

Original article:

**POLYACRYLAMIDE HYDROGEL ENCAPSULATED *E. COLI*
EXPRESSING METAL-SENSING GREEN FLUORESCENT PROTEIN
AS A POTENTIAL TOOL FOR COPPER ION DETERMINATION**

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ABSTRACT

A simple, inexpensive and field applicable metal determination system would be a powerful tool for the efficient control of metal ion contamination in various sources e.g. drinking-water, water reservoir and waste discharges. In this study, we developed a cell-based metal sensor for specific and real-time detection of copper ions. *E. coli* expressing metal-sensing green fluorescent protein (designated as TG1/(CG)6GFP and TG1/H6CdBP4GFP) were constructed and served as a metal analytical system. Copper ions were found to exert a fluorescence quenching effect, while zinc and cadmium ions caused minor fluorescence enhancement in the engineered bacterial suspension. To construct a user-friendly and reagentless metal detection system, TG1/H6CdBP4GFP and TG1/(CG)6GFP were encapsulated in polyacrylamide hydrogels that were subsequently immobilized on an optical fiber equipped with a fluorescence detection module. The sensor could be applied to measure metal ions by simply dipping the encapsulated bacteria into a metal solution and monitoring fluorescence changes in real time as a function of the metal concentration in solution. The sensor system demonstrated high specificity toward copper ions. The fluorescence intensities of the encapsulated TG1/(CG)6GFP and TG1/H6CdBP4GFP were quenched by approximately 70 % and 80 % by a high-dose of copper ions (50 mM), respectively. The level of fluorescence quenching exhibited a direct correlation with the copper concentration, with a linear correlation coefficient (r) of 0.99. The cell-based metal sensor was able to efficiently monitor copper concentrations ranging between 5 μ M and 50 mM, encompassing the maximum allowed copper contamination in drinking water (31.15 μ M) established by the WHO. Furthermore, the cell-based metal sensor could undergo prolonged storage for at least 2 weeks without significantly influencing the copper sensitivity.

Keywords: green fluorescent protein, metal sensor, copper, optical sensor

INTRODUCTION

Copper is an essential trace element playing many vital roles in living organisms, being involved in processes such as electron transfer for cellular respiration, the initiation of neurotransmitter production, estrogen metabolism, and acting as a cofactor for oxidative scavenging enzymes (Kim et al., 2008). However, free copper ions are extremely toxic. Exposure to low dose copper ions (> 8 mg/l) causes the typical symptoms of food poisoning (headache, nausea, vomiting and diarrhea) (Abdul et al., 2012). High dose ingestion or prolonged exposure to copper ions produces severe anemia, neurodegenerative disorders and liver and kidney dysfunction (Ocaña et al., 2013). Copper exposure to the public often occurs through the waste products from several human activities, such as mining, agriculture and industry. Copper often contaminates drinking-water due to the corrosion of interior copper plumbing. Copper can experience prolonged accumulation in soil and water, allowing transfer to humans *via* the food chain. The World Health Organization (WHO) declared a maximum limit for copper ions in drinking water of up to 2 mg/L (31.15 µM) (Fitzgerald, 1998; Olivares et al., 1998). Therefore, early monitoring of copper contamination is necessary to control health risks and the environmental pollution caused by copper. The traditional quantification of copper and other heavy metals relies on atomic absorption spectroscopy, inductively coupled plasma mass spectroscopy or X-ray fluorescence spectroscopy (Beck et al., 2002). Unfortunately, these aforementioned techniques are sample destructive and normally limited to specialized laboratories due to their intricate operation procedures and sophisticated instrumentation. Therefore, many efforts have been geared toward the development of simple, inexpensive, portable and suitable devices for on-line metal monitoring.

Whole bacterial cells have become potential candidates for metal sensor development. Bacteria have been genetically

modified to carry metal reporter molecules, whose signals can be triggered or diminished by metal ions. Recently, fluorescent proteins from various organisms have been widely applied as potential reporters for metal ions, including green fluorescent protein (GFP) and its variants from the Pacific Northwest jellyfish *Aequorea victoria* (Barondeau et al., 2002; Isarankura-Nayudhya et al., 2010; Prachayasittikul et al., 2000; 2001; Richmond et al., 2000; Tansila et al., 2007); red fluorescent protein (DsRed), from the tropical coral *Discosoma* spp. (Eli and Chakrabartty, 2006; Sumner et al., 2006b); and luciferase, from the firefly (Jouanneau et al., 2012). Generally, whole cells carrying fluorescent proteins can sense metal ions based on 2 approaches:

- (1) a reporter gene is fused with an upstream metal-inducible promoter, which controls the production of the reporter protein upon activation by metal ions;
- (2) and a reporter protein autonomously expresses and interacts with metal ions, causing a change in the reporter protein signal.

Several metal-inducible promoters have been used to develop whole cell-based metal sensors by fusing with a downstream reporter gene. Examples of regulatory elements include *cusC*, *zraSR* (Ravikumar et al., 2012), *cadR* (Wu et al., 2009), *merR* (Branco et al., 2013; Pellinen et al., 2004; Roda et al., 2001; Rouch et al., 1995) and *arsR* (Jouanneau et al., 2012; Liao and Ou, 2005), which respond to copper, zinc, cadmium, mercury, chromium and arsenic, respectively. The detection limits using this approach are in the micromolar range. However, the implementation of this technique requires live organisms, which have specific environmental and energetic needs. Therefore, several attempts have focused on the utilization of direct interactions between metal ions and reporter molecules. GFP and DsRed are widely applied for the determination of various metal ions. Copper ions exert a strong quenching effect on the fluorescence of both GFP (Prachayasittikul et

al., 2000; Richmond et al., 2000) and DsRed (Rahimi et al., 2007, 2008; Sumner et al., 2006b). Millimolar levels of copper ions cause a nearly 100 % fluorescence loss. Minor fluorescence quenching of fluorescent proteins can also occur due to other heavy metals, including zinc, cadmium, nickel, iron and cobalt (Barondeau et al., 2002; Eli and Chakrabarty, 2006; Prachayasittikul et al., 2000). Notably, the introduction of a metal-binding motif into the GFP molecule has enhanced metal sensitivity. Chimeric GFP carrying N-terminal hexahistidine gives better performance for the quantification of copper, zinc, cadmium and nickel than does wild type GFP (Prachayasittikul et al., 2000). In the same manner, the introduction of 2 histidine residues in close proximity to the GFP chromophore enhances its sensitivity to copper ions. Thus, the detection limit in this approach has been improved from millimolar to micromolar levels (Richmond et al., 2000). GFP mutants (F165G) containing a solvent-exposed analytical channel on their topological surface exhibit the highest sensitivity toward copper ions at physiological pH, with a dissociation constant of $\sim 4.9 \mu\text{M}$ (Tansila et al., 2007). *E. coli* expressing GFP fused with an N-terminal cadmium-binding peptide have been applied for the determination of metal ions (Prachayasittikul et al., 2001). Cadmium and zinc ions enhanced the fluorescence emission of the engineered cells in a dose-dependent manner, while a rapid decline in fluorescence was observed in the presence of copper ions. The limit of detection was in the range of $0.5\text{-}5 \mu\text{M}$. Furthermore, *E. coli* co-expressing a surface exposed zinc-binding motif and hexahistidine-tagged GFP are able to monitor intracellular zinc ion mobility in real-time (Isarankura-Na-Ayudhya et al., 2005).

Whole cell-based metal detection is simple, inexpensive and field applicable. It is able to sense metal concentrations down to a few hundred nanomolar, which is sensitive enough to detect the maximum al-

lowed copper concentration in drinking water. However, whole cell-based metal sensors in suspension form may be inconvenient to handle and implement for online detection. Therefore, the immobilization of whole cells in a solid matrix may help to address this limitation. Bacteria have successfully been entrapped in various encapsulation materials, such as silica, polyacrylamide, calcium alginate, agarose, gelatin and liposomes. These can widely be applied for biocatalysis (Westman et al., 2012; Zajkoska et al., 2013), analytical (Ben-Yoav et al., 2011; Depagne et al., 2011) and agricultural (John et al., 2011) purposes. For example, *Moraxella* spp. cells expressing recombinant organophosphorus hydrolase on the cell surface have been encapsulated in a sol-gel and used as an organophosphate sensor. The organophosphorus hydrolase activity of the encapsulated cells remained stable for at least 20 days (Yu et al., 2005). Encapsulated engineered bioluminescent *E. coli* containing a stress responsive promoter displayed stable viability and luminescent activity for several months. They could be used either as disposable or multiple use sensors for sensing stress inducers (Premkumar et al., 2002). Metal-binding GFP has been encapsulated in a sol-gel and equipped with a fluorescence detection unit for use as a potential copper sensor (Isarankura-Na-Ayudhya et al., 2010). The encapsulation of GFP in a silica matrix does not significantly affect its fluorescence emission and stabilizes the GFP against proteases, heat and denaturants (Cai et al., 2011).

It is noteworthy that *E. coli* expressing metal-sensing GFP have not been integrated with a detection unit and applied for sensing metal ions while maintaining sample integrity. Therefore, this study aimed to develop a whole cell-based metal sensor by monitoring the fluorescence changes in *E. coli* expressing metal-sensing GFP in real time as a function of the metal ion concentration. The sensor was designed for ease of use and to provide a non-destructive sample platform. Engineered *E. coli* were encapsu-

lated in transparent matrixes, including a polyacrylamide hydrogel and a silica sol-gel, then incorporated into a fluorescence detection unit for the instantaneous detection of metal ions.

MATERIALS AND METHODS

Chemical and biological reagents

Escherichia coli strain TG1 (lac-pro), Sup E, thil, hsd D5/F'tra D36, pro A⁺ B⁺, lacI, lacZ, M15; (ung⁺, du⁻) was used as the bacterial host for the cloning and expression of the chimeric genes. pGFPuv (Clontech Laboratories, USA) served as the parent plasmid for the construction of the chimeric metal-sensing GFP. Restriction endonucleases, T4 DNA ligase and the Lamda DNA/HindIII marker were purchased from New England Biolabs Inc. (USA). 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), isopropyl-β-D-thiogalactopyranoside (IPTG), tetramethylorthosilicate (TMOS), acrylamide, N,N'-methylenebisacrylamide and hydrochloric acid were obtained from Sigma-Aldrich Chemical, Inc. (USA). Metals and other reagents were analytical grade and used without purification. Synthetic oligonucleotides were synthesized by the Bioservice Unit (BSU), National Science and Technology Development Agency (NSTDA), Thailand.

Construction of chimeric GFP providing avidity to metal ions

We developed a whole cell-based metal sensor using *E. coli* expressing chimeric metal-sensing GFP as a metal sensing element. Cysteine-rich GFP and histidine-rich GFP were constructed in the same way as previously described (Prachayasittikul et al., 2000). To construct cysteine-rich GFP, a pair of complementary oligonucleotides (5'AGCTTAATGAGCTCTTGTGGTTGTGGTTGTCCTTGTGGTTGTGGTGTGCGGTAC3' and 5'CGCACAACCACAACCACAAGGACAACCACAACCACAAGAGCCATTA3') encoding six repeats of a cysteine and glycine sequence were hybridized and inserted be-

tween the *Hind*III and *Kpn*I restriction sites of pGFPuv, resulting in the generation of a plasmid coding for cysteine-rich GFP, denoted as (CG)6GFP. Histidine-rich GFP, denoted as H6CdBP4GFP (a chimeric GFP carrying N-terminal hexahistidine in combination with 4 repeated cadmium-binding peptides) (Prachayasittikul et al., 2004), was applied for comparison. Plasmids coding for each chimeric GFP were transformed into *E. coli* strain TG1 and then cultivated in LB broth supplemented with 100 mg/l ampicillin. The expression of chimeric GFP was induced by the addition of 1 mM IPTG at the OD₅₅₀ of 0.1. The expression level could easily be monitored by following the fluorescence intensity of the engineered bacteria.

Fluorescence intensity of the metal-sensing bacterial suspension in the presence of metal ions

E. coli expressing either (CG)6GFP (TG1/(CG)6GFP) or H6CdBP4GFP (TG1/H6CdBP4GFP) were grown in LB broth supplemented with 1 mM IPTG overnight at 37 °C with an agitation of 150 rpm. The recombinant bacteria were harvested by centrifugation with a speed of 6,000 rpm at 4 °C for 5 minutes. The cell pellet was re-suspended and washed twice with HEPES buffer (5 mM HEPES, 0.85 % NaCl, pH 7.1). The suspension of engineered bacteria was freshly prepared to a concentration of 1.5×10⁸ CFU/ml. An aliquot of 100 μl of the bacterial suspension was incubated with an equal volume of Cu²⁺, Zn²⁺ and Cd²⁺ at concentrations ranging between 50 nM and 50 mM. The fluorescence emission in response to metal exposure was monitored with a 96-well plate fluorescence reader (FLx800 fluorometer, BioTek instruments, USA) using a 395 nm excitation filter and a 508 nm emission filter.

Encapsulation of E. coli carrying metal-sensing GFP

To prepare the metal-sensing bacteria for the encapsulation process, either

TG1/(CG)6GFP or TG1/H6CdBP4GFP was cultured and harvested as previously described. Afterward, each type of metal-sensing bacteria was separately encapsulated in 2 types of matrix, including a polyacrylamide hydrogel and a silica sol-gel. For the polyacrylamide encapsulation, the desired concentration of engineered bacteria was thoroughly mixed with 30 % bisacrylamide (20 μ l) and 1.5 M Tris-HCl buffer, pH 8.6 (37.5 μ l). Thereafter, 10 % ammoniumpersulfate (20 μ l) and *N,N,N',N'*-tetramethylethylenediamine (2 μ l) were added, and the solution was finally adjusted to a volume of 150 μ l with 18 M Ω water. The mixture was allowed to polymerize at room temperature for 30 minutes and subsequently washed twice and kept in HEPES buffer at 4 °C until use.

The sol-gel encapsulation was performed in a comparable manner as the polyacrylamide encapsulation. A sol-gel precursor was prepared by mixing concentrated TMOS (98 %) with H₂O and HCl at a molar ratio of 1:5:10⁻² (Kuncova et al., 2004). The mixture was vigorously mixed for 2 minutes by vortexing and then kept at 4 °C for at least 48 hours. The aged sol-gel precursor (37.5 μ l) was mixed with an equal volume of 0.02 N NaOH and immediately mixed with the desired concentration of bacterial suspension (75 μ l). The homogeneous mixture was allowed to solidify in a plastic cylinder mold at room temperature for 30 minutes. After twice washing, the sol-gel was stored in HEPES at 4 °C until use.

Configuration of the cell-based metal sensor

To fabricate the cell-based metal sensor, either the polyacrylamide or sol-gel encapsulated metal-sensing bacteria were placed into a Teflon holder (10 mm diameter and 10 mm length), which was then tightly fitted onto the tip of a Y-shape quartz core fiber optic bundle (5 mm core-diameter and 1 m length). The optical fiber was connected to the excitation and emission units of a

spectrofluorometer (SPEX-Fluorolog 1681, Edison, NJ). The excitation wavelength at 395 nm and the emission wavelength at 508 nm were transmitted between the spectrofluorometer and the metal-sensing compartment *via* the fiber optic bundle.

Optimization of the bacteria-based metal sensor

To achieve the highest metal detection performance, the bacteria-based metal sensor parameters were optimized, including the number of encapsulated bacteria, the thickness of the encapsulation matrix and the strength of the testing buffer. The optimal conditions were achieved by exposing the encapsulated metal-sensing bacteria to 5 mM Cu²⁺ and simultaneously monitoring the fluorescence intensity under a parameter of interest. The quantity of encapsulated metal-sensing bacteria was adjusted within the range between 1.5 \times 10⁵ and 1.5 \times 10⁸ CFU in 10-fold intervals, while the encapsulation thickness and buffer strength were fixed at 1.5 mm and 1x HEPES buffer (5 mM HEPES, 0.85 % NaCl, pH 7.1), respectively. The optimal encapsulation thickness was obtained by varying the thickness of the encapsulation matrix from 1.5 to 4.0 mm in 0.5 mm intervals while keeping the number of bacteria and buffer strength constant at 1.5 \times 10⁸ CFU and 1x HEPES buffer, respectively. In the same manner, the buffer strength was optimized by varying the HEPES buffer ingredients from a 0.25- to 1.5-fold concentration of normal HEPES buffer (1x). The experiments were repeated in triplicate, unless otherwise stated.

Metal selectivity of the bacteria-based metal sensors

The encapsulated metal-sensing bacteria (1.5 \times 10⁸ CFU at 1.5 mm polyacrylamide thickness) were incubated with 5 mM of various divalent metal ions (Cu²⁺, Zn²⁺, Ni²⁺, Ca²⁺, Mg²⁺ and Mn²⁺) for 60 minutes. The metal selectivity was evaluated by comparing the fluorescence intensity of the

encapsulated metal-sensing bacteria under exposure to each type of metal ion.

Determination of copper concentration by the bacteria-based metal sensor

To monitor copper concentrations, the encapsulated metal sensing-bacteria mounted on the optical fiber tip were dipped into 10 ml of HEPES buffer containing various concentrations of copper ions. The fluorescence intensity of the encapsulated bacteria in response to the metal ions was monitored in real time with an integrated spectrofluorometer for 60 minutes at 10 minute intervals. The measurement was performed under the optimal conditions, which comprised 1.5×10^8 CFU of encapsulated metal-sensing bacteria, a 1.5 mm thick polyacrylamide matrix and 1x HEPES buffer. The fluorescence intensity (F) of the encapsulated metal-sensing bacteria in response to the metal ions was expressed in relative fluorescence units (RFU) and derived according to the following equation:

$$F = \frac{F_t}{F_0}$$

where F_t stands for the fluorescence intensity at time t of metal exposure. F_0 refers to the initial fluorescence intensity at time zero of exposure.

Investigation of storage shelf-life

Forty-two samples of encapsulated metal-sensing bacteria (1.5×10^8 CFU at 1.5 mm polyacrylamide thickness) were freshly prepared and stored in 1x HEPES buffer at 4 °C for 14 days. On each storage day, triplicate encapsulated bacteria were selected and exposed to 5 mM Cu^{2+} for 60 minutes. The mean fluorescence of each sampling batch was plotted as a function of the storage duration to explore the stability and variability of the encapsulated metal-sensing bacteria in response to Cu^{2+} after storage.

Assessment of porosity of encapsulation materials via Atomic Force Microscopy (AFM)

The porosities of the polyacrylamide hydrogel and silica sol-gel were elucidated using atomic force microscopy (NanoscopeIIIa Bioscope: Digital instruments, Santa Barbara, USA). Topological images of the gel surfaces were acquired in contact mode using the optimum parameters, as previously described (Prachayasittikul et al., 2005). The mean depth of the surface pores was analyzed by the WSxM software (Nanotec Electronica, Madrid, Spain).

RESULTS AND DISCUSSION

Manifestation of E. coli expressing chimeric metal-sensing GFP

Histidine and cysteine amino acids are known to be strong ligands toward metal ions due to the formation of strong coordination bonds between metal atoms and the imidazole ring of histidine or the thiol side chain of cysteine. We harnessed this property to develop a series of chimeric GFPs for metal sensor applications. Herein, we successfully constructed a cysteine-rich GFP (termed (CG)6GFP) by genetically fusing six repeating cysteine-glycine sequences to the N-terminus of GFP (Figure 1). *E. coli* expressing (CG)6GFP (denoted as TG1/(CG)6GFP) exhibited fluorescence excitation and emission spectra corresponding to those of native GFP. Nevertheless, the introduction of multiple cysteine-glycine sequences caused approximately a 3-fold decrease in the fluorescence intensity of the cysteine-rich GFP compared to that of native GFP (data not shown). This finding agreed with our previous report on the fluorescent deterioration of GFP by an N-terminal foreign cysteine sequence (Isaranakura-Na-Ayudhya et al., 2009). On the contrary, the insertion of a histidine-rich sequence into the N-terminus of GFP has been shown to noticeably enhance the fluorescence intensity (Prachayasittikul et al., 2000; 2004). It has been observed that chimeric GFP carrying either hexahistidine

(H6GFP) or hexahistidine in combination with 4 repeating cadmium-binding peptides (H6CdBP4GFP) gives rise to up to 2-3-fold improved fluorescence emission compared to native GFP (Prachayasittikul et al., 2004).

Fluorescence changes in *E. coli* expressing metal-sensing GFP in the presence of metal ions

To further apply the *E. coli* expressing metal-sensing GFP as a cell-based metal sensor, the fluorescence emission under metal ion exposure was investigated. We observed that the fluorescence intensity of both TG1/(CG)6GFP (Figure 2A and B) and TG1/H6CdBP4GFP (data not shown, similar patterns can be obtained from TG1/(CG)6GFP data) were remarkably quenched by copper ions. The degree of fluorescence quenching was dependent on the copper concentration in the range from 0.5 μ M to 50 mM. Almost 100 % fluorescence quenching was perceived as a result of high copper concentration exposure (50 mM) for approximately 30 minutes. The dose-response curve at 60 minutes of incubation demonstrated a linear relationship between the copper concentration and the fluorescence quenching, with a correlation coefficient greater than 0.95. The quenching of fluorescence emission was possibly mediated by direct interactions between copper and the ground state fluorescent protein chromophore *via* a static quenching process (Isarankura-Na-Ayudhya et al., 2010; Rahimi et al., 2007). On the contrary, cadmium and zinc ions exerted a dissimilar effect compared to the copper ions. The fluorescence intensity of both TG1/(CG)6GFP (Figure 2C and D) and TG1/H6CdBP4GFP (data not shown) was considerably enhanced in response to high-dose cadmium and zinc exposure, in good agreement with previous work (Prachayasittikul et al., 2001). The enhancement of fluorescence was dependent on the cadmium and zinc concentrations, with correlation coefficients (r) higher than 0.98. This

phenomenon may be explained by the improvement in the cyclization process of the immature GFP chromophore upon exposure to metal ions (Heim et al., 1994; Phillips Jr, 1997). Furthermore, Prachayasittikul and colleagues (2004) have reported that chimeric GFP has a tendency to associate with lipid membranes, leading to a fluorescence decrease compared to the free protein.

Construction of bacteria-based metal sensor

To simplify the use of the detection system and enable its use in on-line monitoring, the *E. coli* expressing metal-sensing GFP were encapsulated in a matrix of either a polyacrylamide hydrogel or a silica sol-gel. TG1/H6CdBP4GFP was selected as a model bacteria for system optimization because it emitted brighter fluorescence than did TG1/(CG)6GFP. Various concentrations of polyacrylamide (4 %, 6 %, 8 %, 10 % and 12 %) were prepared for encapsulation of TG1/H6CdBP4GFP (1.5×10^8 CFU). The disc-like encapsulated bacteria were placed into a Teflon holder integrated with a fluorescence detection unit, as schematically depicted in Figure 3. To ensure good exposure of the metal solution to the

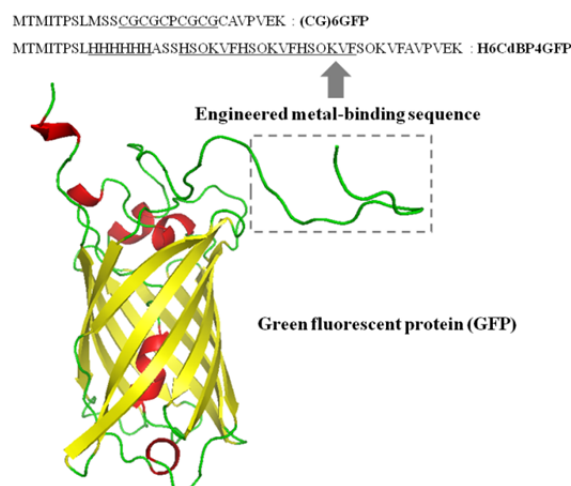


Figure 1: Structural model of the metal-sensing GFP. The model was constructed by the MODELLER protein modeling toolkit (Sali et al., 1995) <http://toolkit.tuebingen.mpg.de/modeller>

encapsulated bacteria, the Teflon holder was designed to possess an open orifice ($\varnothing = 5$ mm) at the distal end and 12 small holes ($\varnothing = 1.5$ mm) at the cylinder wall located around the encapsulated bacteria.

Fluorescence quenching of the bacteria-based metal sensor by copper ions

Upon dipping the sensor unit into a 5 mM Cu^{2+} solution, the fluorescence emission of the encapsulated TG1/H6CdBP4 GFP gradually declined as a function of exposure time. The degree of fluorescence quenching was retarded with an increasing percentage of polyacrylamide (Figure 4A). At 4 % polyacrylamide encapsulation, stable fluorescence quenching was accomplished within 60 minutes of incubation. A longer incubation was needed for higher

amounts of polyacrylamide. This might be because the increasing concentration of polyacrylamide led to the formation of a tightly cross-linked matrix, in which the exposure of the encapsulated bacteria to the metal solution was hindered. In addition to the polyacrylamide hydrogel, the use of a silica sol-gel as the encapsulation material for the cell-based metal sensor was also investigated. Silica sol-gel encapsulated TG1/H6CdBP4GFP (0.74 M TMOS precursor) showed a copper quenching pattern identical to that of 10 % polyacrylamide. Unfortunately, unstable and excessively fragile encapsulated TG1/H6CdBP4GFP were produced if the TMOS precursor concentration was lower than 0.74 M. Although a solid sol-gel could easily be produced by increasing the TMOS precursor,

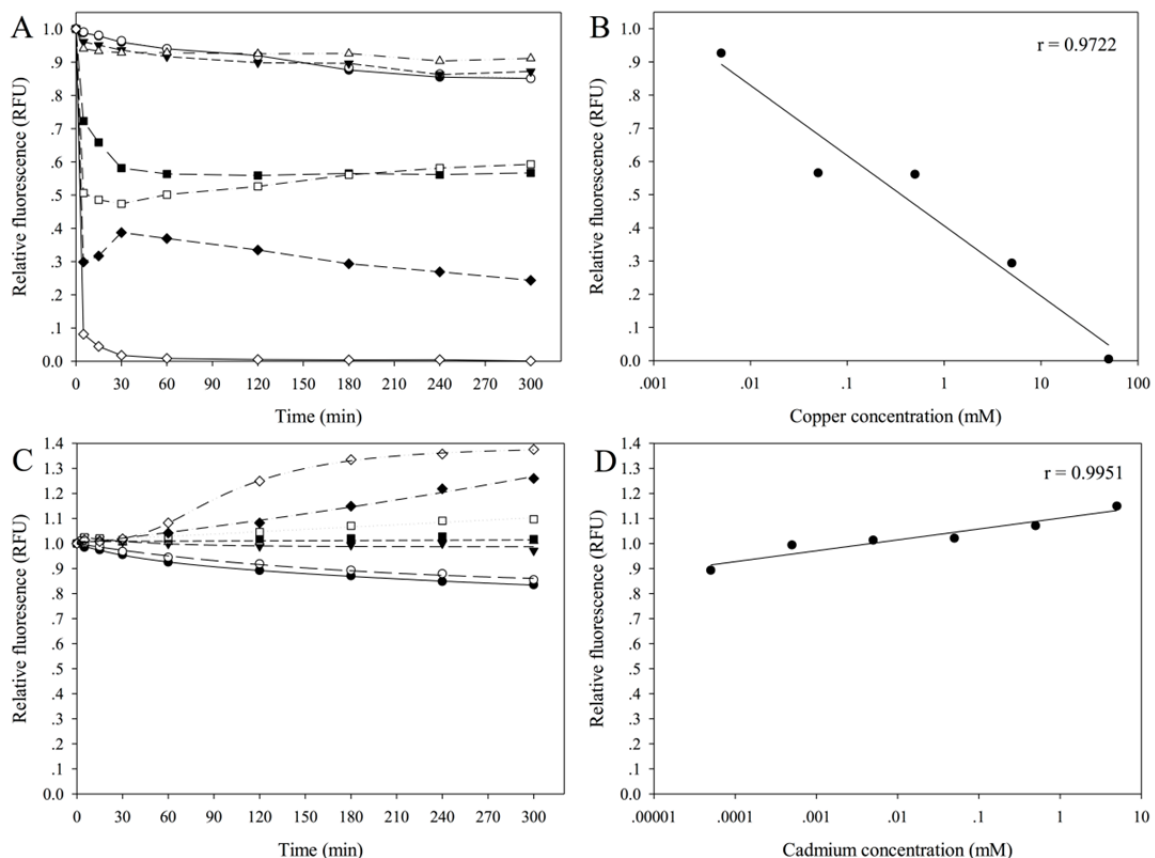


Figure 2: Time-course interaction between TG1/(CG)6GFP and various concentrations of copper (A) and cadmium (C) ions, including 50 nM (○), 0.5 μM (▼), 5 μM (△), 50 μM (■), 0.5 mM (□), 5 mM (◆), 50 mM (◇) and no metal (●). Dose-response curve of TG1/(CG)6GFP against copper (B) and cadmium (D) at 60 minutes of incubation.

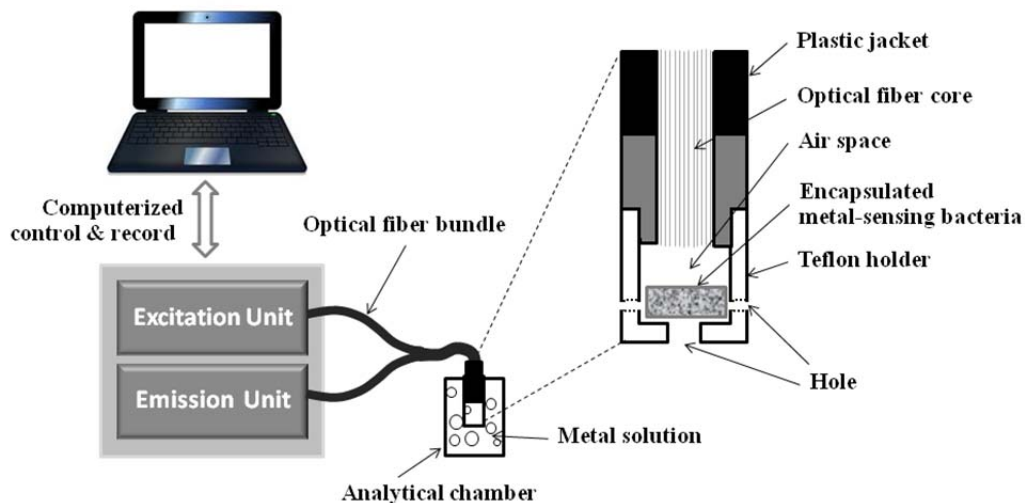


Figure 3: Schematic representation of the cell-based metal sensor system.

this might hinder diffusion of the metal solution to the encapsulated bacteria. Furthermore, surface topology analysis by atomic force microscopy (AFM) revealed that 4 % polyacrylamide encapsulation possessed an almost 3-fold higher degree of porosity than the sol-gel encapsulation, as represented by the 3-fold higher quantity of pores per unit length (Figure 5). The pore dimension in the 4 % polyacrylamide hydrogel was approximately 100 nm in depth and 1 μm in width, while the sol-gel encapsulation contained slightly larger pore dimensions (~250 nm in depth and 1.5 μm in width). This observation led to the conclusion that the higher pore density of the 4 % polyacrylamide hydrogel provided more solvent accessible area than did the sol-gel matrix. Therefore, the 4 % polyacrylamide hydrogel was selected as the most suitable encapsulation material.

Optimization of encapsulated bacteria-based metal sensor

To achieve the highest metal detection performance, the optimal conditions for the bacteria-based metal sensor were investigated. These conditions included the amount of encapsulated bacteria, the thickness of the encapsulation matrix and the ionic strength of the testing buffer. Various amounts of TG1/H6CdBP4GFP (1.5×10^5 ,

1.5×10^6 , 1.5×10^7 and 1.5×10^8 CFU) were encapsulated in a 4 % polyacrylamide hydrogel with a 2 mm thickness and subsequently exposed to 5 mM Cu^{2+} while simultaneously monitoring the fluorescence emission. The level of fluorescence quenching was found to correspond with the amount of encapsulated recombinant bacteria (Figure 4B). The encapsulation of TG1/H6CdBP4GFP at a level of 1.5×10^8 CFU provided the highest level of fluorescence quenching, while an almost undetectable fluorescence change was observed with 1.5×10^5 CFU of encapsulated bacteria. Most likely, lower amounts of encapsulated bacteria produced lower levels of fluorescence emission, which could not be differentiated from the background fluorescence. Furthermore, we can extrapolate from Figure 4B that increasing the amount of encapsulated bacteria above 1.5×10^8 CFU may not be necessary because the level of fluorescence quenching appears to be constant with 1.5×10^8 CFU of encapsulated bacteria. Therefore, the optimal number of encapsulated TG1/H6CdBP4GFP was selected to be 1.5×10^8 CFU. In addition, we also optimized the encapsulation thickness by encapsulating TG1/H6CdBP4GFP (1.5×10^8 CFU) in various thicknesses of 4 % polyacrylamide (1.5, 2, 2.5, 3, 3.5 and 4 mm). The fluorescence quenching in response to

5 mM Cu^{2+} was expressed in terms of T_{50} , where T_{50} refers to the time of Cu^{2+} exposure that produces 50 % fluorescence quenching in the encapsulated bacteria. The result revealed an exponential correlation between the encapsulation thickness and T_{50} (Figure 4C). We therefore concluded that a thinner encapsulation material offers higher sensitivity to metal ions. The optimal thickness for bacteria encapsulation was 1.5 mm. Because metal ions are positively charged, the ionic strength of the testing buffer may influence the metal sensing efficiency of the encapsulated bacteria. Therefore, the optimal buffer strength was explored by varying the HEPES buffer concentration in the range from 0.25x - 1.5x, where the 1x buffer comprised 5 mM HEPES and 0.85 % NaCl at pH 7.1. Varia-

tions in the buffer strength did not significantly influence the Cu^{2+} sensitivity of the encapsulated TG1/H6CdBP4GFP (Figure 4D). Therefore, 1x HEPES buffer was selected as the optimal testing buffer.

Selectivity of the bacteria-based metal sensor to copper ions

To investigate the application of the encapsulated bacteria as a sensor for a broad spectrum of metal ions, various types of metal ions (5 mM), including Cu^{2+} , Zn^{2+} , Ni^{2+} , Ca^{2+} , Mg^{2+} and Mn^{2+} were exposed to polyacrylamide encapsulated TG1/H6CdBP4GFP or TG1/(CG)6GFP for 60 minutes under optimal conditions (Figure 6). Both types of encapsulated bacteria showed high selectivity to Cu^{2+} ions. Cu^{2+} exerted approximately 60 % fluorescence quenching

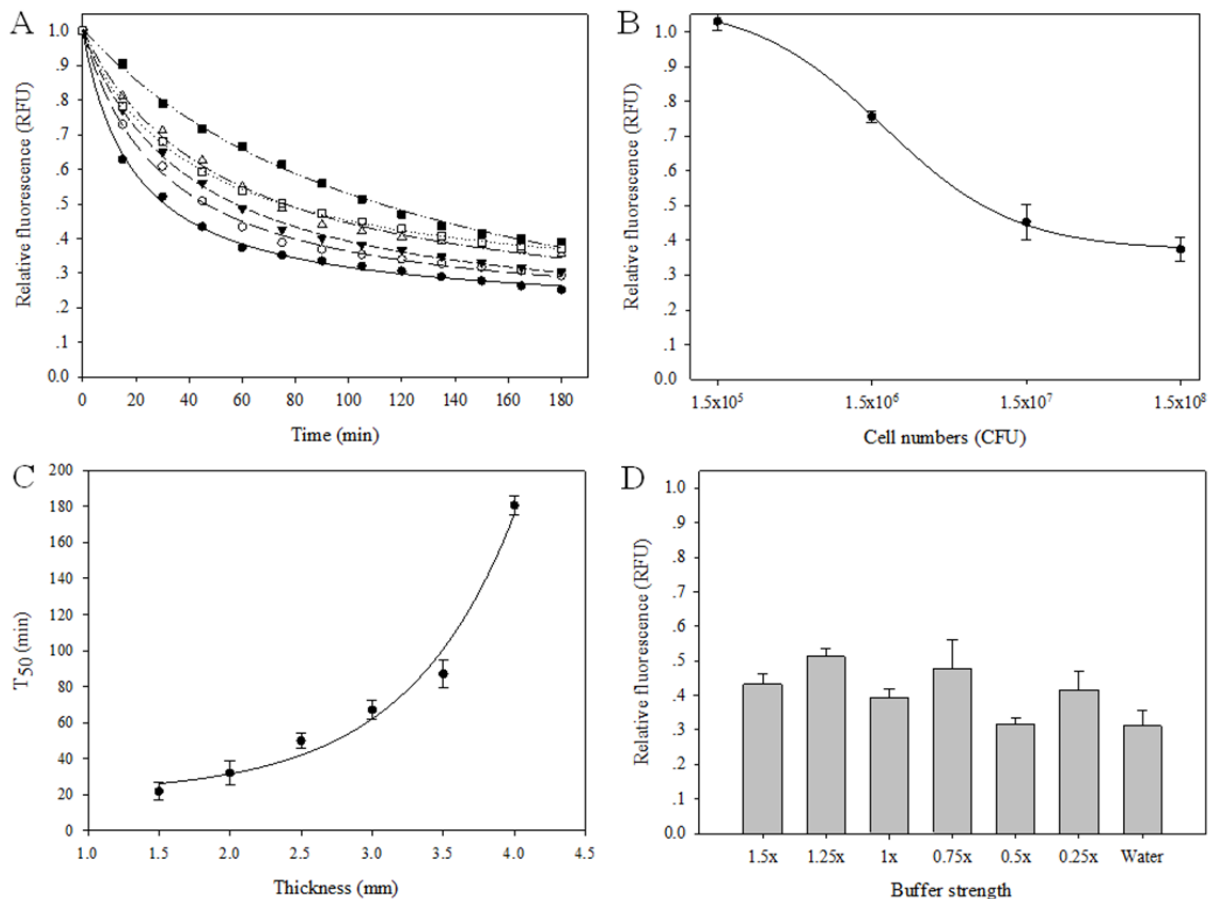


Figure 4: Optimization of the cell-based metal sensor.

(A) The encapsulation material, including 4 % (●), 6 % (○), 8 % (▼), 10 % (△), and 12 % (■) polyacrylamide and 0.74 M silica sol-gel (□); (B) the amount of encapsulated bacteria; (C) the thickness of the encapsulation matrix and (D) the ionic strength of the testing buffer. Notes: T_{50} was the Cu^{2+} exposure time that produced 50 % fluorescence quenching in the encapsulated bacteria. The 1x buffer strength consisted of 5 mM HEPES, 0.85 % NaCl, pH 7.1.

on both types of encapsulated bacteria. High selectivities of fluorescent proteins toward Cu^{2+} ions have also been observed in many previous studies (Isarankura-Na-Ayudhya et al., 2010; Rahimi et al., 2007; Sumner et al., 2006a; 2006b). The other metal ions did not significantly quench the fluorescence of the encapsulated TG1/H6CdBP4GFP, while Mg^{2+} , Mn^{2+} and Ni^{2+} slightly influenced the fluorescence of the encapsulated TG1/(CG)6GFP, with approximately 20 % quenching. In contradiction to the behavior observed in bacterial suspension systems, Zn^{2+} induced fluorescence enhancement of the encapsulated bacteria was not observed. This might have been because the polyacrylamide hydrogel reduced the quantity and diffusion rate of Zn^{2+} , which in turn reduced the degree of interaction between Zn^{2+} and the chimeric GFP. Furthermore, the acrylamide precursor might disrupt cellular processes involve in the Zn^{2+} -chimeric GFP interaction pathway.

Determination of copper concentration using the bacteria-based metal sensor

As a result of the high selectivity toward Cu^{2+} ions, dose-response curves between the Cu^{2+} concentration and the fluorescence intensity of the bacteria-based metal sensor were investigated. The fluorescence emission of the encapsulated TG1/H6CdBP4GFP rapidly decreased and remained stable within 30 minutes of Cu^{2+} exposure. The level of fluorescence quenching was closely associated with the Cu^{2+} concentration (Figure 7A). A similar pattern was observed for the encapsulated TG1/(CG)6GFP (Figure 7B). The dose-response curves at 30 minutes of Cu^{2+} exposure (5 μM - 50 mM) demonstrated excellent linearity, with correlation coefficients of 0.9951 and 0.9929 for the encapsulated TG1/H6CdBP4GFP (Figure 7C) and TG1/(CG)6GFP (Figure 7D), respectively. The detection limit was found to be 5 μM Cu^{2+} for both types of encapsulated bacteria. This sensitivity exceeds the maxi-

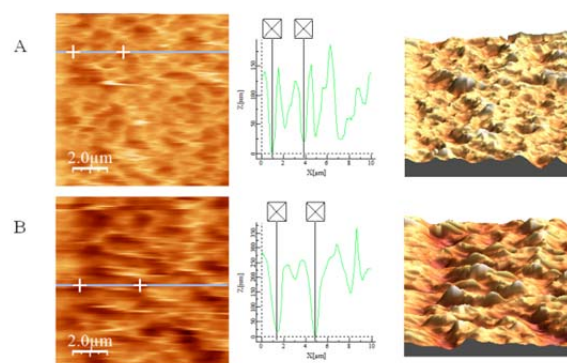


Figure 5: Porosity analysis and surface topography of 4 % polyacrylamide gel (A) and silica sol-gel (B).

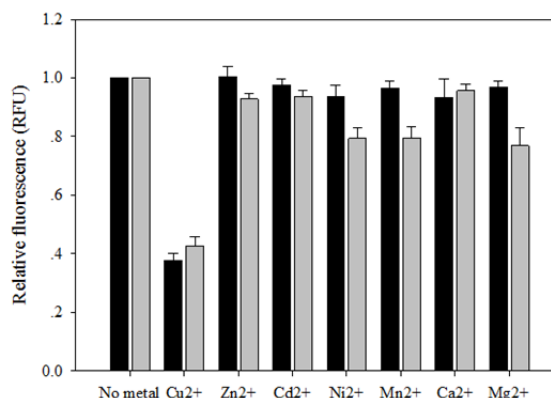


Figure 6: Effect of divalent cations on the fluorescence responses of encapsulated TG1/H6CdBP4GFP (black bars) and TG1/(CG)6GFP (grey bars).

mum allowed copper concentration in drinking water suggested by the World Health Organization (2 mg/l or 31.15 μM) and the US Environmental Protection Agency (1.3 mg/l or 20.25 μM) (Fitzgerald, 1998; Olivares et al., 1998).

Shelf-life of the bacteria-based metal sensor

The encapsulated TG1/H6CdBP4GFP was highly stable under storage conditions (kept in HEPES buffer at 4 °C). Throughout 14 days of storage, all batches gave equivalent fluorescence quenching in response to 5 mM Cu^{2+} compared with freshly prepared encapsulated TG1/H6CdBP4GFP (Figure 8). Encapsulation matrices have been proven to maintain the stability of enzymes and

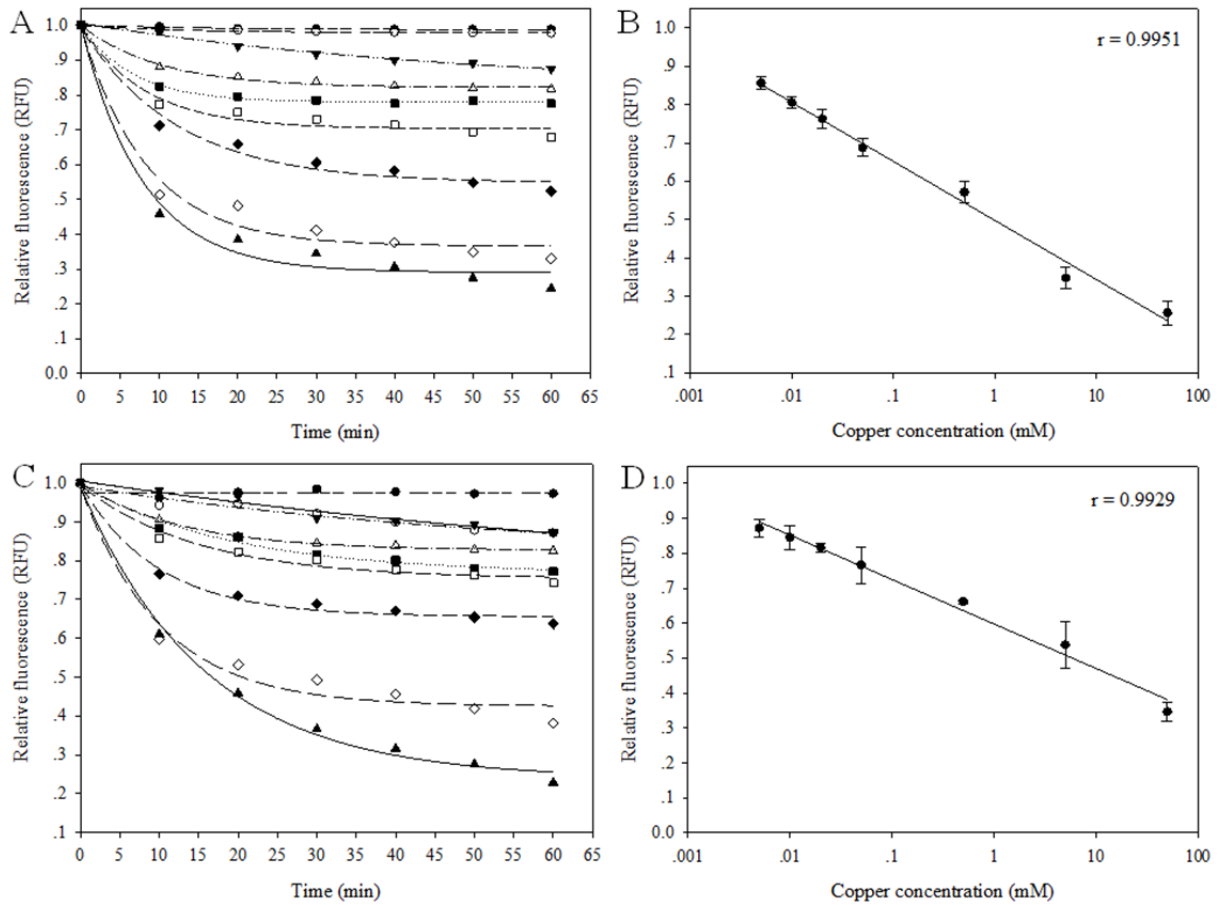


Figure 7: Time-course interaction (A, C) and dose-response curve (B, D) between copper ions, along with the fluorescence intensities of the encapsulated TG1/H6CdBP4GFP (A, B) and TG1/(CG)6GFP (C, D) at 30 minutes of incubation. Symbols represent Cu²⁺ at 50 nM (○), 0.5 μM (▼), 5 μM (△), 50 μM (■), 0.5 mM (□), 5 mM (◇), 50 mM (▲) and without Cu²⁺ (●).

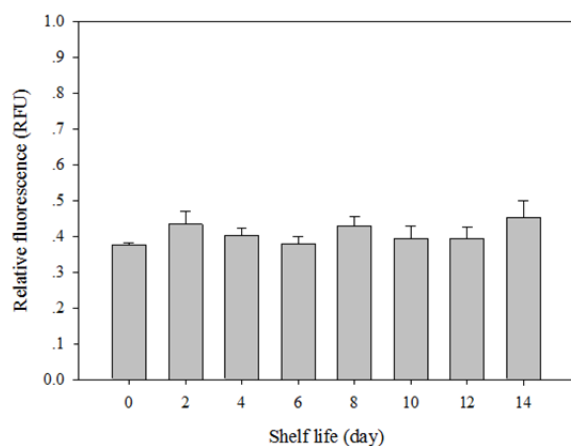


Figure 8: Fluorescence of encapsulated TG1/H6CdBP4GFP in response to 5 mM Cu²⁺ after storage for 2 weeks. The encapsulated bacteria were kept in HEPES buffer (5 mM HEPES, 0.85 % NaCl, pH 7.1) at 4 °C.

living cells in many reports. For example, the encapsulation of engineered bioluminescent *E. coli* containing a stress responsive promoter helped to maintain the viability and luminescent activity of the cells for several months. The sensor could be used either as a disposable or multiple use sensor for sensing stress inducers (Premkumar et al., 2002). The organophosphorus hydrolase activity of encapsulated cells was stable for at least 20 days (Yu et al., 2005).

CONCLUSION

This work demonstrates the successful development of a cell-based metal sensor as a reagentless sensing unit for the quantitation of copper ions. The detection system consists of encapsulated *E. coli* expressing

metal-sensing GFP in combination with a fluorescence detection unit. The fluorescence quenching of polyacrylamide encapsulated *E. coli* expressing either H6CdBP4 GFP or (CG)6GFP displayed a linear relationship with copper ion concentrations ranging from 5 μ M - 50 mM. This cell-based metal sensor was designed as a user-friendly and reagentless detection system. The encapsulated bacteria were observed to be efficient copper reporters, highly stable, easy to transport and manipulate and allow easy removal from the testing sample. Furthermore, GFP is an autofluorescent protein that needs no substrate or cofactor for fluorescence emission. Therefore, this cell-based detection system can potentially be applied to quantify copper ions by simply dipping the metal sensing unit into the analyte solution while simultaneously monitoring fluorescence changes in response to copper ions.

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