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Development of a Cell-Free Optical Biosensor for Detection of a Broad Range of Mercury Contaminants in Water: A Plasmid DNA-Based Approach

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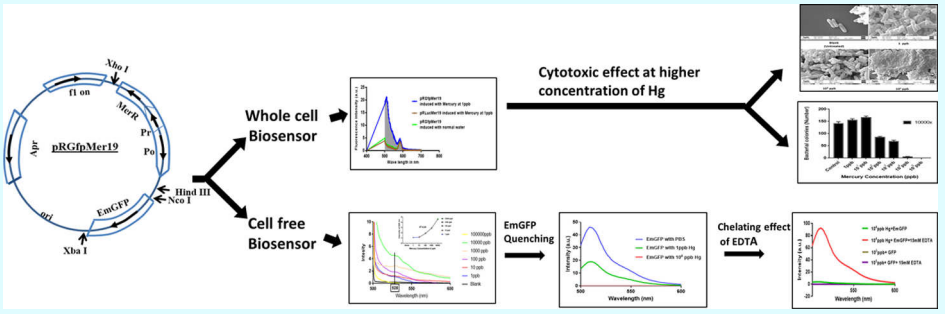
Supporting Information



ABSTRACT: Mercury (Hg) is one of the main water contaminants worldwide. In this study, we have developed both whole-cell and cell-free biosensors to detect Hg. Genetically modified plasmids containing the merR gene were used to design biosensors. Firefly luciferase (LucFF) and emerald green fluorescent protein (EmGFP) genes were separately introduced as a reporter. Both constructs showed a detection limit of 1 ppb (Hg) in *Escherichia coli* and the cell-free system. We found that higher concentrations of Hg become detrimental to bacteria. This cytotoxic effect shows an anomalous result in high Hg concentrations. This was also observed in the cell-free system. We found that EmGFP fluorescence was decreased in the cell-free system because of a change in pH and quenching effect by Hg excess. Once the pH was adjusted to 7 and a chelating agent was used, the EmGFP fluorescence was partially restored. These adjustments can only be done in the cell-free system after the GFP expression and not in whole cells where their number has been decreased because of toxicity. Therefore, we suggest the use of the cell-free-system, which not only reduces the total experimental time but also allows us to perform these postexperimental adjustments to achieve higher sensitivity. We would also recommend to perform more measurements at a time with different dilution factors to bring down the Hg concentration within the measurable limits or to use some other chelating agents which can further reduce the excess Hg concentration.

INTRODUCTION

Mercury and most of its compounds are extremely toxic. In many areas worldwide, it is well-known as a water contaminant. When up taken, it causes health hazards such as gastrointestinal, neurological, and renal organ disorders.[1] Thus, it is necessary to monitor the mercury levels in drinking water regularly. In general, physicochemical methods and high-end analytical techniques are used for the quantitative analysis of mercury,[2−4] although frequent use of these methods is not possible because they are costly and non-eco-friendly.[5] Atomic absorption spectroscopy or inductively coupled plasma mass spectrometry (Agilent Technologies, USA) can be used as alternative methods, but they are very expensive and need special operational expertise. Thus, there is a stern need for an effective, low-cost, and user- and eco-friendly monitoring system for the detection of mercury contaminants at low concentrations. Biosensors can be a better option offering a reduced cost and quick measurement. So, it is apparently the most favorable way to assess the bioavailability of mercury in water samples. Biosensors can have an electronic or an optical readout system. Bacteria propose plethora for the development of whole-cell and cell-free biosensors for detection of different contaminants in the environment.[6] A different strategy for making a microbial biosensor has been developed using a reporter gene connected to a regulatory promoter, where the regulatory gene has been isolated from the genetically encoded resistant system that allows the microbes to grow in an unfavorable environment.[6]

In recent years, cell-free transcription/translation has been used as an important tool to perform protein expression-based sensing.[7,8] The key advantage of this technique lies in the faster expression of recombinant proteins, and additionally, it allows

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for rapid screening and product development without performing time-consuming bacterial culture methods.[9,10] A cell-free biosensor can have many advantages over a whole-cell biosensor, as it simplifies and optimizes the construction of operon circuits for detection of specific heavy metals.[11] It is less time-consuming because in a whole cell biosensor, various enzymes have to be tested, and genetic modifications are always tricky and require considerable time.[12] As the cellular growth and expression are correlated, the analyte-dependent changes in growth kinetics can be overruled by using a cell-free system. In cell-free expression systems, different pathways can also be encoded by adding DNA (plasmid/genomic) at relative concentrations.[13] In this study, the cell-free expression has been used for heavy metal sensing. merR, the transcriptional activator gene of the *Pseudomonas* sp. K-62 strain,[14] mediated expression of the MerR transcriptional activator protein, which has been considered to grow cells on mercury. Active site prediction for mercury and then docking[15] were performed using the MerR protein and Hg^{2+} to confirm their interaction computationally. This study aimed to construct both whole-cell and cell-free (plasmid-mediated) luminescence/fluorescence-based sensors for cost-effective and sensitive detection of mercury, where luciferase (LucFF) and emerald green fluorescent protein (EmGFP genes are used as reporters separately.[6,14] Thus, both recombinant DNA constructs (pRLucMer19 and pRGfpMer19) were synthesized to sense mercury in liquid solutions in both cell-free- and whole-cell-based biosensors. The limit of detection (LOD) for both constructs has been compared in our studies.

RESULTS

Active Site Prediction and Docking Analysis. To confirm the binding of Hg to the MerR protein, a computational study was performed. Active site prediction and docking analysis confirms the wet lab study results. Active site prediction for mercury binding to the MerR protein was performed by PASS algorithm. It was found that Hg^{2+} binds to the MerR protein at cysteine residues. Docking was carried out with autodock 4.2, and a +2 charge was assigned to Hg manually after the protein database (PDB) file preparation. In the case of Hg 201, cysteine residues at the C-terminus (position 146 and 154) show interaction with Hg^{2+} (Figure 1).

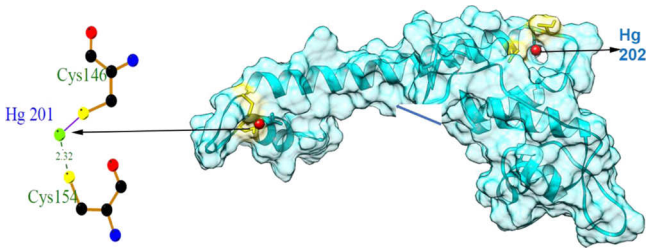

Figure 1. Active site prediction and docking analysis of MerR with Hg^{2+}. Docking was carried out with autodock 4.2, and +2 charge was assigned to Hg manually after PDB file preparation.

The estimated free energy of binding is −5.63 kcal/mol and the final docked energy is −7.33 kcal/mol, whereas in the case of Hg 202, the estimated free energy of binding is +5.11 kcal/mol and the final docked energy is +3.46 kcal/mol.

Construction of pRLucMer19 and pRGFPMer19. The plasmid constructs were prepared according to the schematic diagram (Figures 2 and 3). The designed plasmid constructs

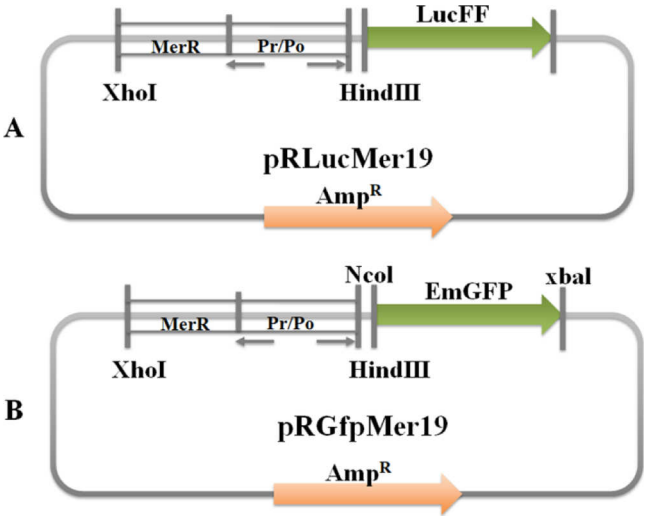

Figure 2. Schematic diagram for plasmid constructs: (A) Luminescence-based whole-cell biosensor pRLucMer19. (B) Fluorescence-based whole-cell biosensor pRGfpMer19.

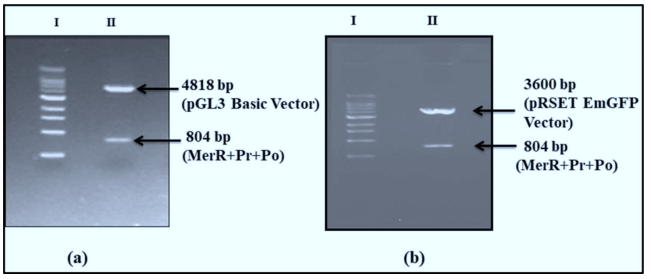

Figure 3. (a) Lane I-I 500 bp ladder and Lane II Xho-I and Hind III-double digestion pattern of pRLucMer19. (b) Lane I-I 500 bp ladder and Lane II NcoI and Xbal-double digestion pattern of pRGFPMer19.

were used as whole-cell biosensors, where the merR gene and its promoter and operator were placed in such an orientation that the reporter genes (firefly LucFF or EmGFP) can be transcribed. Different restriction enzymes were incorporated in pRLucMer19 and pRGfpMer19 (Figures 2 and 3). The restriction digestion (Figure 3) pattern confirmed the measure of the inserts.

Whole-Cell Biosensing. *Luminometric Response of pRLucMer19 to Mercury.* In this experiment, the pRLucMer19 plasmid-containing *Escherichia coli* cells were grown in the presence of different mercury concentrations as an inducer, and their luminescence was measured. The LucFF activity showed linearity in the range of 1 to 10^4 ppb (ppb denotes parts per billion). These results revealed that the MerR/mercury complex effectively binds to the LucFF operator and activates the gene. The signal-to-noise ratio of the luminescence for pRLucMer19 for the lowest mercury concentration (1 ppb) was also found to be more than the required values (>2). Figure 4 represents the linear graph for mercury induction. This result of the microbial whole cell biosensors shows that the detection of mercury contaminants can be used at low or medium concentrations. After adding the substrate, the LucFF activity has to be measured immediately, a fact that restricts multiple sample measurements. To avoid this and get rid of substrate addition, EmGFP has been introduced as a reporter. This will help us avoid manual intervention and automatization.

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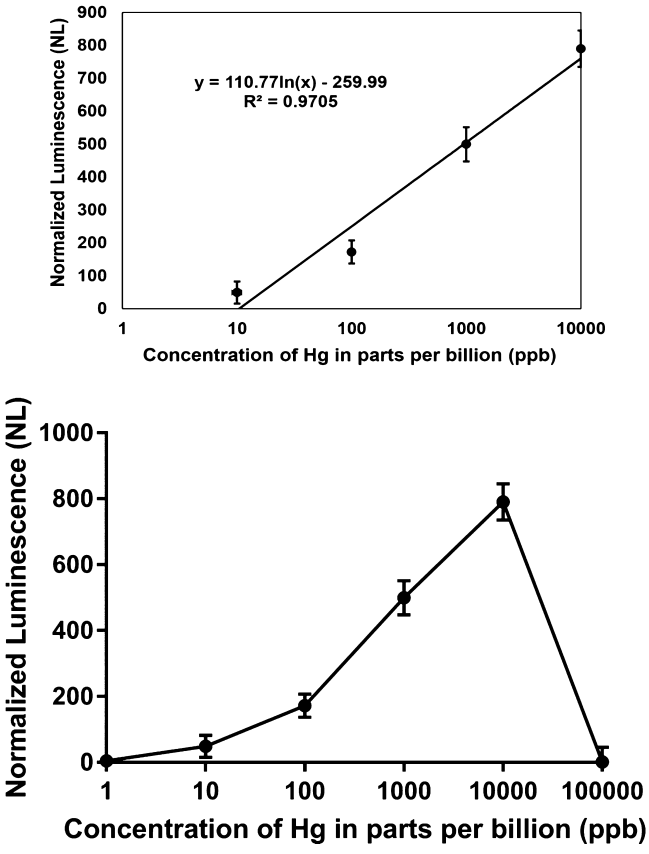

Figure 4. Mercury concentration-dependent induction and expression in *E. coli* containing pRLucMer19. Luminescence was plotted against mercury concentration. The mean and standard deviation of three separate experiments have been plotted. The *X*-axis indicates the concentration of Hg (ppb) on the log scale. The inset picture shows the linearity curve (R^2 = 0.97) with the values from 10 to 10 000 ppb.

Fluorometric Responses of pRGfPMer19 to Mercury. We confirm the proper orientation and placement of gene in pRGFPMer19. As a result, the MerR protein is activated in the presence of mercury, and the GFP gave a fluorescence signal even at 1 ppb (Figure 5). A linearity curve was obtained for the range of 1−10^4 ppb (Figure 6). The expression of the reporter is always associated with cell growth and the total number of cells. At higher mercury concentrations, the cells were damaged, and hence fluorescence started to reduce. Figure

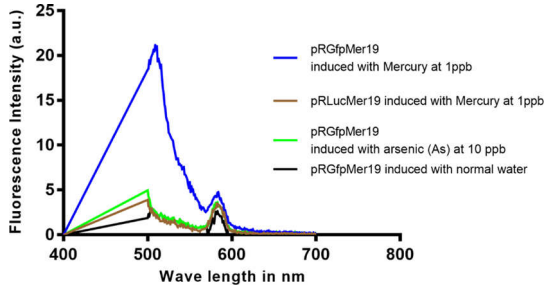

Figure 5. EmGFP expression and its measurement using fluorescence spectroscopy when *E. coli* cells containing pRGfpMer19 plasmid was induced by 1 ppb mercury concentration in the whole-cell biosensor system. The EmGFP expression was compared with the expression of pRLucMer19 to confirm the specificity when incubating with mercury and water.

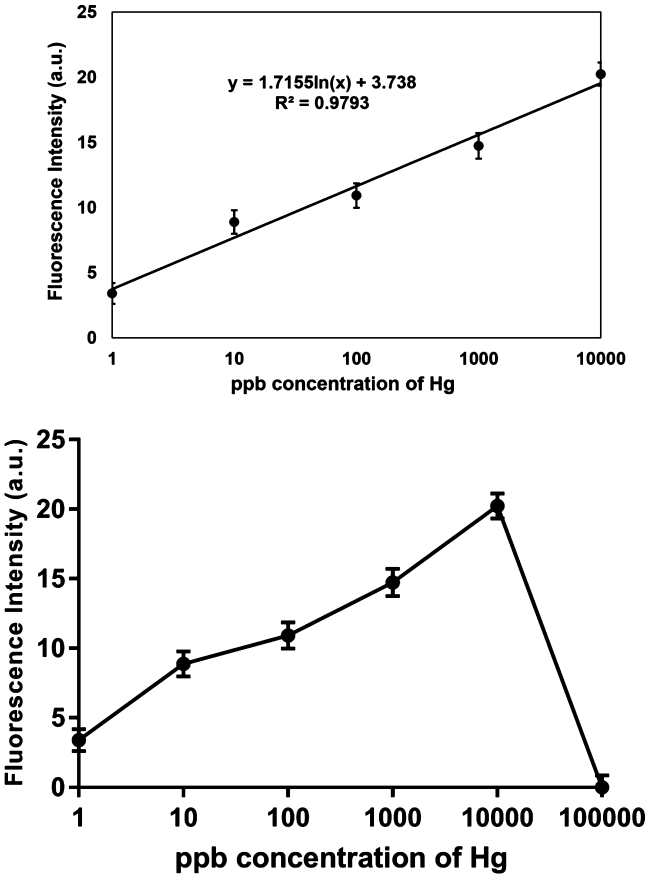

Figure 6. Mercury concentration-dependent induction and expression in *E. coli* containing pRGfpMer19. Fluorescence intensity was plotted against the mercury concentration. The mean and standard deviation of three separate experiments have been plotted. The inset picture shows about the linearity range of mercury (R^2 = 0.99). The *X*-axis indicates the concentration of Hg (ppb) on the log scale.

S2 shows the fluorescence expression of the reporter gene (EmGFP) when both the whole-cell biosensor (Figure S2A) and the cell-free biosensor (Figure S2B) were induced with other divalent cations (Cu^{2+} and Cd^{2+}). The fluorescence emission in the presence of Cu^{2+} and Cd^{2+} was found to be much lower than that of Hg^{2+}. The response was found to be selective against Hg^{2+}.

Cell Fate Study at Higher Concentration of Mercury. *Fluorescence-Activated Cell Sorting.* Figure S3 shows the dot plot of forwarding and side scatterings of treated (with mercury) and untreated samples by using the FlowJO (BD, Bioscience, USA) software at pH 7. Figure S3 denotes samples untreated (A) and treated with 1 to 10^5 ppb of mercury (B to H). An additional experiment at different pH values was performed to check whether the toxic effect was given by low pH conditions or by mercury (Hg). The Hg purchased from Sigma comes in nitric acid (HNO_3) solution. So, to keep mercury soluble at higher amounts, the pH was reduced to around 1. To rule out the effect of pH shock on the bacterial cell, we performed the following experiment. Under three pH conditions (pH = 1, 4, and 7), fluorescence-activated cell sorting (FACS) was performed to check the cell killing/viability. Propidium iodide dye was used for the assay (Figure S4).

From the flow cytometric results, it is clear that side scattering, which indicates the internal complexity or

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granularity, is increased significantly when treated with a higher concentration of mercury. At the same time, the Forward Scattering (FSC) population of the samples markedly decreased at the higher concentration of mercury, indicating a decrease in the size. In the case of different pH conditions (pH = 1, 4, and 7), the side scattering values were increased at pH = 4 and pH = 7 but not in the case of pH = 1. This comparative data (for different pH ranges) reveals the effect of mercury toxicity and the pH effect. At very low pH values (pH = 1), it seems that there is an effect on the viability of the cells, whereas at pH = 4 and 7, only mercury toxicity effect was observed on the bacterial cell.

Scanning Electron Microscopy. The cells were incubated in the presence of different concentrations of mercury, and then their structures were analyzed using scanning electron microscopy (SEM). The electron micrographs (Figure S5) show us that there is a clear change in the surface morphology of *E. coli* cells with the increase of the mercury concentration.

Cell Killing Assay. The bacterial cell killing assay was performed on Luria−Bertani (LB) agar plates, and the results are summarized in Figure S6. Different concentrations (1 to 10^5 ppb) of mercury were incubated with *E. coli* cells containing the recombinant plasmid pRLucMer19 and pRGfpMer19. It was fully in concurrence with the data obtained through the SEM analysis. An initial (1 to 10^3 ppb) increase was seen in a number of colonies, which were observed and counted after incubation. Afterward, a regular decrease was seen up to 10^4 ppb, and no colony was found at 10^5 ppb concentration. It is clear that the toxicity of Hg^{2+} is the main reason for the decreased expression of the reporter gene in the whole-cell-based sensors. It is not clear why a similar effect was observed in the cell-free system too, where no cell growth is associated with the expression.

To avoid this dependency and to reduce the total experimental time, we have used the cell-free transcriptional-translation system.

Cell-Free Biosensing. *Fluorometric Responses of pRGfPMer19 to Mercury.* Experiments were performed to check the fluorometric response of the pRGfpMer19 plasmid only using a cell-free expression system, and it also showed sufficient induction in the presence of a concentration regime of 1 ppb to 10^5 ppb (Figure 7). At the higher concentration of Hg, the whole cell and cell free systems showed a decrease in reporter gene expression. We wanted to investigate the reason behind the decrease in both cases.

Fluorescence Quenching of EmGFP Because of Hg Concentration and pH Difference. To analyze the decreased fluorescence with the increased concentration of mercury, we took the purified EmGFP and studied the quenching effect of the mercury itself. As the concentration of mercury was increased, the fluorescence intensity of the EmGFP molecule decreased (Figure 8). To resolve this, we looked at the changes associated with Hg^{2+}solution addition. At first, we checked the pH of the resultant solution. At higher concentrations (10^4 and 10^5 ppb), the pH of the solutions was shifted to 1.5. Thus, we neutralized the pH and was able to recover the EmGFP fluorescence even at a higher concentration (10^4 ppb) of Hg (Figure S7). Moreover, the metal ion-induced quenching of fluorescence is known.

Removal of Excess Hg Using EDTA as Chelating Agent. So, in the next phase, we wanted to remove the Hg excess to recover the GFP fluorescence at higher concentrations of Hg. We thus added ethylenediaminetetraacetic acid

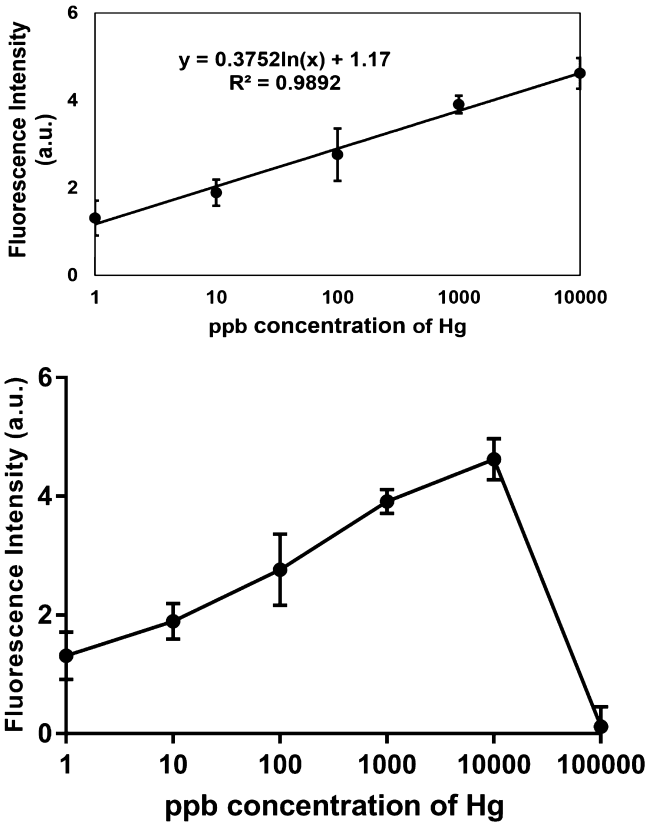

Figure 7. EmGFP expression and its measurements using fluorescence spectroscopy when the pRGfpMer19 plasmid was induced by different mercury concentrations (1−105 ppb) in the cell-free transcription and translation system (Promega). A universal peak of fluorescence was observed at 530 nm. The inset picture shows about the linearity range of mercury (R^2 = 0.99).

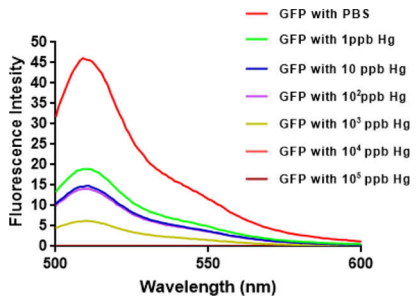

Figure 8. Fluorescence quenching of EmGFP in the presence of different concentrations of mercury (Hg). Purified EmGFP was kept in PBS (0.01 M, pH = 7.4) buffer. Purified green fluorescent protein (EmGFP) was incubated with mercury at different concentrations to check the quenching effect of the EmGFP expression.

(EDTA) to chelate out the untreated Hg from the solution (Figure 9). The fluorescence intensity was recovered significantly, and it was found that 15 mM EDTA was optimum for maximum EmGFP fluorescence.

DISCUSSION

Every day there is an increase in environmental pollution because of the dispense of heavy metals such as mercury (Hg), lead (Pb), copper (Cu), cadmium (Cd), nickel (Ni), chromium (Cr), arsenic (As), antimony (Sb), and so forth. Mostly, they contaminate the groundwater and subsequently

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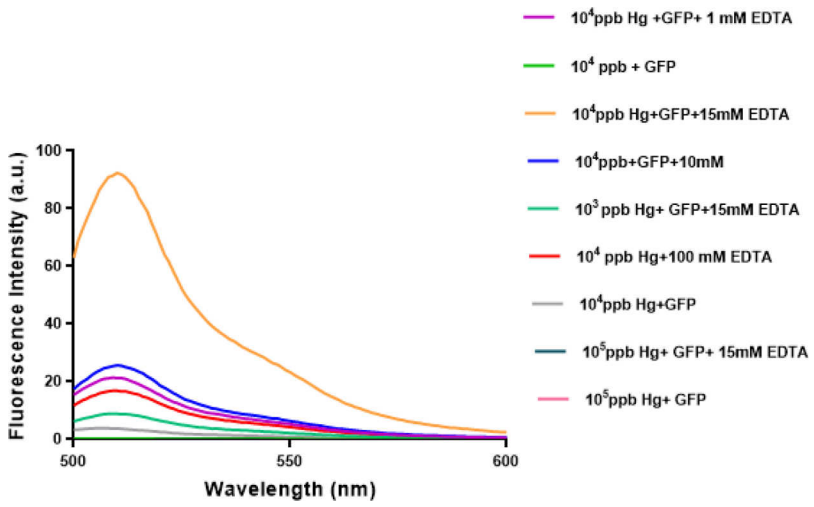

Figure 9. Chelating effect of EDTA for removal of excess amount of Hg in solution. EDTA was added to chelate out the untreated Hg from the solution. The fluorescence intensity can be recovered significantly, and it was found that 15 mM EDTA was optimum for maximum EmGFP fluorescence.

play a key role in causing human health hazards. Among these, mercury is one of the major contaminants. Many new technologies have been developed in the last decade for the detection of mercury.[16,17] Previously, mercury-based whole-cell biosensors were reported with different reporter genes capable of detecting different mercury concentrations.[18] Among these, the microorganism-based method has gained popularity, where the contaminant-induced expression of the LucFF gene and subsequent luminescence production is measured for sensing applications. Still, there are many disadvantages of the whole-cell and LucFF-based biosensors.[19] So, the aim of our work is to overcome the drawbacks. Thus, we started with the previously reported merR gene.[14] Before the wet experiments, we performed the theoretical and computational studies on Hg^{2+} binding to the MerR protein. The docking results showed that due to a negative (low) free energy, Hg^{2+} binds to Cys residues between positions 146 and 154, whereas due to a positive (high) energy, Hg^{2+} is unable to bind to the MerR region in the case of Hg 202. These in silico results confirm the interaction between MerR and Hg^{2+} in the *Pseudomonas* sp. K-62 strain. As the MerR protein structure from *Pseudomonas* sp. K-62 strain is not available in the PDB, this computational study will help recognize the functional and binding capabilities of the same proteins/genes from different biological sources.[15] The detection system is based on induction of the merR gene that depends on the microbial growth, which in turn activates the reporter gene (LucFF). Firefly LucFF requires both luciferin and adenosine 5′-triphosphate as substrates to produce a luminescent signal. Firefly LucFF is very sensitive and has broad dynamic ranges. Using the merR regulatory gene and LucFF, we can achieve the detection limit of 1 ppb. Cell lysis is required to allow the substrate and LucFF enzyme to react under cytosolic conditions. The main demerit of LucFF is, unlike fluorescent reporter proteins, it does not allow to develop live cell assays and is restricted to a single data point in each experiment.[20] The automation is hindered because of the step required for the addition of substrate for the LucFF assay. Moreover, the signal associated with luminescence measurement is transient, and it is very critical to measure just after the addition of the substrate. The time delay for the later samples results in the diminished signal. Thus, it restricts the use for multiple measurements. So, we have replaced our LucFF reporter gene by EmGFP and reconstructed the plasmid pRGfpMer19 from pRLucMer19, where we have replaced the Luc gene with EmGFP.

While studying the sensitivity at higher mercury concentrations, we found that after a certain value, the cell-based biosensors show much lower effective concentration. It was reported that this was due to the mercury-induced cell death.[21,22] It was supported by the cell-killing assay, FACS analysis, and the deformed granular structures of the cells were visualized by SEM. This has been overlooked for a long time where everyone is interested to achieve a lower detection limit,[23] but still, there are constraints or limitations as the microbes tend to behave differently at higher concentrations. In the whole cell, the Hg^{2+}-induced toxicity showed a killing effect. To check whether the effect of cell killing is due to very low pH or due to Hg, FACS at different pH levels was performed, which clearly indicated that bacterial cells deteriorated at high Hg concentrations (10^5 ppb) (Figure S3).

Growing the cells is also time-consuming and requires multiple steps. To minimize the total time of production and detection and to eliminate the dependency of cell growth, we used a cell-free transcription and translation system. Both the plasmids in the cell-free medium also showed linearity and a LOD of 1 ppb. The cell-free system reduced the measurement time drastically, as the laboratory-based cell culture was completely avoided by this method.

Thus, we have reduced the total assay time from 4 to 1 h. In this continuation, our study reports the new strategy based on green fluorescent and LucFF-based whole cell and a cell-free biosensor (Figure S1) for detection of mercury, where we can achieve the sensitivity of 1 ppb with 1 h time for reporter gene or incubation. EmGFP with increased sensitivity also provided stability to the measurement system (Table S1).[11,24−26]

GFP is very sensitive, and its fluorescence is dependent on many factors; but at higher concentration of Hg, the GFP fluorescence was still at a lower level than expected, and linearity was lost. Therefore, we continued to work on purified GFP protein and evaluated the effect of Hg^{2+} on it and how its fluorescence can be recovered.[27] The pH adjustment partially recovers the EmGFP fluorescence where the use of EDTA as a

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chelating agent recovered further by removing the excess Hg ions from the solution[27] (Figure S2). Thus, the metal ion-induced quenching[28] of GFP was minimized. This method would be helpful as it could be used to remove Hg from industrial or clinical waste after quantification by the Luc- and GFP-based biological sensors.[29−32] We, therefore, present a complete process of biomonitoring and bioremediation in a single protocol. Further in continuation, we would like to design a portable device for heavy metal detection, in which we can detect various heavy metals simultaneously by varying different specific cell-free constructs. As the cell-free transcription/translation system does not require any cellular culture at all and allows for postexperimental changes in pH and removal of Hg^{2+} excess, it can be revised as an automated system.

CONCLUSIONS

The designed microbial and cell-free biosensor presented here using the merR regulatory gene is one of the new strategies to detect mercury contaminants by tailoring microbial genetic systems that opens up an excellent platform for microorganism-based biosensing. Two recombinant DNA constructs, pRLucMer19 and pRGfpMer19, were designed and synthesized to sense mercury contaminants in water. Both the constructs showed high sensitivity to detect mercury. The GFP-based construct reduced the substrate addition step in comparison to the LucFF-based system. While using this technique, we have considered the cellular responses to the concentration of the contaminants, which we generally overlook. The contaminant itself becomes toxic to the cellular system, which results in false estimation. To avoid this, we have come up with the cell-free system, which has been found to be a better choice for the quantification of the contaminant, as it is independent of the cellular response against the contaminants. Moreover, this cell-free system allows us to get rid of the cell culture, and thus the total experimental time is drastically reduced. In summary, we can say that merR gene-based biosensors can be developed for the detection of a broad concentration range of mercury contaminants in water using these constructs and a cell-free system.

METHODS

Proposed Binding Sites and Docking. Prior to wet lab experiments, an initial bioinformatic study was done to ensure binding of the MerR protein (from *Pseudomonas* K-62 strain) to Hg^{2+}. PASS algorithm was used to calculate the center of mass of the active site, and docking was carried out with autodock 4.2, and a +2 charge was assigned to Hg manually after file preparation. Cysteine residues at the C-terminus (positions 146 and 154) show interaction with Hg^{2+}.

Bacterial Strains and Media. Plasmids were maintained in *E. coli* DH5α[33] and were cultured at 37 °C in LB media (Himedia, USA), which includes yeast extract (0.5%) along with tryptone (1%) and NaCl (0.5%) with antibiotic ampicillin (Merck, USA) (100 mg/mL) as a selection marker. *Pseudomonas* sp. K-62 was a gift from Masako Kiyono (Tokyo, Japan). The plasmid containing *E. coli* strain was grown at 25 °C in the defined media of tryptic soy broth. Subculturing was done by adding the primary culture from the log phase having an optical density (OD) of 0.4.

Construction of pRLucMer19 and pRGfpMer19. Isolation of plasmids, including *Pseudomonas* Sp. K-62 plasmids, were done using the Qiagen miniprep kit (CA, USA). The mammalian vector, pGL3 basic, was procured from Promega (WI). Polymerase chain reaction (PCR) amplification, ligation, transformation, and other required techniques were performed using standard protocols.[34] PCR was performed according to Bio-Rad using the forward primer 285Xho forward-5′-ATATACTCGAGAGATCTTGGTGCAGGCCGA-3′ and the reverse primer-719Hindrev-5′-TATTAAGCTTATACGTTGGCCCTTTTGA-3′ to generate an insert, which includes the merR gene, promoter, and the operator region. This insert was finally cloned into the pGL3 basic vector (Promega) to construct pRLucMerR19.

Second, PCR was performed according to Bio-Rad using the forward primers 5′-ATACCATGGATGGTGAGCAAGGGCGAGGAGCT-3′& 5′-ATTATCTAGATTACTTGTACAGCTCGTCCAT-3′as reverse primer to get an insert, which was cloned into the EmGFP vector (Promega) for construction of pRGfpMer19. The whole work plan and sensing principle are shown in (Figure 1), as a pRSET-EmGFP vector was double-digested with NcoI and Xba I (New England Biolabs, USA) to make compatible ends to ligate with the pGL3-basic vector (Promega) to make pRGFPMer19. Then, this construct was double-digested with XhoI and HindIII to clone the merR gene along with the promoter and operator. Chemical analyses of mercury compounds were done, and the standard methods of the American Public Health Association[35] were followed.

Treatment of Mercury and Activity Assays for LucFF. *E. coli* DH5α cells having the constructs, pRLucMer19 and pRGfpMer19, were cultured in 5 mL of 100 mg mL^{-1} ampicillin-containing LB media for 16 h at 37 °C. Subculturing was done, and mercury solutions (Merck, USA) of different concentrations (1 to 10^5 ppb) were added to the media of the subculture at the log phase (OD = 0.4) at 600 nm. After 1 h, the cells were withdrawn and lysed with the lysis buffer as provided by the manufacturer (Promega). Thereafter, centrifugation was performed to get the supernatants. Finally, luminescence was measured using a luminometer (Berthold, Germany).

Calculations.[36] Induction of the plasmid construct (sensor) by mercury can be expressed as the normalized luminescence given by the following equation

$$NL = SL_S/SL_B \times CF$$

where luminescence produced by the plasmid construct (pRLucMer19) in different mercury concentrations is denoted by SL_S, and uninduced luminescence of the same plasmid is defined by SL_B. The correction factor is denoted as CF, and it accounts for the possible interference of the sample turbidity, color, and change in microbial growth kinetics due to the addition of the substrate, which has to be quantified. The CF is determined by the luminescence produced by the microbial culture (termed as the control) without (LB) and with (LS) induction of the substrate and denoted by the expression by CF = LB/LS.

Fluorescence Analysis. To check the effect of mercury exposure on the EmGFP activity, a Cary Eclipse fluorescence spectrophotometer (Agilent, USA) was used. An excitation wavelength of 488 nm was used for scanning, and an emission wavelength range of 500−600 nm was used to check EmGFP activity. The filter bandwidth was adjusted at 0.5 nm. The bandwidth of the excitation and emission filters was set up to ±5 nm. For copper and cadmium ions, cadmium acetate

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(Qualigens) and cupric sulphate extrapure (SD fine chemicals) were used.

Cell-Free Protein Expression. Cell-free induction of MerR was done by PURExpress Kit (NEB, USA) at different concentrations of mercury solution. Plasmid pRLucMer19 was induced with six concentrations of mercury (Merck, USA) solutions (1, 10, 100, 1000, 10 000, and 100 000 ppb). Concentrations of plasmids and reagents were kept according to the manufacturer manual (New England Biolabs). These experiments were also followed for pRGfpMer19.

SEM. The treated (with mercury) and untreated *E. coli* recombinant cells were prepared for the SEM study. For SEM samples, the thickness may be up to 1 cm^3. Fixation was done in 2.5% glutaraldehyde (Merck, USA) and 2% paraformaldehyde (Merck, USA) made in 0.1 M sodium phosphate (Merck, USA) buffer (pH 7.2). After that, the samples were dehydrated by ethanol series by immersing in 50% and 70% ethanol for 10 min and in 100% ethanol for 20 min. After dehydration, the samples were gold coated and viewed under a Zeiss EV040 scanning electron microscope.

FACS. The internal complexity in terms of granularity of bacterial cells was measured by a flow cytometer (BD FACSVERSE, BD Bioscience, USA) after treatment with increasing concentration of heavy metal. The *E. coli* cells (10^9 cfu/mL) were grown overnight and treated with mercury at a final concentration range of 1 to 10^5 ppb for 1 h. Then, the forward and side scatterings of the treated cells were analyzed by a flow cytometer. The untreated bacteria were used as the control here.

Cell Killing Assay. LB agar plates were used to check the growth of recombinant *E. coli* cells after addition of mercury (Hg) at a broad range of concentrations (1 to 10^5 ppb). All plates were incubated for 10 h followed by colony counting for each plate. Four dilutions (10×, 100×, 1000×, and 10 000×) of bacterial culture were maintained to get distinguished bacterial colonies. Colonies were counted manually.

Fluorescence Quenching of EmGFP Due to Mercury. To check the fluorescence quenching of EmGFP by mercury, fluorescent spectroscopy was performed. Purified EmGFP was kept in phosphate-buffered saline (PBS, 0.01 M, pH = 7.4) buffer. Purified green fluorescent protein (EmGFP) was incubated with mercury at different concentrations to check the quenching effect of the EmGFP expression. The protein (1 μg) was incubated with different concentrations from 1 to 10^5 ppb for 1 h of incubation time.

pH Effect. Hg solutions were made in three pH buffers: first was in very low pH (1 because of HNO_3), second in pH 4, and third in neutral pH 7. All pH conditions were used to take fluorescence data to compare.

Removal of Excess Hg Using EDTA as the Chelating Agent. EDTA was used to chelate the excess amount of Hg in the solution. Different concentrations (1, 10, 15, and 100 mM) of EDTA were used. Out of these concentrations, significant data was obtained at a 15 mM concentration of EDTA.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b00205.

Comparative table for existing bacterial biosensors for mercury, luminescence measured by mercury concentration-dependent induction and expression with pRLucMer19 plasmid using cell-free transcription and translation kit, EmGFP expression and its measurements using fluorescence spectroscopy when induced with different cations (Hg^{2+}, Cu^{2+}, and Cd^{2+}) at 100 μM concentration to whole-cell and cell-free biosensors, dot plot of forward and side scatterings of treated (with mercury) and untreated samples, dot plot of forward and side scatterings of treated (with mercury concentration of 10^5 ppb) samples, scanning electron micrographs for mercury incubated different cells, bacterial cell killing assay with an initial increase (up to 10 ppb) followed by a decrease (up to 10^5 ppb) in a number of bacterial cells, effect of pH on GFP expression shown at low pH (1.5) and neutral pH (7) as neutralization of the pH was done, and recovery of the EmGFP fluorescence even at higher concentration (10^4 ppb) of Hg (PDF)

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Notes

The authors declare no competing financial interest.

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