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Genetically encoded red fluorescent copper(I) sensors for cellular copper(I) imaging

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ABSTRACT

Copper ranks among the most important metal ions in living organism, owing to its key catalytic effect in a range of biochemical processes. Dysregulation of *in vivo* copper(I) metabolism is extremely toxic and would cause serious diseases in human, such as Wilson's and Menkes. Thus, it would be highly valuable to have a proper approach to monitor the dynamics of copper(I) *in vivo*, as it is directly related to the onset of human copper(I)-related diseases. Under these circumstance, developing fluorescent protein based copper(I) sensors is highly demanded. However, these established sensors are mostly based on green or yellow FPs. Fluorescent copper(I) sensors with a spectra in the red range are more desirable due to lower phototoxicity, less auto-fluorescent noise and better penetration of red light. In the present work, we grafted a special red FP into three different location of a copper(I) binding protein, and generate a series of red fluorescent copper(I) sensors. Despite their limited *in vivo* sensitivity toward copper(I), these sensors are viable for cellular copper(I) imaging. Furthermore, these red fluorescent copper(I) sensors are a good starting point to develop superior copper(I) biosensors capable of imaging copper(I) fluctuations within a truly biologically relevant concentration, and further effort to realize this endeavor is under way.

1. Introduction

Copper, as a trace element, is an essential metal ion that is required by nearly every living organisms, because its biochemical role as catalyst in a variety of biological processes, ranging from oxygen transportation, hormone maturation, signal transduction etc [1,2]. However, an excess of copper is extremely toxic, due to unfaithful competition for proteinaceous metal binging site and excessive ROS resulted from some copper catalyzed reactions [3]. As such, failure to properly control copper homeostasis could have fatal consequence and result in severe disease in human. Understandable, the development of methods to imaging *in vivo* copper is a reasonable pursuit, as this would lead to extra knowledge regarding the onset of copper-related disease in human, which would in turn provide new insights for human health.

A number of synthetic copper sensors have been developed [4–8], but most of these sensors lack a high enough affinity for copper to compete with intrinsic metalloproteins. Besides, specificity, biocompatibility, and stability are all issues yet resolved with synthetic sensors. Hence, it is much needed that alternative copper imaging methodology could be developed.

Fluorescent protein based sensors have been tremendously successful in indicating cellular metal ions metabolism due to their genetic encodability and good readability [9], ever since Dr. Roger Tsien and colleagues pioneered the field with Cameleons [10,11] for Calcium sensing. Encouraged by these pioneering researches, we and others have developed FP based copper(I) sensors which are suitable to dynamically imaging copper(I) in live cells [12–15]. These copper(I) sensors demonstrate a good sensitivity and selectivity for copper(I) imaging in cell, but these sensors are mostly constructed on green or yellow FP. So, phototoxicity, confusion with auto-fluorescence, and tissue obstruction are all potential issues to be addressed with short wavelength light [16]. As a result, it could be advantageous to construct a fluorescent copper(I) sensor with a red-shifted spectra. The red FP templates in other reported red fluorescent metal ion sensors could be a reasonable scratch line to engineer a red FP based copper(I) sensor, as these RFP templates should already possess the right general properties to be considered in a biosensor, such as photostability, brightness, maturation speed etc. Here, we adopted the red FP template developed by Dr. Robert Campbell and co-workers [17,18], and sub-cloned this RFP into three different location of a copper(I) binding protein Amt1, to deliver a series of red fluorescent copper(I) sensor.

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2. Materials and methods

2.1. Chemicals

NaCl, KCl, $ZnCl_2$, $CaCl_2$, $MgCl_2$, $NiCl_2$, $MnCl_2$, $CdCl_2$, $CoCl_2$, $FeCl_2$, $FeCl_3$, $AgNO_3$, $CuSO_4$, were from Shanghai Zhenxin Reagent Company. NaCN was from Dr. Jinlin Zou's lab at Department of Chemistry, Nanjing University. Tetrakis(acetonitrile) copper(I) hexafluorophosphate was from J&K Chemical Ltd.

2.2. Construction of expression plasmids and preparation of sensor proteins

The red fluorescent protein was sub-cloned into three different position of copper binding domain of Amt1 using AVAI respectively, which is between residue 18 and 19, between residue 41 and 42, between residue 59 and 60. Sensors are referred to as AR-18, AR-41 and AR-59, for RFP after residue 18, 41 and 59 of Amt1. All the genes were cloned into pQE80L plasmid between BamHI and KpnI sites.

2.3. Expression plasmids were transformed into Escherichia coli XL1-Blue

Each clone harboring a corresponding plasmid was grown in 2.5% LB medium containing 100 mg/L ampicillin under vigorous stirring and induced with 0.5 mM IPTG when its optical density at 600 nm reached 0.8. Protein expression continued for 8 h at 20 centigrade. Bacterial cells were harvested by centrifugation and resuspended in pre-cooled lysis buffer (20 mM Na_3PO_4, 500 mM NaCl, 4 mM DTT, pH 7.2) further lysed by sonication of 15 min. Then the cell lysate was centrifuged at 12,000 rpm for 25 min at 5 centigrade. The protein is purified using Ni^{2+}-NTA protein resin and eluted in elution buffer (20 mM Na_3PO_4, 500 mM NaCl, 500 mM imidazole, 4 mM DTT, pH 7.2).

2.4. Fluorescence measurement

The fluorescence measurement was done on a fluorescence spectrophotometer (JASCO FP-6500), with an excitation wavelength of 550 nm. Proteins' concentration was kept at 1 μM for all measurements. The molar ratio of copper(I) over sensor proteins were set at 2 and 5. The excitation and emission bandwidth were 3 and 5 nm, respectively.

The normalized fluorescence intensity refers to the fluorescence of the sensor at various wavelength divided by the maximum fluorescence intensity.

2.5. UV–Vis measurement

UV–Vis spectra of sensor protein in the absence and presence of copper(I) were taken using a UV/Vis spectrophotometer (Jasco, V-550). The sensors were kept at 20 μM in a buffer containing 200 mM Na_2H/NaH_2PO_4, 4 mM DTT (pH 7.2). For the measurement of sensor bound with copper(I), additional 100 μM copper(I) was present.

2.6. Determination of Cu(I) dissociation constant

To estimate the dissociation constant of copper(I) to sensors, 1 μM holo sensor proteins were prepared. Then different amount of NaCN is added into the samples to displace the bound Cu(I), thus returning the fluorescence. The equilibrium constants in this solution are based on NIST Critical Stability Constants of Metal complexes. And the log beta of sensors with Cu(I) was estimated using HySS2009 software.

2.7. Mammalian cell fluorescence imaging

Human embryonic kidney 293T cells were incubated at 37 centigrade under 5% CO_2 in DMEM (Hyclone) supplemented with 10% FBS. Then, the cells were seeded overnight into culture chambers to obtain 85–90% confluency. The following day, the cells were transfected with pcDNA3.1(-) carrying AR-18 or AR-41 or AR-59 or RFP template by using lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After allowing a 36 h expression period, the cells were used for imaging. Images were taken with a con-focal microscope (Zeiss, LSM710) equipped with a 100-fold objective, NA 1.35.

3. Results and discussion

3.1. Design and construction of a new family of copper(I) sensors

To extend the spectra range of copper(I) sensor, we borrowed a red FP which was previously used as a template in a calcium sensor [17], and utilized the same copper(I) binding protein Amt1 as in last generation of copper(I) biosensors [12,13]. Given this red FP template being a circular permutation of original mApple, we initiated the sensor design using a strategy different from that we had used previously. Instead of inserting Amt1 into the RFP, we sub-cloned this RFP into Amt1, so as to circumvent the disadvantage of declined stability of CP construct (Fig. 1). And we envisioned that split Amt1 would reassemble in the presence of copper(I), owing to the binding of copper(I) and the formation of a tetracopper(I) cluster [19]. This enormous conformational change

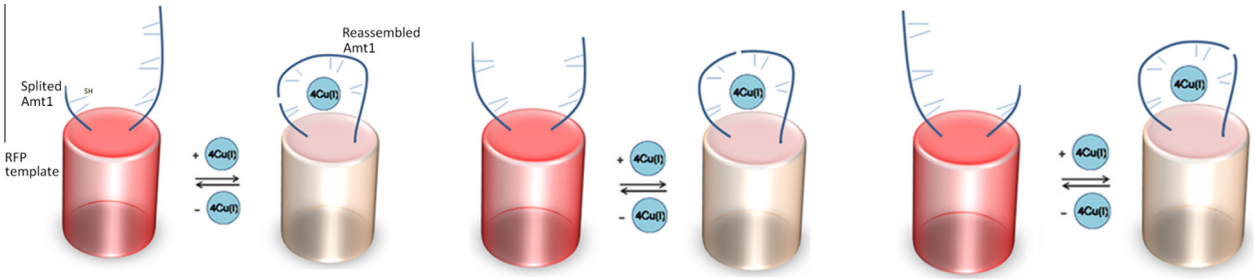

Fig. 1. Schematic of design concept for this series of copper(I) sensors. RFP template was sub-cloned into three different position of Amt1, the resulted sensors were named AR-n, where n stand for the residue in Amt1 after which RFP was inserted. Cloning site was varied to screen for the different sensitivity and *in vivo* response time. The presence of copper(I) mediate the forming of a tetracopper(I) cluster and reassembly of splited Amt1, the intra-molecular rearrangement of which would lead to a corresponding fluorescence drop.