Fluorescent indicators for live-cell and in vitro detection of inorganic cadmium dynamics

Shulin Hu1,2 · Jun Yang1,2 · Anqi Liao1,2 · Ying Lin1,2 · Shuli Liang1,2

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Abstract

Cadmium contamination is a severe threat to the environment and food safety. Thus, there is an urgent need to develop highly sensitive and selective cadmium detection tools. The engineered fluorescent indicator is a powerful tool for the rapid detection of inorganic cadmium in the environment. In this study, the development of yellow fluorescent indicators of cadmium chloride by inserting a fluorescent protein at different positions of the high cadmium-specific repressor and optimizing the flexible linker between the connection points is reported. These indicators provide a fast, sensitive, specific, high dynamic range, and real-time readout of cadmium ion dynamics in solution. The excitation and emission wavelength of this indicator used in this work are 420/485 and 528 nm, respectively. Fluorescent indicators N0C0/N1C1 showed a linear response to cadmium concentration within the range from 10/30 to 50/100 nM and with a detection limit of 10/33 nM under optimal condition. Escherichia coli cells containing the indicator were used to further study the response of cadmium ion concentration in living cells. E. coli N1C1 could respond to different concentrations of cadmium ions. This study provides a rapid and straightforward method for cadmium ion detection in vitro and the potential for biological imaging.

Keywords Inorganic cadmium · Fluorescent indicator · Live-cell · In vitro · Dynamics

Introduction

Cadmium is a harmful heavy metal element. It is widely distributed in water, soil, and agricultural products, and spreads through the food chain to accumulate in the human body[1–3]. It causes great harm to the human body and has aroused widespread concern[4]. The intake of cadmium can adversely affect the kidneys, lungs, bone, and nervous system, with a biological half-life in the range of 17–30 years in the human body[5]. The long-term presence of cadmium causes renal dysfunction, calcium metabolism disorders, pancreatic dysfunction, and an increased incidence of various diseases[6–8]. For these reasons, it is vital to detect and quantify trace amounts of cadmium in environmental and food samples.

The traditional methods used to detect cadmium are mainly inductively plasma mass spectrometry (ICP-MS), atomic absorption spectroscopy (AAS) and electrochemistry techniques for sensitive detection[9, 10]. ICP-MS can quantify heavy metal ions at trace concentration in food[11] and environmental samples[12], thus it is usually used to compare with other novel methods[13]. In addition, AAS is widely used as the standard method for metals test. For example, cadmium ions in samples were extracted using artificially synthesized nanocomposites and then detected by atomic absorption spectroscopy with detection limits under nanomolar level[14]. However, these methods have disadvantages such as high cost, high technical difficulty, time-consuming sample pretreatment, inability to perform real-time detection, and the need for precision instruments[15–17].

A method based on the circularly permuted fluorescent protein (cpFP) gene-encoded fluorescent indicator can obtain a highly responsive sensor to make up for these shortcomings[18, 19]. The construction of the SoNar sensor is to detect NADH by inserting cpFP inside the Rex protein[20].
To generate a high response to mercury ions, the GEIM sensor inserts cpFP near the main binding site of mercury ions in the MerR protein[21]. The two ends of the cpFP, which is sensitive to small conformational changes and has a large fluorescence change range[19], are connected to the binding protein that can bind a specific substance through a linker to obtain a new fluorescent protein so that the specific substance is bound to the binding protein and the conformation changes. The binding protein can easily drive the conformational change of the fluorescent protein, thus producing fluorescence changes[20, 21].

Therefore, To obtain a sensor that is highly responsive to trace amounts of cadmium ions, the anti-cadmium operon CadR protein from Pseudomonas putida is a promising candidate for constructing a cadmium-specific sensor[22, 23]. CadR is a homodimer composed of a DNA binding domain, coiled coil domain, and a metal-binding domain (MBD). The unique triangular planar coordination formed by the three conserved cysteine residues of two monomers provides high selectivity and sensitivity to cadmium binding[22]. Under cadmium exposure, large conformational changes will occur in the MBD[24].

In the present study, we fused cpFP with the MBD of CadR to develop genetically encoded biosensors for inorganic cadmium. Then, we proved their excellent reversibility, high dynamic range, specificity for cadmium detection, and studied the response kinetics of cadmium in E. coli, which was helpful in determining the content of cadmium in vivo.

### Materials and methods

#### Materials and reagents

CdCl₂, CoCl₂·6H₂O, HgSO₄, CuCl, and meso-2,3-Dimercaptosuccinic acid (DMSA) were all purchased from Macklin (Shanghai, China). KOD FX was purchased from Toyobo (Shanghai, China), and homologous recombinase was purchased from Clone Smarter (USA). T4 PNK ligase was purchased from Takara (Japan). In this study, other unspecified reagents were of analytical grade. CadR (NCBI: AF333961) was synthesized by GENEWIZ (Suzhou, China). pRSETB was from Invitrogen.

#### Construction of cadmium sensor

We ligated CadR[22] to pRSETB by homologous recombination, and then performed reverse PCR between the C112 to C119 amino acids of pRSETB-CadR (Table S1), and using the same method amplified cpYFP[25] with the homology arms at both ends of the insertion point of CadR the linkers SAG and GTG were added[26] (Table S2). Finally, the homologous recombinase was used for ligation for 15 min at 50 °C, and then transferred to Escherichia coli Top10 competent strain for amplification, and then extracted and sequenced. The plasmids identified by sequencing were introduced into E. coli BL21(DE3) competent cells, incubated overnight at 37 °C, a single colony was picked to inoculate 100 ml of LB medium, and 0.1 mg/ml of ampicillin was added. Following culture for 8–12 h at 37 °C and 220 rpm to an OD₆₀₀ of 0.4–0.6, 1 mM Isopropyl-beta-D-thiogalactopyranoside was added and incubated after 20–24 h at 18 °C. The fermented strains were collected into HEPES buffer (100 mM HEPES, 100 mM NaCl) via ultrasonication, and the cell lysate supernatant was diluted with HEPES buffer, 0.5 μM, and 5 μM CdCl₂ were added, and the fluorescence intensity was immediately detected at F₄₈₅/₅₂₈ and F₄₂₀/₅₂₈.

To further optimize the cadmium sensor, we firstly knocked out the N-terminal methionine of cpYFP, and then used traditional truncation methods to shorten the amino acid linker between cpYFP and CadR to improve the response of CadR₁₆ (Table S3 and S4). First, we used reverse PCR to remove the amino acid linker at the N-terminus of cpYFP in CadR₁₆. The C-terminal linker was then reduced by the same method. In our nomenclature, N and C are the abbreviations for N-terminal and C-terminal, respectively. Therefore, N1C1 means that one amino acid at the N-terminal and C-terminal of the cpYFP linker have been removed from CadR₁₆. All these truncated mutants were screened as described above. In addition, we changed the key cysteines C77, C112, and C119 of N0C0 to serine by site-directed mutagenesis (Table S5).

#### Protein expression and purification

We dissolved E. coli containing N0C0, N1C1, and C112S proteins in HEPES buffer and sonicated them on ice. These fusion proteins all had 6x His tags when they were constructed, and were purified by nickel-column affinity chromatography (Cytiva) on the AKTA pure system. The eluents were incubated with 10 mM EDTA at 4 °C to remove possible metal ions, such as nickel. Then, indicator proteins were concentrated using the Amicon Ultra centrifugal filter device (Millipore). A 5 ml desalting column (Cytiva) was then used to remove the imidazole and chelating agent contained in it, and was equilibrated with HEPES buffer (100 mM, 100 mM NaCl, pH 7.4) and then HEPES buffer (10 mM, 100 mM NaCl, pH 7.4). Protein concentrations were determined by the Bradford method using Coomassie Protein Assay Reagent with bovine serum albumin as the standard.
In vitro characteristics of the cadmium sensors

For spectrum measurement, the purified 0.2 µM N0C0 and N1C1 were added with or without 0.5/5 µM CdCl₂, and a microplate reader (TECAN infinite M200) was then used to measure the absorption spectrum at room temperature. The extinction coefficient was calculated with the Beer-Lambert equation according to the absorption spectrum. The fluorescence spectrum was detected with the emission wavelength fixed at 530 nm and the excitation wavelength at 400–550 nm; the fluorescence wavelength was fixed at 485 nm, the emission wavelength was 485–600 nm, and the scanning interval was 1 nm. To determine the quantum yield of purified N0C0 and N1C1, excitation was performed at the ultraviolet absorption peak, the emission spectrum was measured with a fluorescence spectrophotometer (Shimadzu RF-6000), and the integrated fluorescence value was calculated. EGFP (QY 0.60, pH 7.4) was used as a control to calculate the quantum yield of N0C0. Similarly, the quantum yield of N1C1 was measured and calculated using the Brightness and Fluorescence Changes[28].

For all microplate experiments, the recombinant protein was diluted to a final concentration of 0.1–0.2 µM, and the fluorescence was detected using the same settings as those for screening. In the Cd²⁺ titration experiment, 100 µL of different concentrations of Cd²⁺ and 100 µL of protein were mixed in a 96-well flat-bottomed plate, and the fluorescence change was immediately measured. All fluorescence intensities were normalized to a signal of 1 in the absence of cadmium at pH 7.4 and data were fitted to the Hill1 equation[29].

To determine the specificity of N0C0 and N1C1 against other ions, 100 µL of buffer containing different ionic components and 100 µL of purified protein were used for the reaction with or without 0.5/5 µM CdCl₂. The ion concentrations are listed below: 300 mM Na⁺ or K⁺, whereas other ions were 100 µM (Fe³⁺, Mg²⁺, Ca²⁺, Ni²⁺, Mn²⁺, Fe²⁺, Li⁺, Cs⁺, Ba²⁺, Co²⁺) or 0.5 µM (Ag⁺, Zn²⁺, Cu²⁺, Pb²⁺, Hg⁺, Cu⁺). To determine the sensitivity of N0C0 and N1C1 to temperature, a 25–40 °C temperature program in a microplate reader was performed and the fluorescence change was detected every 20 s. To determine the dependence of N0C0 and N1C1 on the pH value, HEPES buffer with a pH value ranging from 6.8 to 8.4 was prepared at 0.2 pH unit intervals.

At the same time, to study the in vitro response kinetics of N0C0 and N1C1, 0.5/5 µM Cd²⁺ and 2 mM DMSA were sequentially added, and the fluorescence changes were monitored every 20 s.

Measuring inorganic cadmium in E. coli

E. coli cells after fermentation were washed and dissolved in HEPES buffer, the bacterial solution was diluted to an OD₆₀₀ of 1, the microplate reader was set to 37 °C and incubated for 5 min, and 0.5/5 µM Cd²⁺ and 2 mM DMSA were sequentially added. The fluorescence changes were detected every 60 s.

Data analysis

Unless otherwise specified in this study, data processing was normalized, and the ratio of excitation at 485 nm, excitation at 420 nm, and emission at 528 nm (R₄₈₅/₄₂₀) are presented. The data are presented as a representative example of a single experiment repeated three or more times. The data obtained are expressed as mean ± SD or mean ± SEM.

Results and Discussion

Synthesis of cpFP-based cadmium sensor

To obtain a cadmium ion sensor, we firstly amplified CadR onto the vector pRSETB and then inserted cpYFP between the key residues Cys112 and Cys119 of the MBD domain of the CadR protein with two flexible linkers, SAG and GTG, by homologous recombination; thus, 28 chimeras were produced (Fig. 1a).

After fermenting and expressing each chimera, the chimera (CadR₁₆) with cpYFP inserted between the Ala114 and Ala117 of the MBD showed an approximately 200% increase in the ratio of fluorescence when excited at 485 nm and 420 nm when 5 µM Cd²⁺ was added (Fig. 1b). Compared with the other 27 chimeras, CadR₁₆ had a higher
response level to Cd^{2+}, so we used CadR_{16} as our subsequent optimization model.

Therefore, to further expand the dynamic range of the detection of cadmium ions, we deleted the starting amino acid methionine of cpYFP, and based on this, reduced the number of amino acids in the linker, and constructed a total of 16 truncation variants (Fig. 1c). We used the same method and found that N0C0/N1C1 in the presence of 0.5 µM/5 µM Cd^{2+}, when excited at 485 nm and 420 nm, showed an approximately 230% increase and 167% decrease in fluorescence, respectively (Fig. 1d). Compared with the FRET model Met-cad 1.57 cadmium ion sensor, N0C0 and N1C1 suggested a larger dynamic range[30].

After the three key cysteine residues (C77/C112/C119) were mutated to serine, no obvious fluorescence changes were observed for the non-functional control, under cadmium exposure (Fig. 1d). These results indicated that the response of N0C0 and N1C1 to cadmium ions was caused by the binding of cadmium to cysteine in the MBD domain of the CadR protein[24]. These data indicated that N0C0 and N1C1 are sensors with high response and high sensitivity to cadmium ions, and they are promising tools for in vitro detection.

**In vitro characterization of the cadmium sensor based on cpFP**

**Spectral and biochemical properties of cadmium sensor**

The spectral and biochemical properties of purified N0C0 and N1C1 in cadmium and cadmium-free environment were characterized. N0C0 and N1C1 had excitation peaks near 500 nm and emission peaks near 515 nm in the fluorescence spectrum, and absorption peaks near 410 and 500 nm in the ultraviolet spectrum, respectively (Fig. 2; Table 1). In addition, we measured the extinction coefficient and quantum yield of N0C0 and N1C1, the quantum yield of N0C0 and N1C1 was about 3.3% and 30% of EGFP when excited at 410 and 500 nm. The molecular brightness change in N0C0 and N1C1, defined as the ratio of the brightness change with or without Cd^{2+}, increased 229% and decreased 151%, respectively (Table 1).

**Sensitivity of cadmium sensor**

Protein titration experiments showed that by fitting the Hill1 equation, the apparent Cd^{2+} dissociation constants (K_{d}) of N0C0 and N1C1 were 0.12 µM and 0.10 µM, respectively (Fig. 3a-b; Table 1). Data of titration experiment were fitted using linear fitting model. N0C0 and N1C1 had a linear range from 0.01 µM to 0.05 µM and from 0.03 µM to 0.1 µM, and the limit of detection (LOD) was 10 nM and 33 nM, respectively. LOD of this sensor was compared with other methods, and the result was listed in Table 2. Although the LOD of this sensor was higher than some sensors reported previously but lower than the colourimetric,

| Sensor | CdCl_{2} (µM) | λ_{abs} (nm) | ε | λ_{em} (nm) | QY | Brightness | Change | K_{d} (µM) | LOD (nM) |
|--------|--------------|--------------|---|-------------|----|------------|--------|-----------|---------|
| N0C0   | -            | 410          | 18.8 | 511         | 0.0197 | 0.37 | 2.29 | 0.12 | 10       |
|        | +            | 499          | 10.6 | 517         | 0.1613 | 1.71 |
|        | +            | 410          | 16.7 | 512         | 0.0219 | 0.37 |
|        | +            | 499          | 20.2 | 516         | 0.1939 | 3.92 |
| N1C1   | -            | 412          | 18.7 | 511         | 0.0182 | 0.34 | 0.66 | 0.10 | 33       |
|        | +            | 497          | 13.5 | 517         | 0.1519 | 2.05 |
|        | +            | 412          | 28.4 | 513         | 0.0173 | 0.49 |
|        | +            | 497          | 12   | 517         | 0.1622 | 1.95 |

![Fig. 2](image-url) The optical properties of the cadmium sensor in vitro. (a and b) Fluorescence spectra of purified N0C0 (a) and N1C1 (b). The fluorescence spectrum intensity of purified and desalted N0C0 and N1C1 were measured under blank conditions and after the addition of 0 µM and 0.5 µM CdCl_{2}. The fixed wavelength of excitation was 485 nm, and the fixed wavelength of emission was 550/530 nm, and the scanning interval was 1 nm. The fluorescence maximum value was used for normalization. (c and d) UV spectra of purified N0C0 (c) and N1C1 (d). The purified N0C0 and N1C1 were scanned for UV spectra at 300–600 nm. The scanning interval was 1 nm.
In addition, N0C0 and N1C1 could detect Cd^{2+} at different temperatures (25–40 °C), which showed that they had temperature adaptability (Fig. 4a-b). Similar to other cpFP fluorescent probes\[19, 28\], the fluorescence of NOC0 and N1C1 was affected by pH. With an increase in pH, the fluorescence level of F_{485} increased continuously (Fig. 4c–d).

Kinetic studies showed that the addition of Cd^{2+} to NOC0 resulted in an immediate reaction and reached a maximum within 200 s (Fig. 5a), while N1C1 reached a maximum immediately (Fig. 5b). When DMSA was added, it responded immediately and returned to the normal level (Fig. 5a–b), which showed its reversibility in real-time detection.

The detection time of most methods listed in Table 2 was more than 10 min. The detection time of whole-cell cadmium sensors p2T7RNApmt-68 and TCM constructed using CadR was more than 8 h [35, 42], while NOC0/N1C1 could be performed within 5 min. This indicated that NOC0/N1C1 exhibited a faster detection speed.

Sensitivity of the cadmium sensor to cadmium in E. coli

The cadmium sensing properties of NOC0 and N1C1 expressed in E. coli was investigated. Similarly, titration experiments with E. coli containing NOC0 and N1C1 were carried out. The 0.5/5 µM Cd^{2+} was added to E. coli firstly, and then the final concentration 2 mM DMSA was added. The NOC0 E. coli cells did not respond to cadmium ions in both the titration and kinetic experiments (Fig. 5c and e). The N1C1 E. coli cells showed similar responsiveness to different cadmium ion concentrations in the titration experiment as the purified protein (Fig. 5d). The N1C1 E. coli cells supplemented with Cd^{2+} produced changes in fluorescent signals. N1C1 continued to combine with Cd^{2+} until DMSA was added to combine with Cd^{2+}, thereby releasing part of the sensor (Fig. 5f).

Compared with the titration solution, the different responses of NOC0 and N1C1 to Cd^{2+} may be attributed to the limited entry rate of cadmium through thick cell walls and plasma membranes, as well as the influence of E. coli cadmium resistance and adsorption capacity to cadmium ions\[43\].

Conclusions

In summary, we engineered genetically encoded yellow fluorescent indicators based on the gene encoding of P. putida CadR by constantly changing the insertion point strategies and optimization methods of deleting amino acid linkers. A total of 28 chimeras and 16 deletions were constructed. Their properties as purified proteins were characterized to measure the changes in inorganic cadmium in vitro.
Further, N0C0 and N1C1 displayed the potential to be used in the real-time, quantitative measurement of single cells and subcellular trends. The detection of Cd\(^{2+}\) content in living organisms is very useful for research on the toxicity of cadmium in living cells or in vivo[44]. Moreover, CadR is a class of proteins belonging to the MerR family[45, 46]. This indicator can provide a theoretical basis for the detection of metal ions in other MerR families, including Zn\(^{2+}\), Cu\(^{2+}\), Pb\(^{2+}\).

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**Authors’ Contributions** Shulin Hu conducted the experiment and drafted the manuscript, Jun Yang analyzed the results, Anqi Liao experiment assisted. All authors read and approved the final manuscript.

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**Data Availability** All data generated or analyzed during this study are included in this published article and its supplementary information.

**Declarations**

**Conflict of interest** The authors have no relevant financial or non-financial interests to disclose.

**Ethics Approval** Not applicable.

**Consent to Participate** Not applicable.

**Consent to Publication** Not applicable.

**Conflicts of interest/Competing Interests** The authors declare no conflicts of interest or competing interests.

**Note** Photophysical properties of N0C0/N1C1 with or without cadmium were measured at room temperature. Extinction coefficients (\(\varepsilon, \text{mM}^{-1} \cdot \text{cm}^{-1}\)) were calculated from absorbance (abs) spectra. QYs of N0C0 and N1C1 were measured against EGFP at pH 7.4 (QY 0.6). Brightness is defined as the product of extinction coefficient and quantum yield. Experimental data were fitted to Hill1 equation.

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Fig. 5 Monitoring Cd\textsuperscript{2+} dynamics in vitro and in living bacteria. (a and b) Kinetics of fluorescence response of purified N0C0 (a) and N1C1 (b) to the sequential addition of CdCl\textsubscript{2} and DMSA. (c and d) Fluorescence response of N0C0 (c) and N1C1 (d) in \textit{E. coli} BL21(DE3) treated with exogenous CdCl\textsubscript{2}. Cells were treated with different concentrations of CdCl\textsubscript{2} for 30 min at 37 \textdegree C. (e and f) Fluorescence change of N0C0 (e) and N1C1 (f) in response to 0.5/5 \textmu M CdCl\textsubscript{2} and its chelator DMSA (2 mM) in \textit{E. coli} BL21(DE3)

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