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Bioluminescence-Triggered Photoswitchable Bacterial Adhesions enable Higher Sensitivity and Dual-readout Bacterial Biosensors for Mercury

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Supporting Information Placeholder

ABSTRACT: We present a new concept for whole-cell biosensors that couples the response to Hg^{2+} with bioluminescence and bacterial aggregation. This allowed us to use the bacterial aggregation to preconcentrate the bioluminescent bacteria at the substrate surface and increase the sensitivity of Hg^{2+} detection. This whole-cell biosensor combines a Hg^{2+}-sensitive bioluminescence reporter and light-responsive bacterial cell-cell adhesions. We demonstrate that the blue luminescence in response to Hg^{2+} is able to photoactivate bacterial aggregation, which provides a second readout Hg^{2+} for detection. In return, the Hg^{2+}-triggered bacterial aggregation leads to faster sedimentation and more efficient formation of biofilms. At low Hg^{2+} concentrations, the enrichment of the bacteria in biofilms leads to an up to 10-fold increase in the signal. The activation of photoswitchable proteins with biological light is a new concept in optogenetics, and the presented bacterial biosensor design is transferable to other bioluminescent reporters with particular interest for environmental monitoring.

KEYWORDS: mercury, biosensor, bacteria adhesion, bioluminescence, photoswitchable, dual-readout

Unfortunately, mercury commonly pollutes the environment as a result of both anthropogenic sources and natural processes, posing a danger to all forms of life. Mercury is highly toxic to humans and accumulates through the food chain in the liver, kidneys, and the nervous system.[1, 2] Due to continuing concerns over mercury pollution in the environment and its deleterious effects on human health,[3] new ways to detect mercury with high sensitivity and selectivity in complex environmental samples are necessary, but challenging.[4, 5]

While traditional spectrometry such as atomic absorption spectrometry (AAS)[6] and inductively-coupled plasma mass spectrometry (ICP-MS)[7] are very sensitive and selective, the large and expensive equipment and complex sample preparation limit their practicality in field studies. On the other hand, optical chemosensors[8-11] such as mercury-responsive small molecules,[12-15] polymers[16] and functionalized nanomaterials[17, 18] are simple to use and cost-effective but suffer in many cases from the interference of other heavy metal cations,[16, 19] low compatibility with complex matrices or poor water solubility.[4, 20] Whole-cell bacterial biosensors have emerged as appealing low-cost, water compatible alternatives for environmental detection with high specificity and sensiti. Such bacterial biosensors report bioavailable mercury as well as a variety of environmental pollutants in complex matrices.[21-23] Here, the design principle is that a highly sensitive, selective regulator protein recognizes the analyte and changes the expression of a fluorescent or bioluminescent reporter protein.[5, 24, 25] Few biosensors with other readouts have been developed so far.[5] For example, agglutination assays, in which bacteria express the recognition protein on their surface and the analyte leads to cell aggregation, are observed directly by eye and are easy-to-use and low cost.[26, 27]

In this manuscript, we develop a new concept for whole-cell biosensors with two outputs: bioluminescence and cell aggregation. These outputs enhance each other and provide lower detection limits. As demonstrated with a Hg^{2+} biosensor, we couple the bioluminescence response to the cell aggregation and biofilm formation. First of all, this allows us to measure these outputs independently and cross-validate the concentration of the analyte. Secondly, we take advantage of the cell aggregation to concentrate the bioluminescence signal from the bacteria and increase the signal to noise ratio.

In this new sensor design, we build on two already reported elements; a Hg^{2+} sensitive bioluminescence biosensor based on the metalloregulatory protein MerR and a luciferase reporter protein as well as the blue light-triggered bacterial cell-cell adhesions of bacteria expressing the photoswitchable proteins on their surfaces. The bioluminescence Hg^{2+} reporter (merR-lux) consists of the transcriptional regulatory protein MerR, which regulates the expression of a bacterial luciferase gene cassette (*luxCDABE*).[28] In the absence of Hg^{2+}, the luciferase expression is suppressed, whereas the binding of Hg^{2+} to MerR activates the transcription of the luciferase genes.[29] Consequently, the bacterial luciferase-catalyzed reaction generates luminescence with a maximum around 485 nm.[30]

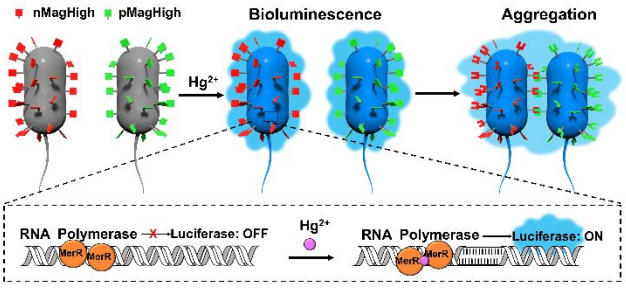

Figure 1. Design principle of the dual output bacterial biosensor for Hg^{2+}. Bacteria are equipped with the bioluminescent Hg^{2+} reporter and express either nMagHigh or pMagHigh on their surfaces. Upon Hg^{2+} binding to the transcriptional regulator MerR, the expression of the bacterial luciferase is induced and the bacteria emit blue luminescence. The luminescence activates the binding of the surface displayed photoswitchable proteins, nMagHigh and pMagHigh, inducing bacterial aggregation.

The blue light-triggered bacterial cell-cell adhesions rely on two different photoswitchable proteins, nMagHigh and pMagHigh, which are expressed on the surface of *E. coli* through fusion with the circularly permutated outer membrane proteins OmpX (CPX).

Cocultures of bacteria displaying nMagHigh and pMagHigh aggregate into large clusters under blue light (450 nm), but not in the dark,[31] [32]due to the blue light-dependent heterodimerization of these two proteins.[33] Moreover, the light-triggered bacterial clustering also alters coupled multicellular processes such as bacterial sedimentation and biofilm formation. In previous studies, an external blue LED light source was utilized, as normally done in the field of optogenetics.[31] We hypothesized that the blue bioluminescence could also be used for photoactivation of the photoswitchable proteins. Indeed, recent reports have demonstrated that nano luciferase,[34] engineered versions of it and *Gaussia* luciferase can activate different light-responsive proteins.[35, 36] In our sensor design, Hg^{2+} recognition would turn on the bioluminescence and in turn, the luminescence generated in the cells would induce the aggregation of bacteria due to the photoactivation of photoswitchable proteins (Figure 1). We foresee that the biological light generated by the bacterial cells would be an ideal internal light source for the localized illumination and the self-control of bacterial adhesions in response to the analyte. Furthermore, the activation of optogenetic systems with biological light opens the possibility to utilize them in places where light does not reach.

To realize the envisioned design, we co-expressed the merR-lux bioluminescence reporter and the surface-displayed nMagHigh (or pMagHigh) proteins in *E. coli* MG1655. In initial experiments, we verified the bioluminescent signal in response to Hg^{2+} ions while co-expressing one of the photoswitchable proteins on the surface in these bacteria. In these bacteria, the bioluminescence signal was detectable at Hg^{2+} concentrations above 1.25 nM and increased linearly with the Hg^{2+} concentration (Figure S1a). Upon the addition of Hg^{2+} the bioluminescence signal increased steadily until it reached a plateau after about 5 hours and remained stable over 18 hours (Figure S1b). The long lasting and stable bioluminescence is important to sustain the activation of the photoswitchable proteins.

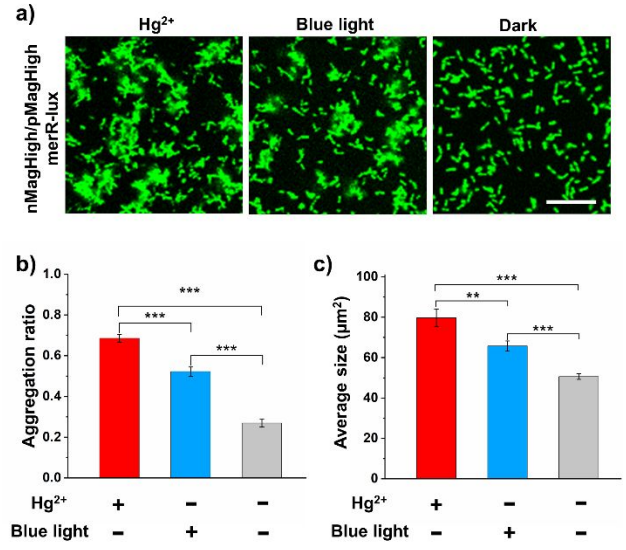

Figure 2. Hg^{2+}-dependent bioluminescence activates bacterial aggregation. a) Microscopy images of nMagHigh and pMagHigh displaying merR-lux *E. coli* in the presence of Hg^{2+} (1.25 μM), under blue light and in the dark. The bacteria were stained with Hoechst 33342 (shown in green), nMagHigh and pMagHigh displaying bacteria were mixed in a 1:1 ratio (OD_{600} = 0.15) and incubated for 2 hours. Scale bar is 20 μm. b) Aggregation ratio, and c) the average cluster size of cultures shown (a). The aggregation ratio was defined as the area of bacterial aggregates (objects with an area > 30 μm^2) divided by the area occupied by all bacteria. The error bars are the standard error from 25 images. $^{**}p < 0.01$. $^{***}p < 0.001$.

Next, we tested our hypothesis that the blue bioluminescence output of the Hg^{2+} sensor could activate the photoswitchable bacteria aggregation. For this purpose, we mixed merR-lux bacteria that either displayed nMagHigh or pMagHigh in a 1:1 ratio (OD_{600}=0.15) and incubated them with Hg^{2+} (1.25 μM). The same cocultures were kept in the dark and under blue light illumination in the absence of Hg^{2+} as negative and positive controls, respectively. For better visualization, the bacteria were pre-stained with Hoechst 33342 and after 2 hours of incubation, imaged under the microscope (Figure 2a). Large bacterial aggregates were observed in samples incubated with Hg^{2+} or kept under blue light, but not in samples kept in the dark in the absence of Hg^{2+}. To quantify the bacterial aggregation, the aggregation ratio and the average aggregate size were determined. As a parameter, the aggregation ratio was defined as the area occupied by clustered bacteria (only objects bigger than 30 μm^2 were considered clusters, the average area of a single *E. coli* is 2.5 μm^2) divided by the total area occupied by all bacteria (Figure 2b). The aggregation ratio showed that in samples incubated with Hg^{2+}, 70% of the bacteria were integrated into clusters, which was even higher than the aggregation ratio of samples kept under blue light (55%) as a positive control. On the other hand, only 27% of bacteria were clustered in the dark in the absence of Hg^{2+}. Parallel to the aggregation ratio, the average cluster size (only objects with an area > 30 μm^2 were counted as clusters) was also significantly larger in the sample with Hg^{2+} (80 μm^2) than the clusters in samples kept under blue light (positive control, 70 μm^2) or in the dark (negative control, 50 μm^2) (Figure 2c). It is also remarkable that not only did the Hg^{2+}-treated bacteria cluster, but both the aggregation ratio and the size of these clusters were larger than for the bacteria kept under blue light. These findings suggest that the bacteria-generated bioluminescence is a more effective inducer of nMagHigh-pMagHigh interactions than external blue light. A potential reason for this is that the bioluminescence emitted directly from the *E. coli* is so close to the photoswitchable proteins on the bacteria surface such that light losses from scattering and absorption are reduced.

Taken together, the bioluminescence from the merR-lux sensor was strong enough to activate the photoswitchable bacterial cell-cell adhesions. It should be noted that the absorption maxima of the photoswitchable proteins nMagHigh and pMagHigh at 450 nm and the bioluminescence emission maximum of the bacterial luciferase at 500 nm are spectrally close to each other and both of these peaks are quite broad, leading to a significant overlap (Figure S2). The photoactivation of the photoswitchable proteins by the bacterial bioluminescence is further supported in solution experiments with purified nMagHigh protein. The typical absorbance peak of nMagHigh at 450 nm decreased significantly after 10 min incubation with the bioluminescent bacteria in the presence of Hg^{2+} (Figure S2), which confirms efficient photoactivation.

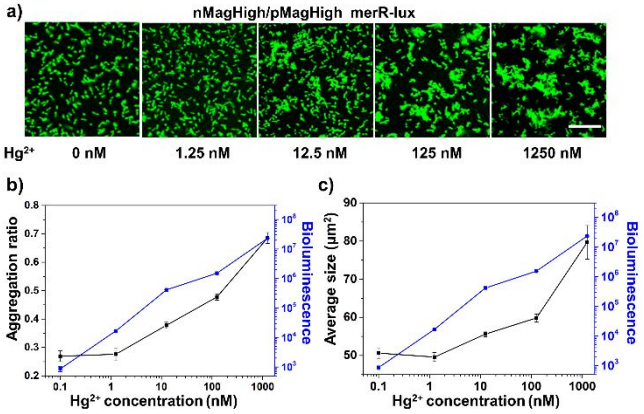

Figure 3. Bacterial aggregation depends on Hg^{2+} concentration. a) Microscopy images of nMagHigh and pMagHigh displaying merR-lux *E. coli* (stained with Hoechst 33342, shown in green) (OD_{600} = 0.15), which were incubated with different concentrations of Hg^{2+} for 2 h. Scale bar is 20 μm. b) Aggregation ratio and bioluminescence, and c) the average cluster size and bioluminescence of cultures in (a). The error bars are the standard error from 25 images.

As previously demonstrated, the activation of photoswitchable bacteria-bacteria adhesions depends on the light intensity when an external light source is used.[32] This suggests that the bacterial bioluminescence signal, and thus the Hg^{2+} concentration, could alter the bacterial aggregation. To examine this, we quantified the aggregation nMagHigh and pMagHigh-displaying merR-lux bacteria at varying Hg^{2+} concentrations after 2 hours (Figure 3a). At the Hg^{2+} concentration of 1.25 nM, there were no more visible bacterial aggregates compared to the dark control in the absence of Hg^{2+}. Yet, when the Hg^{2+} concentration was increased to 12.5 nM, the bacterial aggregation was partially activated and 37% of the bacteria integrated into clusters with an average size of 56 μm^2 (Figure 3b-c). The bacterial aggregation ratio increased linearly with the logarithmic Hg^{2+} concentration (Figure 3b, Figure S3) and clusters with a larger average size formed with increasing Hg^{2+} concentrations. Thus, the aggregation ratio is a second readout for Hg^{2+} concentrations with a detection limit of 12.5 nM. It should be noted that the aggregation ratio correlates differently to the mercury (II) concentration than the bioluminescence, which increases linearly with Hg^{2+} concentration over the same range (Figure 3b). Moreover, the biosensor was also be used to detect doped in mercury in environmental samples as shown with water taken from a local lake. We observed similar aggregation and bioluminescence read-outs (Figure S4), which opens the possibility for environmental detection.

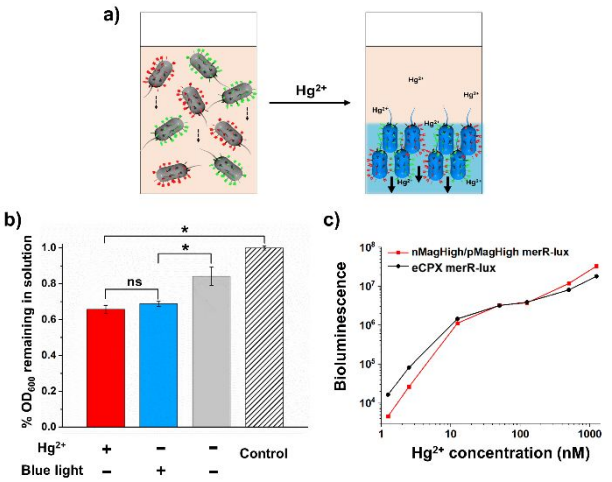

Figure 4. Bacterial sedimentation for increased sensitivity in Hg^{2+} detection. a) Bacterial aggregation of bacterial displaying nMagHigh and pMagHigh due to the Hg^{2+} triggered bioluminescence leads to faster sedimentation from the solution. This should lead to a preconcentration of the bioluminescent bacteria in the lower part. b) Bacterial sedimentation in co-cultures of nMagHigh and pMagHigh displaying merR-lux *E. coli* (initial OD_{600}= 1.0) for 2 h in the presence of Hg^{2+} (1.25 μM), under blue light or in the dark. OD_{600} in the top 25 % of the culture was used as a measure. *$p < 0.05$. c) Bioluminescence of nMagHigh and pMagHigh displaying merR-lux *E. coli* in the presence of different Hg^{2+} concentrations in the bottom-half of the solution after 2h. The error bars are the standard error from 3 replicates. eCPX merR-lux bacteria not displaying any photoswitchable protein were used as controls in (b) and (c).

The mercury-dependent aggregation of the bioluminescent bacteria opens the possibility to preconcentrate the biosensors upon analyte detection and increase the bioluminescence signal and sensitivity of the biosensor. As demonstrated previously, bacterial cell-cell adhesions change multicellular behavior and enhance bacterial sedimentation as well as biofilm formation.

One approach to locally collect the bioluminescent bacteria is to use the bioluminescence-induced bacterial aggregation and settling out of solution in response to Hg^{2+} (Figure 4a). To demonstrate this, we measured the sedimentation of cocultures of nMagHigh and pMagHigh-displaying merR-lux bacteria in the presence of Hg^{2+} (1.25 μM) by measuring the optical density (OD_{600}) of the upper 25% of cultures unshaken after 2 hours (Figure 4b). The coculture settled faster in the presence of Hg^{2+} and to the same extent as the positive control kept under blue light illumination without Hg^{2+}. Moreover, the bioluminescence alone in bacteria, which do not express photoswitchable proteins at their surfaces (control), did not lead to more sedimentation due to unforeseen interactions or visible bacterial aggregation (Figure S5). Likewise, the expression of photoswitchable proteins on the bacteria does not change the bioluminescence signal in response to Hg^{2+} compared to the control bacteria (Figure S1).

The remaining question is if the Hg^{2+}-induced bacterial sedimentation can be used to achieve higher sensitivity Hg^{2+} detection. To answer this question, cocultures of nMagHigh and pMagHigh-displaying merR-lux bacteria were exposed to different concentrations of Hg^{2+} for 2h, and their response was compared to control bacteria that did not express photoswitchable proteins on their surface. During this time before the luminescence of the bottom half of the samples was measured, the bacteria were allowed to sediment (Figure 4c). We observe a difference in bioluminescence compared to the control bacteria without photoswitchable proteins on their surfaces only at Hg^{2+}

concentrations higher than 500 nM. Although the bacterial aggregation was already significant at 12.5 nM Hg^{2+}, the impact on sedimentation is only large enough at concentrations above 500 nM to result in significant accumulation of bacteria.

The second option for concentrating the bioluminescent signal is to rely on the increased biofilm formation when bacteria adhere to each other, as has already been reported for the photoswitchable bacterial cell-cell adhesions.[32] To test if biofilm formation can be enhanced in the presence of Hg^{2+}, we seeded a mixture of nMagHigh- and pMagHigh-displaying merR-lux bacteria into glass chambers, incubated these for 48 h at 37 °C and acquired confocal fluorescence microscopy images of the biofilms stained with the Hoechst 33342 dye (Figure 5a). Bacterial mixtures incubated without Hg^{2+} but kept under blue light and in the dark were used as positive and negative controls, respectively. These images showed that a biofilm similar to the positive control sample formed in the presence of Hg^{2+}, while little biofilm formation was observed in the negative control in the absence of Hg^{2+}. Quantification of the biofilm thickness showed that the biofilm was thicker in the presence of Hg^{2+} (22 µm) than that in the negative control (12 µm), and comparable to that in the positive control (18 µm) (Figure 5b). These findings were further supported with on the crystal violet staining of the biofilms (Figure S6).

The mercury-induced biofilm formation represents a good way to concentrate the bioluminescence signal on a surface, especially for long term monitoring the environmental status of mercury. To demonstrate the potential signal amplification and increase in sensitivity, we compared the bioluminescence signal of biofilms formed by merR-lux bacteria with and without the photoswitchable proteins on their surfaces. For this purpose, different concentrations of Hg^{2+} were added to the bacteria during the biofilm formation over 48 h and subsequently the planktonic bacteria were washed away. The biofilms formed from the co-culture of nMagHigh- or pMagHigh-displaying bacteria showed much higher bioluminescence signals than the bacteria without these proteins (Figure 5c). The increase in sensitivity of bacteria displaying nMagHigh and pMagHigh was particularly pronounced at low concentrations close to the detection limit of the bioluminescence biosensor (Figure 5d). At 1.25 nM Hg^{2+}, the bioluminescence signal was already 4.4-fold higher in merR-lux bacteria displaying the photoswitchable proteins than the control bacteria. This increase in sensitivity even grew to 10.9-fold at 2.5 nM Hg^{2+}. These findings clearly show that bioluminescence-induced biofilm formation can preconcentrate the bacteria on a substrate upon analyte detection and improve the sensitivity of Hg^{2+} detection.

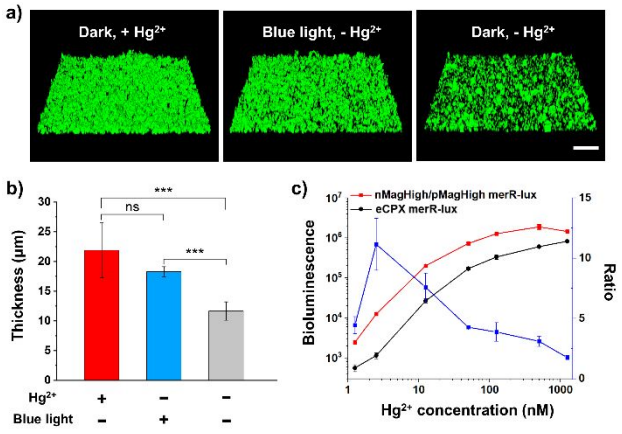

Figure 5. Hg^{2+} activated bioluminescence enhanced biofilm formation for increased sensitivity in Hg^{2+} detection. a) Confocal laser scanning microscopy images of biofilms formed with nMagHigh and pMagHigh displaying merR-lux bacteria (stained with Hoechst 33342, shown in green) after 48 hours, in the presence of Hg^{2+} (1.25 µM), under blue light or in the dark. Scale bar is 100 µm. b) Biofilm thickness in (a). The error bars are the standard error from 3 replicates. ***$p < 0.001$. c) Bioluminescence signal from biofilms of merR-lux *E. coli* with or without (eCPX, control) nMagHigh and pMagHigh at their surfaces in the presence of different Hg^{2+} concentrations and the ratio of the bioluminescence signals of the two samples. The error bars are the standard error from 3 replicates.

In this work, we presented a novel design concept for bacterial biosensors with two outputs, bioluminescence and bacterial aggregation, in response to the analyte. The new biosensor design, as tested for Hg^{2+}, combines an existing bioluminescent biosensor and photoswitchable bacterial cell-cell adhesions. We demonstrated that the blue bioluminescence output in response to Hg^{2+} could be used to activate the blue light-triggered bacterial adhesions and lead to bacterial aggregation. This results in a dual-output biosensor, where the bacterial aggregation can also be used to preconcentrate bioluminescent bacteria in aggregates, as well as biofilms, and increase the sensitivity of Hg^{2+} detection. The synergistic effect of the two outputs is especially beneficial to detect low concentrations of Hg^{2+} with biofilms with up to 10-fold signal enhancement. This approach is transferable to bioluminescent bacterial sensors for other heavy metals and environmental pollutants.

The activation of the blue light-responsive bacterial cell-cell adhesions with bacterial luciferase provides a new mode of photoactivation in the field of optogenetics. The concept of using biological sources of light is appealing for photoactivation in deep tissue or other places where physical light sources cannot reach. As bioluminescence is widely used as a readout for reporter assays in various types of cells, this provides a great opportunity for coupling to existing optogenetic systems to self-regulate intracellular events. These bioluminescence-activated bacteria-bacteria adhesions provide an alternative tool in the study of bacterial cell biology and the regulation of bacterial consortia. Therefore, we anticipate that this intersection of optogenetics with bioluminescence for photoactivation will be of interest for the biosensor, optogenetics and synthetic biology communities.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org. Experimental details, bioluminescence assay for Hg^{2+} detection; photoactivation of nMagHigh protein with bioluminescent bacteria; kinetics of bioluminescence upon the addition of Hg^{2+}; Hg^{2+} detection in an environmental sample; crystal violet stain of biofilms.

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Notes

The authors declare no competing financial interests.

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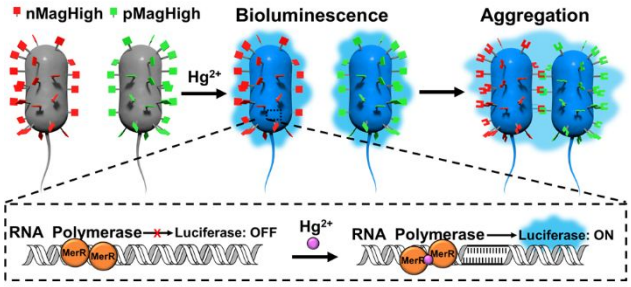
For TOC only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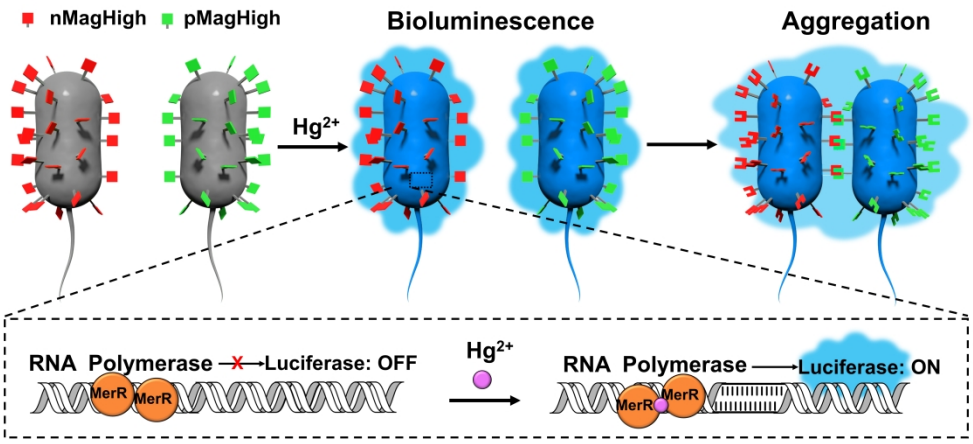

Fig. 1

558x304mm (300 x 300 DPI)

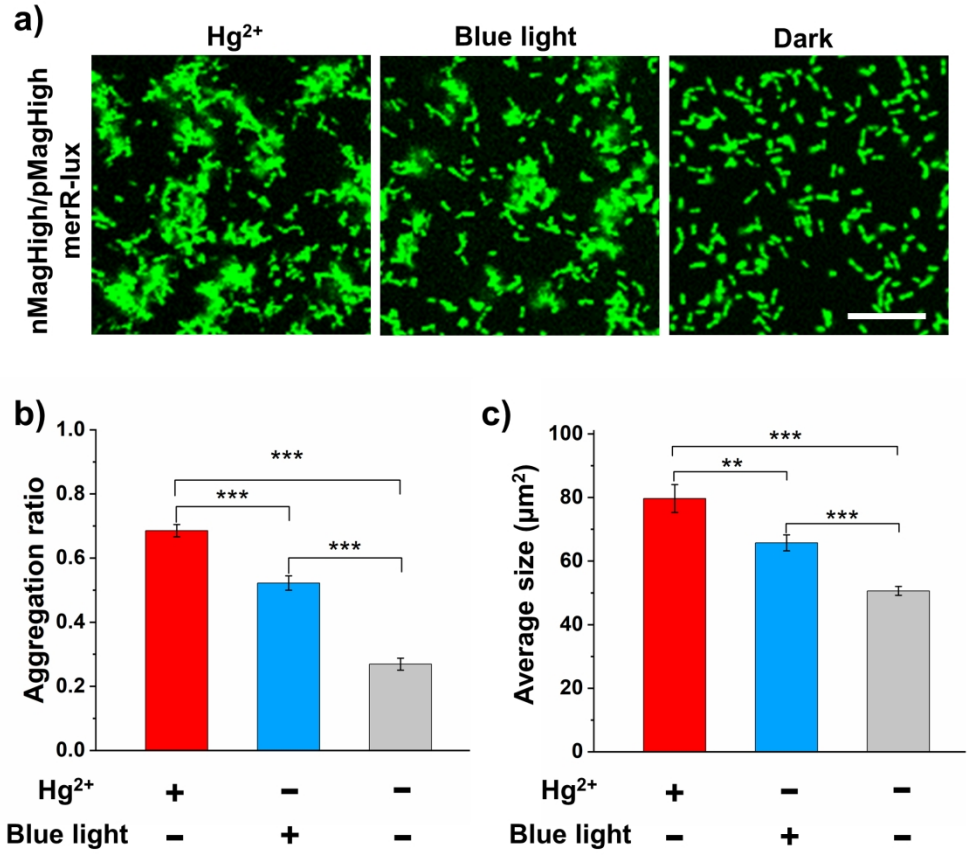

Fig. 2

181x159mm (300 x 300 DPI)

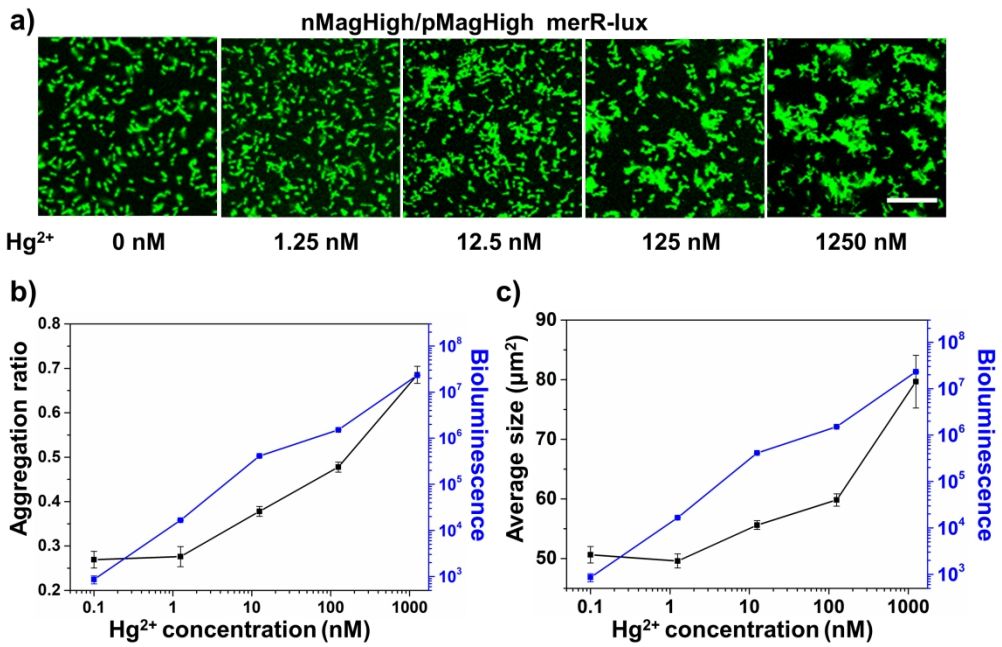

Fig. 3

241x160mm (300 x 300 DPI)

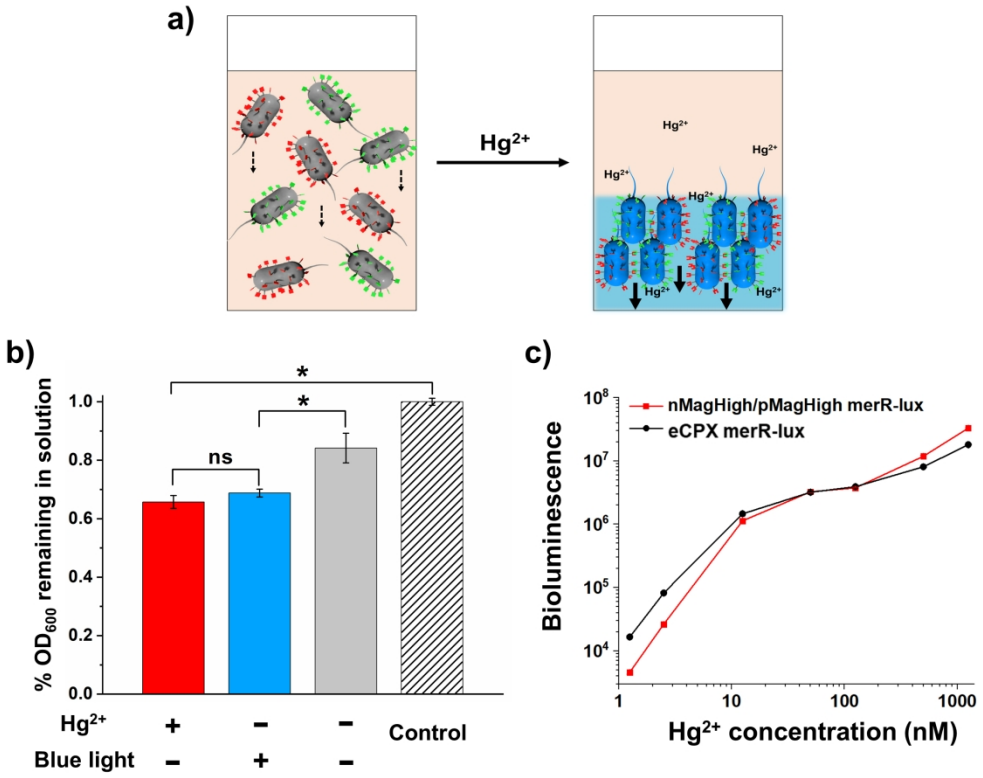

Fig. 4

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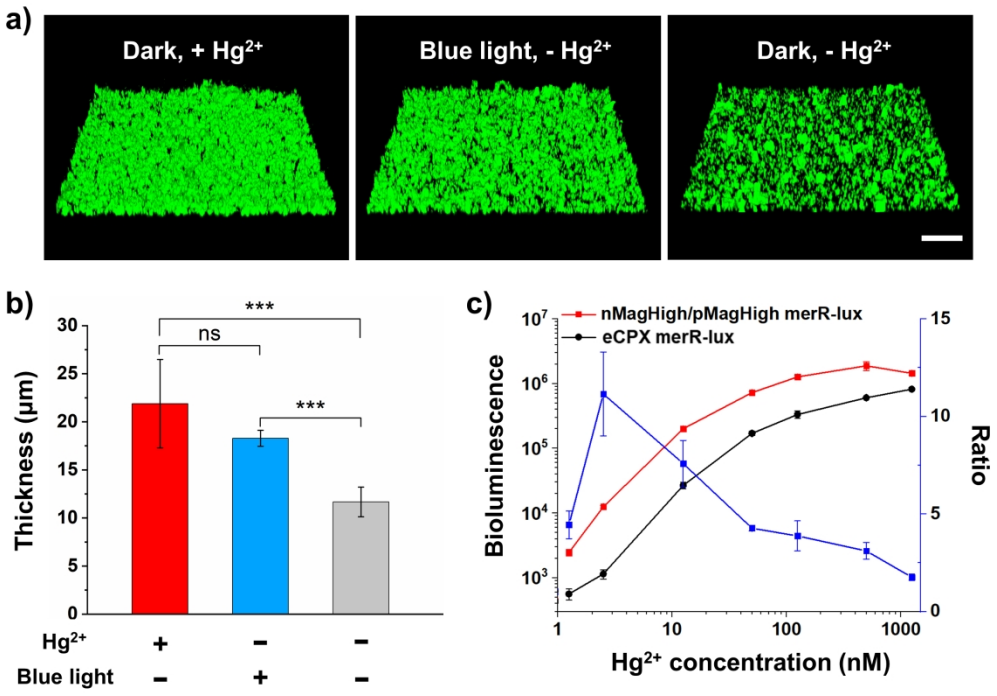

Fig. 5

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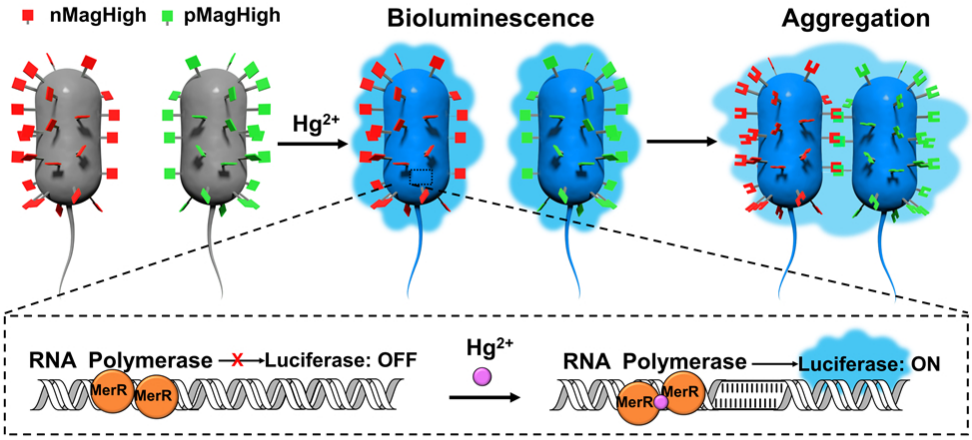

TOC

84x47mm (300 x 300 DPI)