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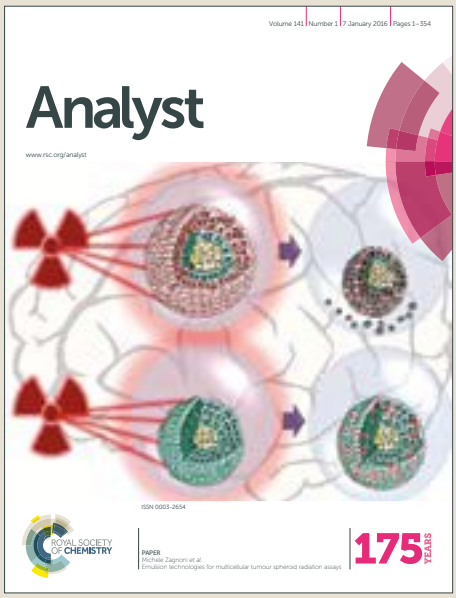

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Engineered Highly Sensitive Whole-Cell Mercury Biosensors Based on Positive Feedback Loop from Quorum-Sensing System†

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Mercury contamination represents a global threat. A simple, sensitive, and rapid means of detecting trace mercury is urgently needed. Herein, we have developed a series of mercury biosensors by combining quorum sensing-based positive feedback systems with a mercury-specific operon, merR. Our results have demonstrated that sensitivity and fluorescence intensity of the engineered *E.coli* cells were greatly improved thanks to the positive feedback system. In addition, by fitting the fluorescence signals to the classic Hill equation, we discovered that the responses of the engineered *E.coli* cells were close to ultrasensitive curves. Our work highlights quorum-sensing system as a powerful tool in biosensor designs.

Introduction

Mercury, as a heavy metal, is widely used for the manufacture of industrial chemicals or electrical and electronic applications. However, it has strong biological toxicity and can be easily absorbed through the skin and mucous membranes, which causes damage to central nervous system, immune system, and vital organs such as the kidney and liver.[1,2] Concern over the toxicity of mercury means researchers constantly require efficient methods in order to detect mercury in environmental samples.

Although conventional methods based on physical-chemical analysis are available, these approaches such as atomic absorption spectrometry (AAS) and inductively coupled plasma-mass spectrometry (ICP-MS) generally rely on expensive instruments and complicated procedures, rendering them incompatible with practical use outside of laboratory settings.[3] What is more, chemosensors generally use chemical-chelating agents or porous polymeric materials and the wasted metal-complexed materials are often difficult to degrade and become secondary pollutants.[4,5] Thus, the process of distinguishing mercury sensitively represents a thorny problem. The development of a cost-efficient, easy-to-operate, and environmentally friendly biological method for mercury detection is urgently needed.

Recently, some novel mercury-specific, biosensor-based oligonucleotides or DNA-protein interactions have been reported.[1,6,7] On the other hand, by studying a diverse mechanism of microbes that maintain homeostasis and resistance to heavy metals, researchers have developed some whole-cell biosensors for detecting heavy metals, including mercury.[8,9] For example, Karp *et al.* developed a luminescence biosensor in order to detect inorganic mercury by taking advantage of a mercury-regulated metalloprotein.[10] Thouand *et al.* reported the identification of four heavy metals, including mercury in environmental samples using bioluminescent bacteria.[11] Moreover, we also have reported developing a series of heavy metal biosensors for cell-based selective visual detection by engineering the bacterial regulons.[12,13] However, for environmental samples monitoring applications, as the mercury detection limit in drinking water below 10 nM (the maximum contamination level defined by US EPA (Environmental Protection Agency) and 5nM(European standards in water according to the European directive 98/83/CE), the sensitivity and response time of these existing whole cell microbial biosensors do not satisfy requirements for application purposes and therefore still need to be optimized.

In nature, the bacterial quorum-sensing system constitutively secretes certain signaling molecules and expresses the receptor of the signal molecules to regulate a diverse array of physiological activities both in Gram-positive and Gram-negative bacteria.[14–17] There is a formation of a positive feedback loop that occurs in the system when the concentration of the signaling molecules passes a threshold and then, the signal receptor becomes fully

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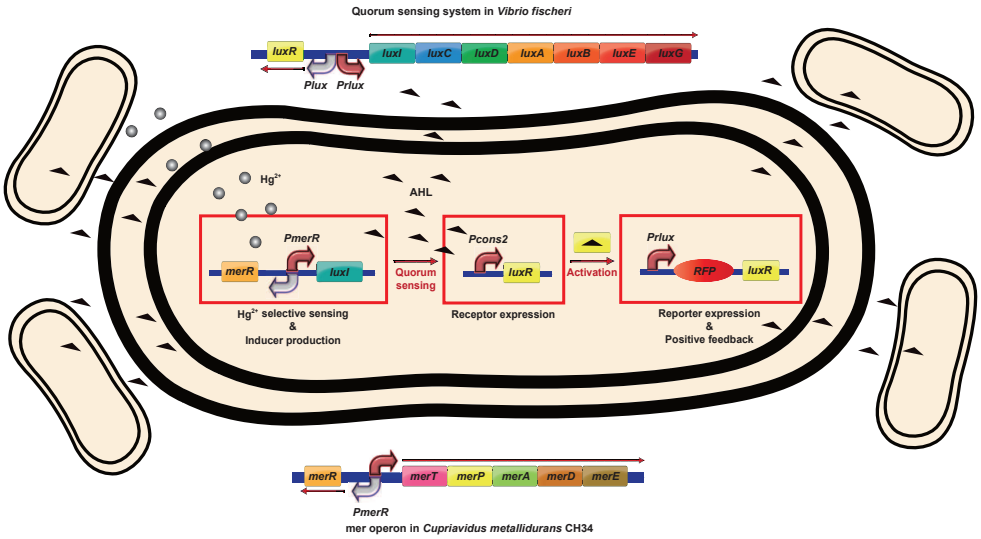

Fig. 1 Engineered whole-cell mercury biosensors based on positive feedback loop from quorum sensing system.

activated. Positive feedback is a wide-spread and well-known mechanism and has been broadly applied in the expanding field of synthetic biology.[18,19] The mechanism plays vital regulatory roles in diverse pathway, involving in signaling, oscillatory constitution, intracellular polarization processes, switches designing and whole-cell biosensor.[20–22] In the application of synthetic biology, positive feedback systems have contributed to a number of behaviors including bistability and hysteresis and a clearly defined one, amplification.[23–26] Serrano *et al*, for example, developed a eukaryotic gene switch using a simple positive feedback loop.[27] Xiong and Ferrell showed that a positive feedback system could be used to engineer memory and regulate cell maturation.[28] Sun L *et al.* determined that positive feedback loops based on the quorum-sensing system could regulate transcriptional activity, which makes the luxR regulator more sensitive to the autoinducer.[29] All of these pioneer researches have focused on the role of positive feedback employed in bistable switch, accelerator or delay, and amplifier. In light of these results, we set out to explore the feasibility of this strategies for optimizing the mercury biosensors. Herein, by introducing the positive feedback loop of luxI-luxR type quorum-sensing system from *Vibrio fischeri* into the whole-cell heavy metals biosensor, we have developed a mercury-specific whole-cell biosensor with engineered Hg^{2+} regulatory MerR proteins shown in Figure 1.[30,31] This approach represents a general strategy for improving the sensitivity of existing whole cell biosensors.

Results and Discussion

First, we engineered the mercury-specific operon merR including the MerR protein in order to control the expression of LuxI, which can secrete the signal molecular AHL to bind with LuxR. The expression of LuxR is under the control of Pcons2, the strongest constitutive promoter of *E.coli*.[32] Consequently, while LuxR-AHL forms, the complex will in turn activate Prlux and express RFP. Depending on different assemblies of the DNA fragments encoding LuxR, LuxI or nothing downstream RFP protein, three strategies have been designed in order to verify the actual output signals enlarging effect of these biosensors, named as QS1, QS2, QS3, respectively. (Figure 2(a)). These three circuits were constructed with the primers (Table S1) in the Biobricks vector psB1A2 and transformed in *E.coli* cells, respectively.[33] And the original merR operon which is reported by Karp *et al.* was engineered only with RFP and transformed as control[10].

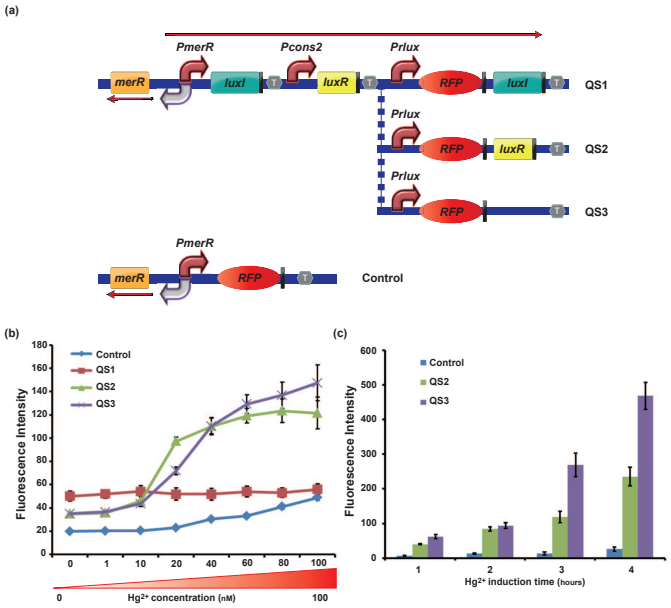

Fig. 2 (a) Different genetic organization of the mercury-specific inductive RFP expression plasmid with positive feedback genes of quorum sensing system (QS1, QS2, QS3) in *E.coli* cells, and genetic organization of the original merR operon engineered only with RFP expression plasmid as control. (b) Fluorescence measurement of *E.coli* cells containing QS1, QS2, QS3 and Control plasmid in gradient concentrations of 0∼100 nM Hg^{2+} in one hour. All cells were resuspended in PBS buffer (pH 7.4). (c) Fluorescence measurement of *E.coli* cells containing QS1, QS2, QS3 and Control plasmid for different induction times in final concentration of 100nM Hg^{2+}.

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Next, we began to determine the fluorescence intensity of biosensors contain QS1, QS2, QS3 respectively in the present of mercury. The response results of QS1 exhibited almost no change, which implies the expression of RFP and LuxI was not activated by Prlux (Figure 2(b)). But the fluorescence intensity of biosensors containing QS2 and QS3 increased gradually to a varied concentration of Hg^{2+}. In order to determine the consequences of these two systems in signal amplification, fluorescence responses of QS2 and QS3 were compared with the control plasmid in presence of 100nM Hg^{2+} for different culture times. As Figure 2(c) shows fluorescence intensities of QS2 and QS3 significantly increase with time, and this result implies that during cultivation of the whole-cell biosensors, the concentration of AHL increased under Hg^{2+} induction and the output fluorescence signal was then enhanced through cell-to-cell quorum sensing. Thus, preliminary conclusions would indicate that biosensors containing positive feedback loop show more sensitivity than control.

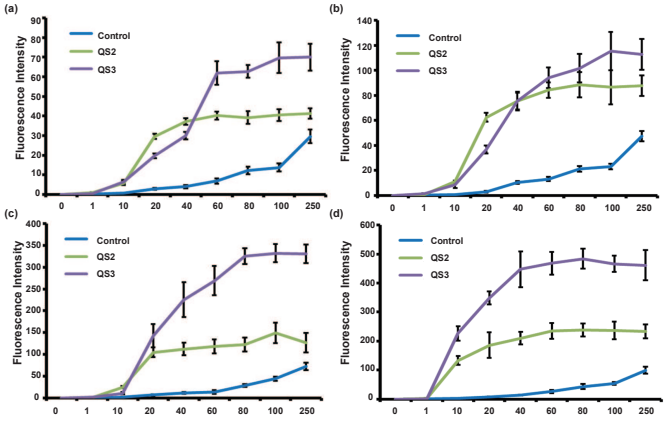

Fig. 3 Fluorescence measurement of *E.coli* cells containing QS2, QS3 and Control plasmid induced by gradient concentrations of 0∼250 nM Hg^{2+} from one hour (a), two hours (b), four hours (c) and six hours (d). All cells were re-suspended in PBS buffer (pH 7.4), and the mean values of three independently performed experiments are reported. Error bars correspond to the standard deviations.

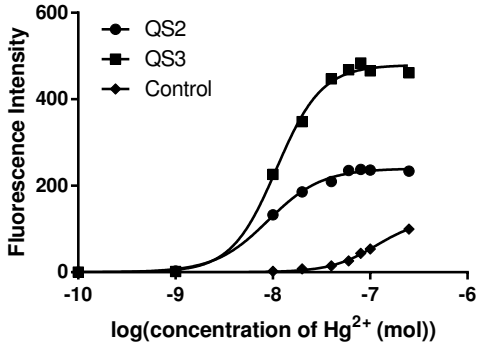

Fig. 4 The fitted curve of QS2, QS3 and control for six hours. The concentration is transformed to log-domain.

Furthermore, the fluorescence responses of QS2, QS3 in range of 0∼250 nM Hg^{2+} with 1, 2, 4 and 6 hours incubation was examined, respectively. To better show the sensitivity, the fluorescence intensities in Figure 3 does not include a baseline response. The results demonstrate that, with the time extension and increasing Hg^{2+} concentration, both the fluorescence intensities of QS2 and QS3 grew in the present of the mercury concentrations as low as 1nM, eventually reaching a steady state (Figure 3(a-d)). Both QS2 and QS3 showed great fluorescence changes in low and small scope concentrations of Hg^{2+}. The response curves of QS2 and QS3 are close to ultrasensitive curve, which could be fit by equation(1) [19]. As ultrasensitivity functions is a kind of biochemical switch in the cell cycle and has been involved in many important cellular events, this phenomenon reveals that quorum-sensing communications among the *E.coli* cells of QS2 and QS3 indeed enhances the biosensors' sensitivity.[34,35]

We tested the responses of the *E.coli* cell containing the plasmids of QS1, QS2, and QS3 in gradient concentrations of Hg^{2+}. The fluorescence intensities were used to determine the experimental transfer curve and were fit to the Hill equation:

$$y = y_{min} + \frac{y_{max} - y_{min}}{1 + (\frac{EC_{50}}{Hg^{2+}})\eta}, \quad (1)$$

where Hg^{2+} is the concentration of Hg^{2+} and the response y is determined by four parameters: the baseline response (y_{min}), the maximum response (y_{max}), the curve slope (η) and the Hg^{2+} concentration that induces half fluorescence intensity between y_{min} and y_{max} (EC_{50}).

In order to best show the ultrasensitivity, the concentration of Hg^{2+} was transformed to log domain. By simulation, the parameters of response curves are listed in Table 1, in which the fitted results for 6 hours are shown in Figure 4. It can be seen that the final fluorescence intensities of QS3 were found to be over two-fold higher than QS2 in high-level Hg^{2+} concentrations. These intensities mainly result from less energy consumption for the expression of RFP. Comparing to the reported original whole biosensor based on MerR operon, our engineered biosensors can detect the concentration of Hg^{2+} as low as 1nM. The concentration of Hg^{2+} which resulted in half-maximal QS2 and QS3 response for 6 hours are 1.55×10^{-8} M and 3.76×10^{-8} M, respectively. From the table, the data for EC_{50} of QS2 are less than QS3, which illustrates that the cells of QS2 show more sensitivities than QS3 at the beginning in lower Hg^{2+} concentrations. With time increasing, numbers of η declined, accounting for a tendency toward a steady response for both QS2 and QS3. What is more, the intracellular positive feedback loop in QS2 cells further enhances responsibility. But it was inferred that the expression of the intracellular positive feedback loop finally increased the burden and complexity of gene regulation and expression in QS2 cells, resulting in final lower fluorescence intensity.

Conclusion

With increasing concern regarding heavy metal contamination all over the world, the development of effective, rapid, and visual biological tools for toxic heavy metals, especially mercury detection, has been a global challenge as well as an indispensable technology. Our work functions by combining positive feedback based

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Table 1 The fitted parameters for Fluorescence intensity curve.

Time (hrs)	QS2			QS3			Control		
	y_{max}	EC_{50} (nM)	η	y_{max}	EC_{50} (nM)	η	y_{max}	EC_{50} (nM)	η
1	39.95	15.54	3.96	74.43	37.64	2.19	47.21	176.8	1.51
2	85.39	15.84	3.81	116.7	29.73	2.15	76.31	175.2	1.37
4	122.5	13.58	4.38	340.8	27.04	2.12	80.13	98.11	2.44
6	239.9	8.97	1.58	479.2	10.92	1.87	124.1	115.7	1.83

on quorum-sensing system with merR operon in order to develop new mercury specific biosensor and provide a new strategy for metal-specific detection. Future work will be directed towards designing more efficient biosensors by producing large signal response with low concentration of inducer through signal communications among the cells.

Experimental section

Construction of original mercury biosensor

The BioBrick Vector psB1A2 and the gene encoding RFP were constructed following a previously published protocol.[33] First, DNA fragments of merR and mer promoter were amplified from Cupriavidus metallidurans CH34 genomic DNA. This 506bp DNA fragment was digested by XbaII and pstI and then inserted into psB1A2 vector following the order shown in Figure 2(a). After verifying by sequencing, the gene encoding RFP was inserted into the vector using XbaII and pstI. Third, a 163bp gene as a terminator was inserted into the vector afer RFP using XbaII and pstI. Finally, the plasmid was transformed into *E.coli* strain DH5α.

Mercury sensitivity detection with the *E.coli* RFP sensor

For the mercury concentration sensitivity measurements, the *E.coli* DH5α containing the plasmid was cultured in LB medium until OD600 = 0.6-0.8, then induced by various concentrations (500pM, 1, 10, 20, 40, 60, 80, 100, 250 nM) of $HgCl_2$ (Hg^{2+}) overnight in LB medium. All of the cells were normalized to an OD600=0.6 with PBS buffer (pH = 7.4). For fluorescence determinations, 200 ul aliquots of each sample were added in triplicate into a quartz dish. Fluorescence was recorded using a F-7000 fluorescence spectrometer with 558 and 583 nm for the excitation and emission wavelengths, respectively.

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