Figure 4a,b). The slide was then equilibrated with EDTA for 5 min and observed under similar conditions with the fluorescence microscope. The original fluorescence emission of GFPdopa was recovered immediately (Figure 4a,b). Densitometry analysis with the Scion Image PC software package revealed that 94% of the original fluorescence intensity of GFPdopa was recovered (Figure 4b). A Stern–Volmer plot was generated with micropatterned GFPdopa for the addition of Cu^{2+} at different concentrations (Figure 4c). Our results were very consistent, and the plot clearly showed the linear behavior of the protein biosensor. This significant difference in fluorescence according to the amount of Cu^{2+} added confirmed that GFPdopa can function as a sensitive biosensor for Cu^{2+} detection.

In conclusion, we characterized a DOPA-containing protein as a biosensing tool which specifically binds to Cu^{2+} in a reversible manner. In general, the concentration of Cu^{2+} in polluted environmental samples and biological samples was found to be in the millimolar to micromolar range.[3] The biosensor could be adapted to sense Cu^{2+} in polluted environmental samples as well as for the development of a novel molecular diagnostic tool for Cu^{2+} detection. To the best of our knowledge, NCAA incorporation has not been coupled previously with biological microelectromechanical systems (bioMEMS) for metal sensing and protein assembly.

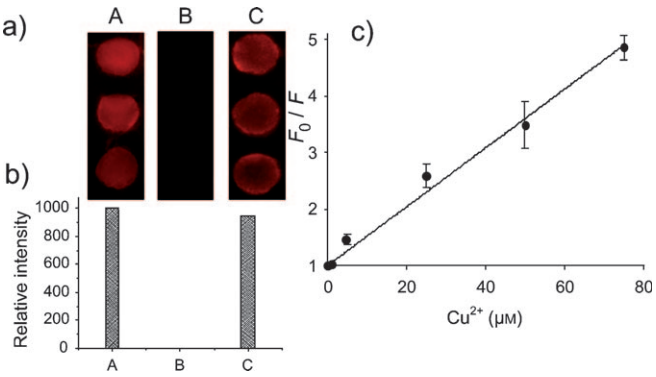

Figure 4. a) Fluorescence images of micropatterns of GFPdopa on the amine-coated surface: A) before treatment with Cu^{2+}; B) after treatment with Cu^{2+} (100 μM); C) after treatment with Cu^{2+}, followed by the addition of EDTA. The micropattern size is 50 μm. The incorporation of L-DOPA in GFP resulted in a red shift of its fluorescence emission. The images were recorded with a Nikon fluorescence microscope. b) Densitometry analysis of the GFPdopa-based protein biosensor. c) Stern–Volmer plot generated for micropatterned GFPdopa by the addition of Cu^{2+} at different concentrations.

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