Determination of Gold ions in Human Urine Using Genetically Engineered Microorganisms on a Paper Device

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Determination of Gold ions in Human Urine Using Genetically Engineered Microorganisms on a Paper Device

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Key words: whole-cell biosensor, paper device, smartphone-based diagnostic system, cueR, Cupriavidus metallidurans

Supporting Information Placeholder

ABSTRACT: This paper presents a whole-cell biosensor that operates in conjunction with a smartphone-based fluorescence diagnostic system on a paper device to monitor the concentration of gold ions in human urine. The heavy metal-tolerant bacteria *Cupriavidus metallidurans* was genetically engineered for use as a chassis in a red fluorescent protein (RFP)-based microbial sensor. The biosensor is highly sensitive to gold ions, with a detection limit of 110 nM. The proposed smartphone-based analysis system provides a user-friendly approach to design tools of personal health monitoring for reporting the presence of gold ions in human urine.

The widespread use of gold compounds such as gold(I) tetraacetylthioglucose (auranofin) and sodium aurothiomalate for the treatment of rheumatoid arthritis, and gold nanoparticles in cancer imaging, drug delivery and disease treatments, has raised concerns with regard to bioaccumulation in the human body.[1-2] Previous studies showed that positive tests of gold ions in urine specimens of patients underwent treatments with aurothiomalate.[1-2] Therefore, the analysis of the gold content in physiological samples is an issue for the development of new type of medicines.[3-4] Inductively coupled plasma mass spectroscopy is one of the most common methods for quantitative metal ion detection but this technique requires the use of expensive instrumentation and laborious pretreatment of samples. The whole-cell reporter system offers an alternatives analytical method that was sufficiently simple and robust.[5-8]

Bacteria respond to environmental changes by sensing signaling molecules via a variety of transcriptional factors, which up-/downregulate distinct sets of target genes.[9-12] Whole-cell biosensors use transcriptional regulators capable of recognizing specific target molecules and inducing the expression of reporter proteins containing detectable, quantifiable output signals.[13] These devices have been developed for the detection of Hg, Cd, As, Pb, or Au ions in environmental samples.[14] Engineered microorganisms have proven highly effective in environmental analyses;[8, 15-21] however, their application in physiologically relevant samples is limited because selectivity and specificity of the biosensor in complex biological matrices remains a major problem for applications.

Most whole-cell biosensors use *Escherichia coli*, as a host, due to the availability of a large expression toolbox, which allows for genetic engineering of high complexity. Nonetheless, the low tolerance of *E. coli* to environmental stress has prompted researchers to adopt the agriculturally/medically relevant species for applications, such as soil bacterium, *Cupriavidus metallidurans* CH34.[7, 22-24] CueR protein is a transcriptional regulator that binds specifically to Cu(I), Ag(I), and Au(I) ions forming linear two-coordination geometries.[9, 25-26] The protein binds to the consensus sequences at the –35 and –10 regions in the promoters of genes to regulate the transcription. Previous biochemical studies have shown that CueR from *E. coli* provides two conserved cysteines (cys112 and cys120) [27] that bind to Cu, Ag, and Au ions forming linear two-coordination geometries. In this study, we therefore sought to develop a multifunctional biosensor for the detection of Cu, Ag, and Au ions in *C. metallidurans* through the expression of the CueR regulon from phylogenetically closely related species *Ralstonia eutropha* H16.

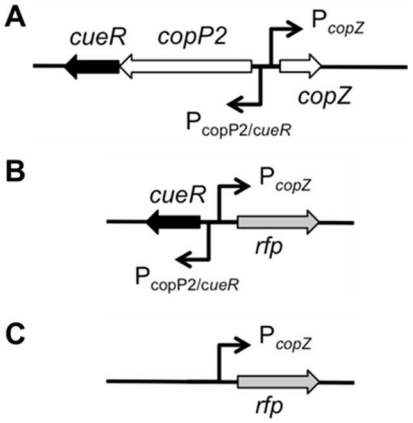

Fig. 1 (A) Schematic representation of the cueR gene cluster in *R. eutropha* H16. Arrows indicate the position and direction of transcription. Biosensor construction of (B) *cueR(rev)-PcopP2/cueR(rev)-PcopZ-rfp* plasmid in *C. metallidurans* and *R. eutropha*, and (C) *PcopZ-rfp* in *C. metallidurans*.

Figure 1A presents a schematic representation of the organization of the cueR gene cluster. We began by constructing a plasmid that carries cueR under its own promoter and the rfp gene under the control of a CueR-upregulated promoter (PcopZ) in both *R. eutropha* and *C. metallidurans* (Fig. 1B). Details of plasmid construction including strains, plasmids, and primers were listed in Table S1, S2 and S3. We then examined the specificity of the whole-cell biosensor toward nine common metal ions (Fig. S1A and Fig. S2A). Au^{3+} was first reduced to Au^{+} and formed complex with CupR/CueR to regulate the transcription of genes due to high redox potential of Au^{3+}complexes upon sorption of Au^{3+} to cell surfaces. In addition, to avoid the precipitation of AgCl or $PbCl_2$, NaCl-free LB (SLB) was used to test the induction of these two metals throughout the rest of the study. Both strains presented strong fluorescence signals in the presence of 10 μM gold ions; moreover, the signal of *C. metallidurans* was approximately 7.8-fold stronger than that of *R. eutropha*. The cell growth condition (OD values) was presented to examine the toxicity of the ions on the sensor cells. Copper and silver ions also induced the expression of RFP; however, none of the other heavy metals had any effect at this concentration (Fig. S2A). Fluorescence microscopy images of sensor cells (Fig. S2B,) in the presence of gold ions were examined to confirm the expression of RFP at the single-cell level.

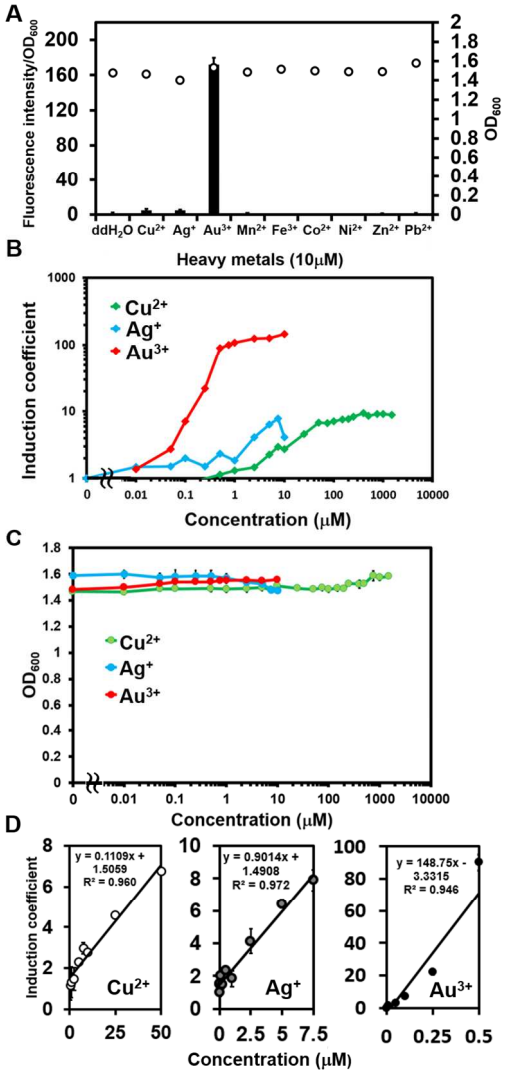

Fig. 2 (A) Fluorescence intensity/OD_{600} and OD values of *C. metallidurans* pcopZ-RFP biosensor cells in response to 10 μM of various metal ions. Data represent the mean (± standard deviation) of three independent experiments, each performed in triplicate. Ag^{+} and Pb^{2+} were performed in SLB to avoid the precipitation. (B) Dose-response curves, (C) OD values, and (D) linear calibration curves for cells in the presence of copper, silver, and gold ions. The background fluorescence intensity/OD_{600} without metals was 1.4 ± 1.2.

We next tested our regulatory sensor with regard to its effect on the heavy metals dose-response/growth conditions to determine the selectivity and sensitivity in *C. metallidurans* (Fig. S2C and S2D). Copper, silver and gold ions revealed transcriptional activation sufficient to induce the expression of RFP and the level of red fluorescence intensity increased steadily with an increase in the concentration of copper, silver or gold ions. The most pronounced effects were observed at concentrations of 1000 μM of Cu^{2+}, 0.1 μM of Ag^{+}, and. 1.0 μM of Au^{3+}. Based on these results, the proposed system should provide an optimal sensing window for copper ions at 5-200 μM, for silver ions at 0.01-0.1 μM, and for gold ions at 0.01-0.5 μM, respectively (Fig. S2E). We then sought to determine whether the CueR regulon would work in the native host organism by conducting similar experiments in *R. eutropha*. The resulting biosensor presented lower induced fluorescence signals (Fig. S1B, C and D) with a narrower dynamic range. We also examined the dose response curves/OD of Mn^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, and Pb^{2+} in *C. metallidurans* to ensure that no significantly fluctuations in fluorescence intensity would occur at higher concentrations (Fig. S3A and B).

The whole-cell biosensor presented far higher sensitivity toward gold ions than toward copper and silver ions. To further optimize the sensitivity for the detection of gold, we

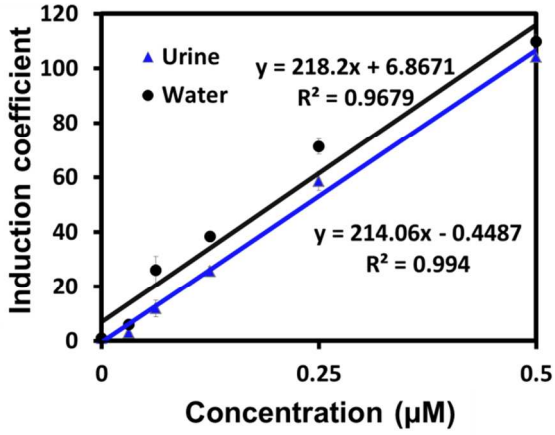

Fig. 3 Induction coefficient of *C. metallidurans* pcopZ-RFP biosensor cells in response to gold ions at various concentrations in urine and pure water samples. Error bars represent the standard deviation from triplicate measurements. The background fluorescence intensity/OD_{600} without metals was 0.8 ± 0.7.

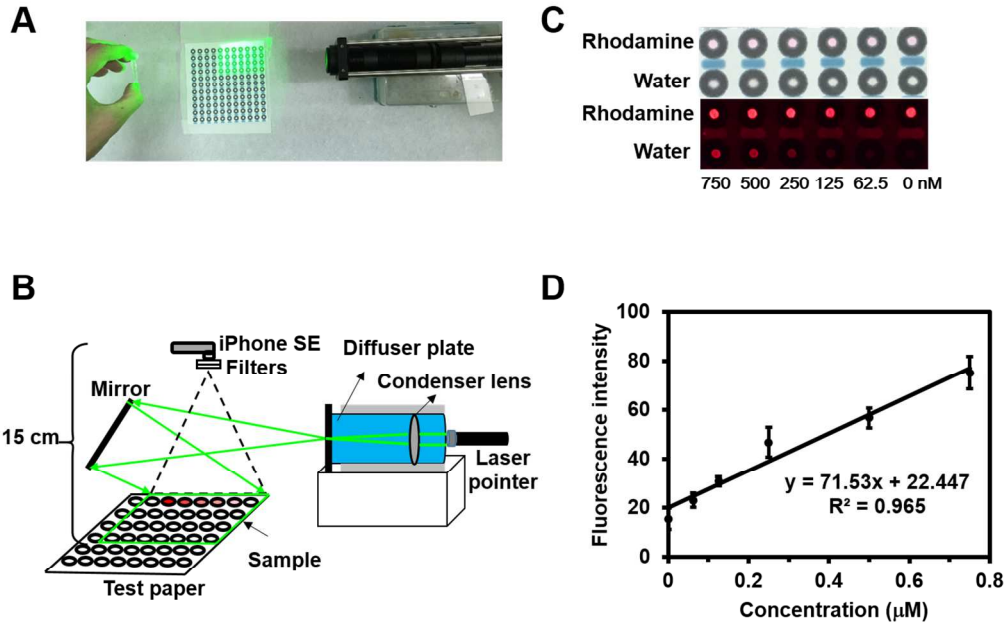

Fig. 4 (A) The assembled setup and (B) schematic illustration of the smartphone-based diagnostic system. (C) Photograph of rhodamine calibration standard and *C. metallidurans* pcopZ-RFP biosensor cells in a cellulose paper-based microplate under white light and green laser pointer light. (D) Fluorescence intensity recorded by smartphone-based diagnostic system of sensor cells in response to gold ions at various concentrations was analyzed by imageJ. Data points represent the average of at least three independent biological replicates.

engineered the strain with the aim of tuning the RFP expression levels. It is interesting to note that the sequence of *R. eutropha* CueR-activated promoter PcopZ is very similar to the consensus operator of CupR (Au-responsive transcriptional regulator) in *C. metallidurans* (Fig. S4A). Specifically, conserved A and T residues at positions 3' and 3 of the operators of pcopZ were identical to those in CupR-activated pCupC.[28] This led us to construct a strain carrying pcopZ-RFP without CueR (Fig. 1C). As shown in Fig. S4B, cells lacking CueR presented 2.7-fold stronger Au-inducible expression, whereas weaker and similar Cu and Ag induction, which suggests that CupR in *C. metallidurans* may cross-regulate the expression of pcopZ. Metals selectivity, does-response, OD values, and linear range of this optimized stain were examined (Fig. 2A, B, C, and D). As shown in Fig.2A, cells presented great specificity and induction toward Au. Regression analysis revealed a linear relationship between fluorescence intensity and the concentrations with good correlation coefficients (R2 = 0.960, 0.972 or 0.946 for Cu^{2+}, Ag^{+}, and Au^{3+}). These results suggest that the *C. metallidurans* pcopZ-RFP biosensor could be activated in the presence of copper, silver or gold ions. Based on the standard error of the linear regression (STEYX) and slope (S), we calculated the limit of detection (LOD) values using the formula 3*STEYX /S. LOD values were 34.51 μM, for copper ions, 0.95 μM for silver ions, and 0.025 μM for gold ions. The limit of quantitation (LOQ) was listed in Table S4.

To eliminate the interference of silver and copper ions, we examine the possibility of introducing pretreatment steps involving i. precipitation of AgCl and ii. EDTA for the removal of copper ions. As shown in Fig. S5A, we obtained a significant decrease of the fluorescence induction upon precipitation of AgCl. EDTA is a chelating agent that binds to copper ion; however, it affects the stability of the cell envelope. We first examined the fluorescence intensity and bacterial growth in the presence of various concentrations of EDTA and 10 μM of copper ion (Fig. S6). At 500 μM of EDTA, the induction of fluorescence was significantly reduced but 50% lowered the OD values. We subsequently examined the fluorescence intensity of sensor cells that had been cultured in the presence of copper, gold, or a mixture of copper and gold with or without EDTA treatment at 500 μM. As shown in Fig. S5B, the fluorescence signals in samples supplemented only with gold were nearly identical to those in samples with a mixture of copper that underwent EDTA treatment. These results suggest that EDTA treatment proved highly effective in eliminating interference from copper ions.

The above results highlight the high sensitivity of pcopZ biosensor in the detection of gold ions. Thus, we then examined if the proposed system can be applied as a personal health monitoring tool for reporting the presence of gold ions in biological matrices. This was achieved by growing sensor cells carrying pcopZ-RFP at a 1:1 ratio with 2X LB medium in samples of human urine spiked with various concentrations of gold ions. In the concentration range of 0-0.5 μM, we observed a linear relationship between the concentrations of gold ions and the fluorescence response (Fig. 3). Urine samples presented a similar increase in fluorescence signals compared with pure water samples, suggesting that the physiological samples did not significantly affect the efficacy and stability of the biosensors.

To facilitate simple, inexpensive, and portable setup with minimal or no instrumentation, we developed a smartphone-

based diagnostic system using the built-in camera of a smartphone (iPhone SE) in conjunction with a green laser pointer and optical filters (Fig. 4A and B). In addition, a wax-printed cellulose paper-based microplate was used to reduce the volume of samples.[29] The fluorescence intensity was calculated from photographs. The fluorescence intensity of a rhodamine calibration standard (50.87 μM) was recorded (Fig. 4C, top panel). The fluorescence intensity of sensor cells supplemented with various concentrations of gold ions was examined by spotting 1 μL of 10X concentrated cells on the paper-based microplate (Fig. 4C). Our results showed that the fluorescence intensity increased linearly with the dynamic range from 0-750 nM of gold ions (Fig. 4C and D). The system showed a good linear correlation (R^2 =0.965). Urine samples spiked with gold ions was examined as well using the same setup (Fig. S7) and showed nearly identical results. The LOD/LOQ information was summarized in Table S4.

We previously designed whole-cell biosensor using *C. metallidurans* CupR for the detection of gold ions. [7] We furthermore compared pCopZ and CupR-based biosensor (Fig. S8). They showed similar performance but pCopZ-based sensor was more significant induced. In summary, this study demonstrates the feasibility of designing a simple, cost-effective whole-cell biosensor in conjunction with a smartphone-based fluorescence diagnostic system for personal health monitoring applications. The paper device provides a simple and effective platform, which enables to achieve the goal of low cost. In addition, concentration of urinary gold ions for patients receiving gold therapy was ranging from 250-500 nM.[3] Therefore, the proposed whole-cell biosensors could be applied to the analysis of urinary gold ions with high efficacy and stability. Enhanced fluorescence response of sensor strains via genetic engineering has been implemented. The optimized strain presented a 50-fold change from 0-0.5 μM of gold ions. Furthermore, our studies proved the possibility to integrate microbial sensors with the paper device.

ASSOCIATED CONTENT

Supporting Information

Supporting Information associated with this article can be found in the online version.

Experimental details, RFP characterizations, detailed of smartphone-based fluorescence assay, and preparation of paper devices.

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TOC graphic specifications.