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A Whole-Cell Bacterial Biosensor for Blood Markers Detection in Urine

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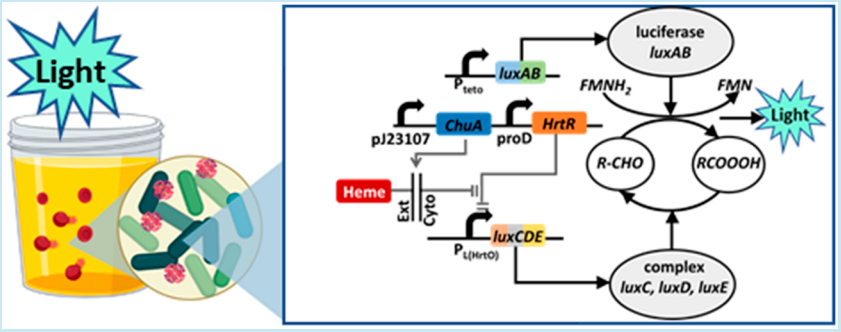

ABSTRACT: The early detection of blood in urine (hematuria) can play a crucial role in the treatment of serious diseases (*e.g.*, infections, kidney disease, schistosomiasis, and cancer). Therefore, the development of low-cost portable biosensors for blood detection in urine has become necessary. Here, we designed an ultrasensitive whole-cell bacterial biosensor interfaced with an optoelectronic measurement module for heme detection in urine. Heme is a red blood cells (RBCs) component that is liberated from lysed cells. The bacterial biosensor includes *Escherichia coli* cells carrying a heme-sensitive synthetic promoter integrated with a luciferase reporter (*luxCDABE*) from *Photorhabdus luminescens*. To improve the bacterial biosensor performance, we re-engineered the genetic structure of *luxCDABE* operon by splitting it into two parts (*luxCDE* and *luxAB*). The *luxCDE* genes were regulated by the heme-sensitive promoter, and the *luxAB* genes were regulated by either constitutive or inducible promoters. We examined the genetic circuit's performance in synthetic urine diluent supplied with heme and in human urine supplied with lysed blood. Finally, we interfaced the bacterial biosensor with a light detection setup based on a commercial optical measurement single-photon avalanche photodiode (SPAD). The whole-cell biosensor was tested in human urine with lysed blood, demonstrating a low-cost, portable, and easy-to-use hematuria detection with an ON-to-OFF ratio of 6.5-fold for blood levels from 5×10^4 to 5×10^5 RBC per mL of human urine.

KEYWORDS: *whole-cell biosensor, bioluminescence, luxCDABE, blood cells, hematuria, single-photon avalanche photodiode*

Recent advances in synthetic biology and bioelectronics have enabled the development of innovative biosensing systems for advanced healthcare by providing fully integrated platforms for the detection of a large set of human disease-related biomarkers. The idea is to create low-cost analytical tools for detecting environmental contamination by toxic chemicals in the environment,[1,2] for biomedical applications in diagnostics,[3] and for health monitoring.[4] Whole-cell bacterial biosensors consist of biological elements, typically genetically modified bacteria, integrated with an electronic detector.[5] Such bacterial cells include sensory components fused with fluorescent proteins (*e.g.*, green fluorescent protein − GFP), bioluminescence (*e.g.*, luciferase), or β-galactosidase (*lacZ*).[6] Among the different reporter proteins, the bioluminescent reporter encoded by the *luxCDABE* cassette has been increasingly adopted due to its high sensitivity, fast response, and independent light generation without using external substrates. Moreover, it has recently been shown that the *luxCDABE* cassette can be engineered to detect and integrate multiple signals.[7]

The recent developments of microelectronics and information technologies have also led to significant improvements in whole-cell bacterial biosensor performance. For example, whole-cell bacterial biosensors have been integrated with a single-photon avalanche photodiode (SPAD) as a real-time environmental sensor for measuring various hazardous chemicals in water.[8] Recently, a state-of-the-art micro-bioelectronic device carrying bioluminescence probiotic

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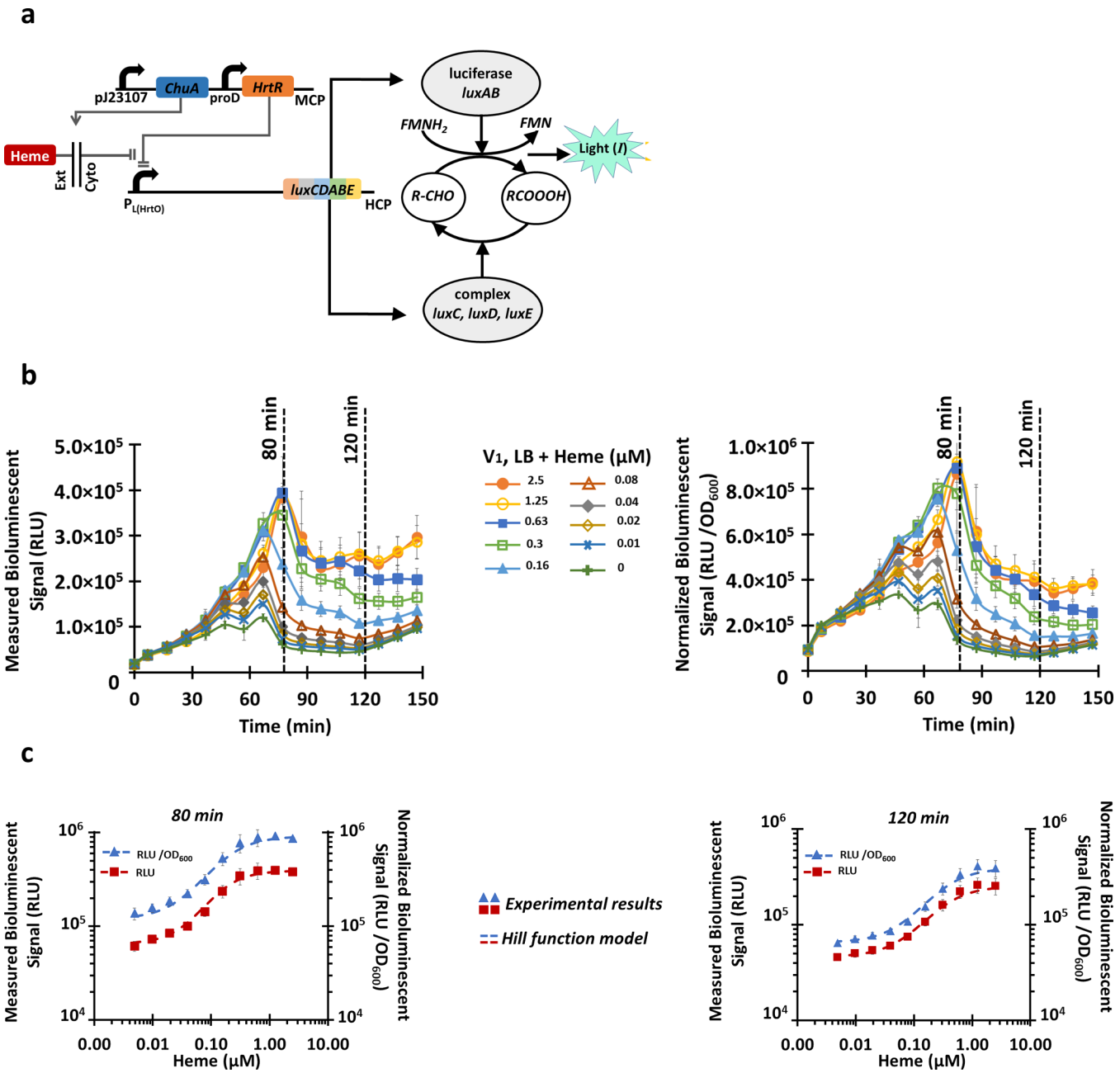

Figure 1. Characterizing the prototype heme-sensing system (V_1). (a) Prototype genetic circuit V_1 includes five lux genes (*luxCDABE*) that are regulated by a synthetic promoter $P_{L(HrtO)}$ located on a high-copy-number plasmid (HCP). The *Lactococcus lactis* heme-responsive transcriptional repressor *HrtR* and an outer-membrane transporter *ChuA* are expressed under constitutive promoters proD and pJ23107, respectively, which locate on a medium-copy-number plasmid (MCP). Extracellular heme enters bacterial cells through the outer-membrane transporter *ChuA* and interacts with the transcriptional repressor *HrtR* to allow for the expression of *lux* genes, which facilitate a luminescence reaction. The reaction involves the oxidation of a long-chain aliphatic aldehyde (R-CHO) and flavin mononucleotide ($FMNH_2$), resulting in the release of excess free energy in the form of light. The *luxCDE* genes encode for three enzymes (reductase, transferase, and synthetase) that are involved as the substrate for the luminescence reaction. (b) Time-response of bioluminescent signals from genetic circuit V_1 (left) and the signals normalized to absorbance (signal/OD_{600}, right). Bacterial cells were mixed with 10 concentrations of heme (0−2.5 μM), and luminescence signals (in RLU) were read at 10 min intervals for 2.5 h. (c) Dose−response curves are represented by the transfer function of heme-input concentration and bioluminescent outputs after 80 min (left) and 120 min (right). The dashed lines are fitted to Hill functions. The average and errors (s.e.m.) shown in the figures were derived from three experiments.

bacteria and wireless electronic communication readout circuits has been developed for *in situ* biomolecular detections of gastrointestinal bleeding.[9]

Taking advantage of the highly evolved biochemical pathways in biological systems, biosensors based on living cells are sensitive to a wider range of analytes than chemical biosensors. Also, bacterial cells can operate over a broader range of temperature and pH values and offer rapid and reliable detection in complex environments such as serum or urine samples.[10] However, developing whole-cell biosensors faces several challenges that could limit their implementations in real-world samples. For example, since analytes of interest

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are often present in low concentrations, a high sensitivity is required for reliable biosensing. Also, cells usually contain a network of metabolic pathways affected by different analytes or substances in the environment, which reduces the specificity of whole-cell bacterial biosensors. For instance, one target analyte can activate several stress-responsive promoters or the same stress-responsive promoter can be activated by different analytes.[1] Therefore, early efforts in synthetic biology focused on solving these challenges and improving bacterial biosensor performance. For example, genetic logic gates integrating multiple inputs have increased biosensor sensitivity[11] or incorporating an analog design has broadened the operating range.[12] A design that can reduce the unwanted cross-talk between chemical substances and stress-responsive promoters has been recently implemented, based on a combined genetic and metabolic crosstalk-compensating circuit.[7] Moreover, a cascaded signal amplification has been implemented to achieve ultrasensitive cellular sensing.[13]

In this study, we designed and built an ultrasensitive bacterial biosensor to detect blood in urine. A large portion of blood cells (about 40%) is red blood cells (RBCs), which carry the hemoglobin for oxygen transportation. Hemoglobin has four subunits, each with one polypeptide chain and one heme group. The detection of high heme levels in urine can indicate the possible presence of free hemoglobin (hemoglobinuria) or blood (hematuria[14]). Several diseases can cause hematuria, such as the infection of the urinary tract, bladder and kidney cancer, polycystic kidney disease, bladder or kidney stones, and schistosomiasis. Nowadays, hematuria is diagnosed in laboratory tests by demonstrating RBCs in the urinary sediment through qualitative and quantitative microscopy[15] or by quick urine dipstick tests. Such tests often yield false-positive results if the urine contains myoglobin from damaged muscle cells or when high doses of vitamin C are digested.[16] Thus, developing sensors for earlier hematuria diagnostics at home or in remote locations is still necessary.

To this end, we developed a new generation of low-cost, portable, and easy-to-use biosensors for hematuria detection that includes two parts: (1) a synthetic gene circuit that detects heme in synthetic urine with a 42-fold change in ON-to-OFF ratio. The indication of blood presence is related to the heme molecules, which are released by lysed RBC. The gene circuit includes a synthetic promoter activated by heme and an engineered bacterial luciferase reporter (*luxCDABE*). (2) The second part is a light detection setup based on commercial SPAD for measuring bioluminescence in terms of the number of photons emitted from bacteria. Our new genetic circuit designs dramatically increased the biosensor sensitivity by reducing its background signal and achieving sufficient maximum output expression without compromising the detection limit and operating range. We demonstrated that our biosensors could detect heme molecules in synthetic urine and blood levels in human urine.

RESULTS AND DISCUSSION

Prototype Biosensor Application for Heme Solution. A prototype heme-sensing system[9] was kindly supplied as a gift by Tim Lu (Massachusetts Institute of Technology, MIT). The biological system that we named as version V_1 (Figure 1a) includes *Escherichia coli* cells carrying a heme-sensitive promoter, which regulates the bioluminescent *luxCDABE* reporter. The bacterial luminescence reaction involves the oxidation of a long-chain aliphatic aldehyde and a flavin mononucleotide that releases excess free energy in the form of light.[17] The luciferase encoded by the *luxAB* genes regulates the forward reaction. The reductase, transferase, and synthetase encoded by *luxCDE* genes regulate the reverse reaction and produce the aldehyde substrate of luminescence. In the V_1 heme-sensing system, both *luxAB* and *luxCDE* genes are regulated by the synthetic promoter $P_{L(HrtO)}$,[9] located on a high-copy-number plasmid (HCP). The *Lactococcus lactis* heme-responsive transcriptional repressor *HrtR*[18] and an outer-membrane transporter *ChuA* from *Escherichia coli* O157:H7[19] are expressed under constitutive promoters, located on a medium-copy-number plasmid (MCP). The extracellular heme is taken up through the outer-membrane transporter *ChuA* and interacts with the transcriptional repressor *HrtR* to control the expression of reporter genes.

The prototype blood biosensor cells (V_1) were mixed with various heme concentrations (0−2.5 μM), dissolved in LB (Figure 1b) or minimal media (Figure S3 of the Supporting Information) with suitable antibiotics, transferred into a 96-well plate, and immediately placed in the plate reader at 37 °C with continuous shaking. Luminescence (in RLU) and absorbance (OD_{600}) were read at 10 min intervals for 2.5 h. Figure 1b illustrates the kinetics of the measured bioluminescent signal (left) produced by bacterial cells. Signal levels were normalized to the optical density to account for the effect of population growth (right). Bioluminescence values increased immediately after exposure to heme and reached the maxima after 60−80 min. A maximal normalized signal value of 9.2×10^5 RLU/OD_{600} was observed at 80 min after exposure for 1.25 μM heme.

The dose−response curve describes the system behavior as an input−output transfer function, in a way that the bioluminescence signal at a specific time point is displayed as a function of heme concentrations. Using the time-response curves values at 80 and 120 min postexposure, we constructed the dose−response curves (Figure 1c). The dose−response curve is used to extract the values of important parameters for biosensor characterization (*e.g.*, operating range, leaky expression, and ON-to-OFF-response). The operating range (OR) is defined as the input's concentrations corresponding to output ranging between 10% to 90% of the maximal output. The OR ranges from 0.01 to 0.3 μM at 80 min and from 0.02 to 0.63 μM at 120 min. The leaky expression (LE) is the reporter level in response to zero heme concentration and is equal to 1.4×10^5 RLU/OD_{600} at 80 min and 6.5×10^4 RLU/OD_{600} at 120 min. The leaky expression is due to the unspecific binding of RNA polymerases to promoters. Sensitive promoters with a high leaky expression often suffer from a narrow dynamic range (DR) and a low ON-to-OFF response ratio. Here, the ON response is the maximum promoter activity and the OFF response is the minimum promoter activity. In the V_1 design, the ON-to-OFF response ratio is about 6.5-fold at 80 and 120 min. The measured transfer functions of heme to bioluminescence signals are well-fitted by Hill functions at both time points by $Y = (Y_{max}X^n)/(K_D{}^n + X^n) + \beta$, where Y is the normalized luminescence output, Y_{max} is the maximum luminescence, X is the heme concentration, K_D is a dissociation constant, n is the Hill coefficient, and β is the leaky bioluminescence (basal level). The functions have dissociation constants (K_D) of 0.15 μM at 80 min and 0.25 μM at 120 min. The Hill coefficient (n) is 1.3 for both transfer functions (Table S5 of the Supporting Information summarizes the fitting parameters).

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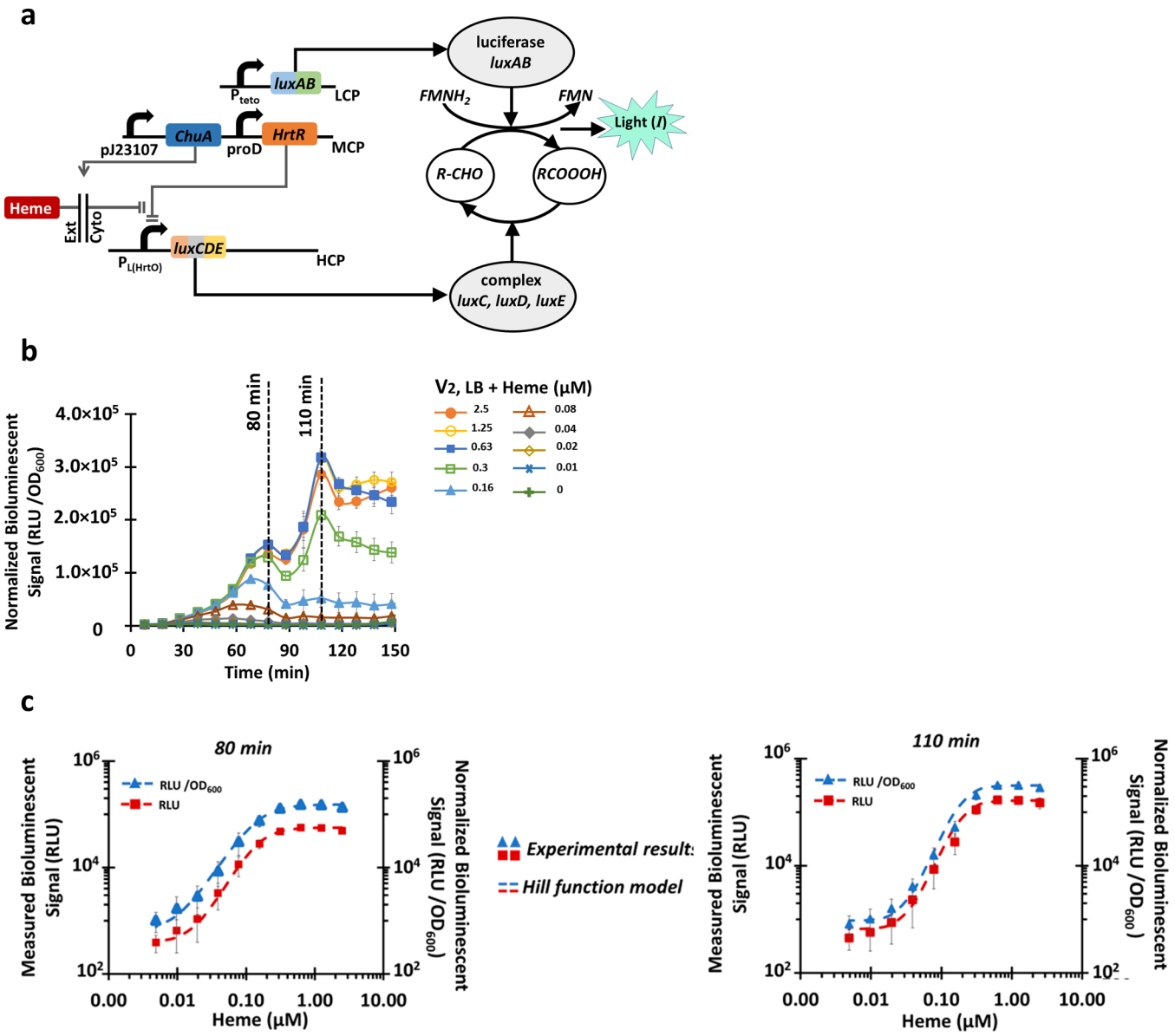

Figure 2. V_2 design of the re-engineered heme-sensing system. (a) Luciferase encoded by genes *luxAB* is constitutively expressed under P_{tetO} on a low-copy-number plasmid (LCP). The substrate-producing enzymes are encoded by the *luxCDE* genes and are regulated by heme input *via* the heme-sensitive promoter $P_{L(HrtO)}$, located on a high-copy-number plasmid (HCP). *HrtR* and *ChuA* genes are expressed under constitutive promoters proD and pJ23107, respectively, located on a medium-copy-number plasmid (MCP). (b) Time-response of V_2 design presented as normalized bioluminescent signals (signal/OD_{600}). Bacterial cells were mixed with varying concentrations of heme (0−2.5 μM), and luminescence signals (in RLU) were read at 10 min intervals for 2.5 h. (c) Dose−response curves are represented by the transfer function between heme-input concentration and bioluminescent outputs after 80 min (left) and 110 min (right). The dashed lines are fittings using the Hill function. The average and errors (s.e.m.) shown in the figures were derived from three experiments.

Further Design and Examination of Heme-Sensing Genetic Circuit Based on Split *luxCDABE* Cassette. To reduce the leaky expression while keeping the maximum luminescence output high and to enhance the dynamic range by increasing the ON-to-OFF-response ratio, we optimized the prototype genetic circuit V_1. First, we split the *luxCDABE* cassette into two subcircuits that are controlled by two different promoters (Figure 2a). In this new design (V_2), the P_{tetO} constitutive promoter, which is located on the low-copy-number plasmid (LCP), regulates the *luxAB* gene expression. The heme-sensitive promoter located on an HCP regulates the *luxCDE* gene expression. *HrtR* and *ChuA* genes are expressed under constitutive promoters and are located on an MCP.

Upon exposure to various heme concentrations, the induction of a significant bioluminescence signal could be observed in as little as 30 min (Figure 2b). The maximal normalized bioluminescence signal of 3.2×10^5 RLU/OD_{600} was observed at 110 min after exposure to 0.63 μM heme. The dose−response transfer functions (Figure 2c) revealed wide operating ranges from 0.01 to 0.16 μM at 80 min after heme exposure and from 0.01 to 0.30 μM at 110 min. The leaky expression was decreased compared to that of the V_1 to a low value of 10^3 RLU/OD_{600}, and the dynamic range was dramatically enhanced to 320-fold at 110 min time response. The Hill function fitted for bioluminescence levels at 80 min has a K_D of 0.15 μM and a Hill coefficient (n) of 2. The Hill

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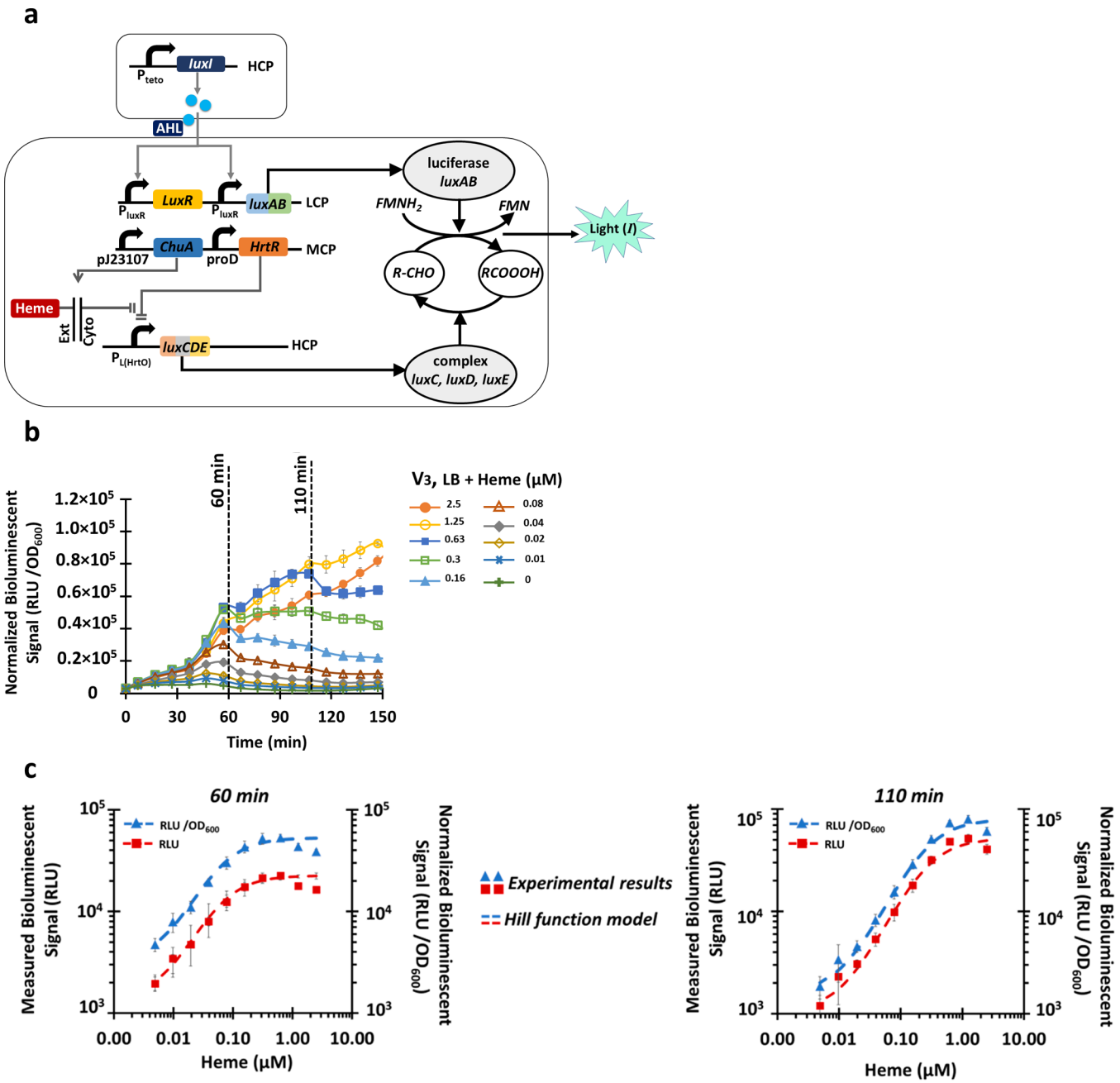

Figure 3. V_3 design of the re-engineered heme-sensing system. (a) Luciferase encoded genes *luxAB* are regulated by an inducible P_{lux} promoter, located on a low-copy-number plasmid (LCP). The P_{lux} is activated by acyl-homoserine-lactone (AHL) binding to LuxR, which is also regulated by a P_{lux} promoter located on an LCP. We used bacterial cells to produce AHL *via* LuxI protein expression from an additional bacterial strain. The AHL molecules diffuse into the reporter cells, leading to the expression of *luxAB* genes. The *luxCDE* genes are regulated by heme input *via* the heme-sensitive promoter $P_{L(HrtO)}$ on a high-copy-number plasmid (HCP). *HrtR* and *ChuA* are expressed under constitutive promoters proD and pJ23107, respectively, located on a medium-copy-number plasmid (MCP). (b) Time-response of V_3 represented by normalized bioluminescent signals (signal/OD_{600}). Bacterial cells were mixed with different concentrations of heme (0−2.5 μM), and luminescence signals (in RLU) were read at 10 min intervals for 2.5 h. (c) Dose−response curves are represented by the transfer function between heme-input concentration and bioluminescent outputs after 60 min (left) and 110 min (right). The dashed lines are fitted to Hill functions. The average and errors (s.e.m.) shown in the figures were derived from three experiments.

function fitted for bioluminescence levels at 110 min has a K_D of 0.25 μM and a Hill coefficient (n) of 3.

Next, we built a V_3 design by replacing the P_{tetO} promoter that regulates *luxAB* in the V_2 design with an inducible P_{lux} promoter, located on an LCP (Figure 3a). In the new V_3 design, the acyl-homoserine-lactone (AHL) binds to transcription factor LuxR, forming a complex that activates the P_{lux} promoter. LuxR is self-regulated by a P_{lux} promoter located on an LCP. Instead of using an external AHL supplement, we used additional *E. coli* MG1655 bacterial cells that constitutively produce AHL *via* LuxI protein expression (see Figures S4 and S5 of the Supporting Information for additional information). First, both strains were grown separately overnight, and then, one volume of cells carrying the AHL production circuit was mixed with 10 volumes of cells carrying the heme-sensitive reporting circuit. The produced AHL

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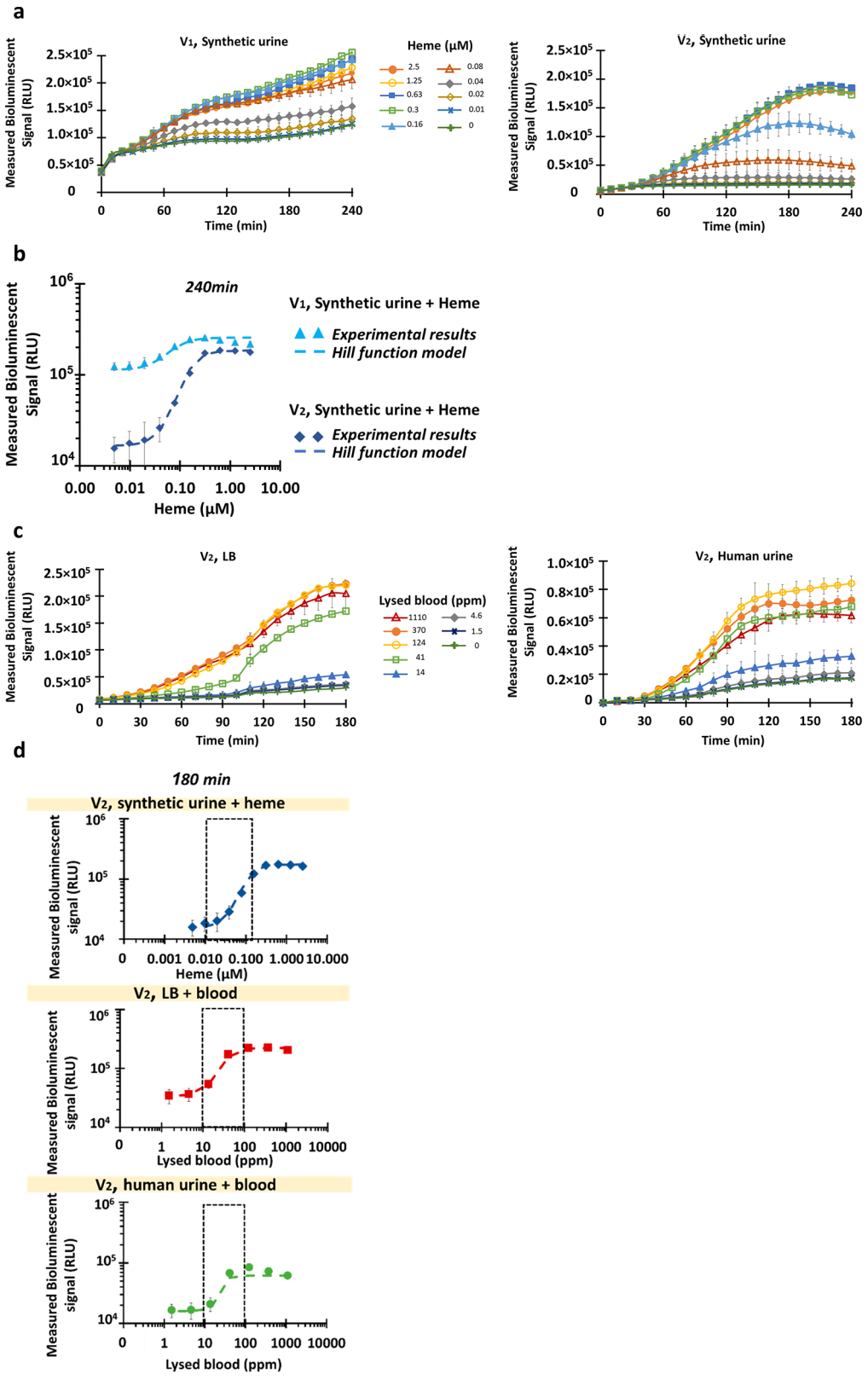

Figure 4. Activity of bacterial biosensors in urine samples. (a) Time-responses of the prototype (V_1) and redesigned (V_2) heme-sensing genetic circuits, respectively, represented by the measured (RLU) bioluminescent signals after exposure to heme dissolved in synthetic urine (0−2.5 μM). (b) Comparison of the dose−response curves for the V_1 and V_2 heme-sensing genetic circuits relative to the same levels of heme concentration at 240 min after exposure to heme. (c) Time-responses of V_2 after exposure to lysed blood in LB and human urine (0−1110 ppm). (d) Comparison of the dose−response curves for the V_2 circuit at 180 min after exposure to different levels of heme solution in synthetic urine and lysed blood in LB and human urine. The dashed lines are fitted to Hill functions. The represented data show the measured signals without normalization by optical density. The heme and blood concentrations are the urine samples' concentrations. The average and errors (s.e.m.) shown in the figures were derived from three experiments.

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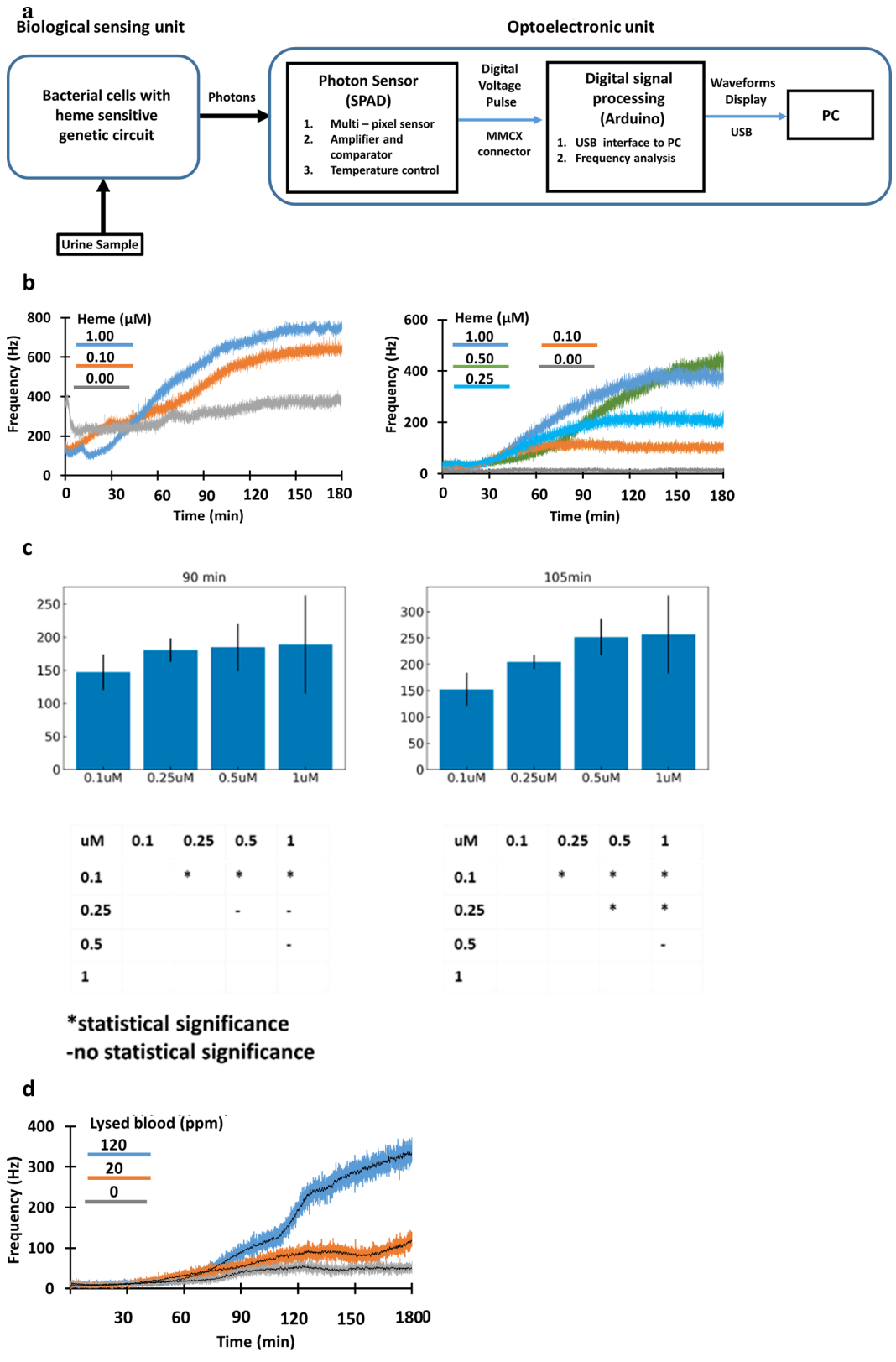

Figure 5. Whole-cell bacterial biosensor for detecting heme/blood in synthetic/human urine. (a) Flowchart consists of a biological sensing unit and an optoelectronic unit. Photons are emitted from engineered bacteria cells as a result of exposure to heme. The photons are detected by the photodetector, generating a digital voltage pulse per time that is proportional to the rate of generated photons. The frequency of the output signal indicates the emitted light level. (b) Time-responses of the whole-cell biosensor based on the V_1 (left) and V_2 (right) heme-sensing genetic circuits tested in synthetic urine. The V_1 sensing cells were exposed to 0, 0.1, and 1 μM heme and the V_2 sensing cells were exposed to 0, 0.1, 0.25, 0.5, and 1 μM heme. (c) Pairwise *t* test comparisons for V_2 between conditions with different heme concentrations. Data from time points 90 and 105 min were compared. (d) Two blood samples (20 and 120 ppm) were dissolved in human urine and were tested with the V_2 whole-cell bacterial biosensor. The black lines were estimated after applying a digital low pass filter with a cutoff frequency of 20 mHz.[8]

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molecules diffuse outside the first strain cells and then enter the bacterial cells carrying the heme-sensitive system. The *luxCDE* genes are regulated by heme input *via* the heme-sensitive promoter, located on an HCP. *HrtR* and *ChuA* genes are expressed under constitutive promoters located on an MCP.

The time-response curves show an immediate moderate induction of bioluminescence signals (Figure 3b) with a sharp rise starting at 40 min. For heme concentrations below 0.3 μM, the maximal level was observed at 60 min after exposure; for higher heme levels, the signal kept increasing during 150 min of measurement to a maximal value of 9.3×10^4 RLU/OD_{600} in response to 1.25 μM heme. The operating range was calculated at 60 and 110 min from dose−response functions (Figure 3c) for heme concentrations of 0.01−0.16 and 0.01−0.63 μM, respectively. The leaky expression value at 110 min was the same as that of our first design (2×10^3 RLU/OD_{600}). The dynamic range that was calculated by the ratio of maximal output value (8.0×10^5 RLU/OD_{600}) to leaky expression was equal to 40-fold. The Hill function was fitted for bioluminescence levels at 60 min with a K_D of 0.06 μM and at 110 min with a K_D of 0.25 μM. The Hill coefficient (n) was 1.3 for both transfer functions.

Sensor Design and Implementation for Urine Samples. To demonstrate the application of bacterial blood sensors in physiological samples, we selected the V_2 design that exhibits the highest ON-to-OFF-response ratio. This design combined with SPAD enables us to accurately detect heme levels in synthetic urine, commonly used to mimic human urine,[20] as well as lysed blood in human urine samples. As a preliminary step, we evaluated the activity of both V_1 and V_2 designs in physiological samples represented by synthetic urine using a plate reader. The cells were grown in LB for 2 h (to an OD_{600} of 0.3) before being dissolved in synthetic urine diluent with various heme concentrations (0−2.5 μM) for immediate measurement in a plate reader. This specific experiment was performed without shaking to mimic conditions in real-world applications. The time-response curves showed a continuous induction of bioluminescence signals when cells were exposed to different heme concentrations (Figure 4a). The curves start to increase around 40 min after exposure to urine samples. The signal in V_1 grows continuously during 4 h to a maximum of 2.5×10^5 RLU in response to 0.3 μM heme. In comparison, the signal in V_2 reaches a maximum signal of 1.8×10^5 RLU at 3 h after exposure to 0.6 μM heme. Compared to the control measurements in LB (Figures 1 and 2), the dose−response transfer functions in urine are characterized by a narrower operating range from 0.01 to 0.08 μM for V_1 and from 0.01 to 0.16 μM for V_2 at 240 min after exposure (Figure 4b). The responses of V_2 still show a lower leaky expression (1.5×10^4 μM) compared to those of V_1 ($\approx 10^5$ μM), leading to a dynamic range that is wider compared to V_1 (12-fold and 2.5-fold, respectively).

Subsequently, we tested the V_2 biosensor after exposure to different levels of human blood (Figure 4c). The cells were grown in LB for 2 h (up to an OD_{600} of 0.3), then dissolved in LB or human urine diluent with various lysed blood levels (0−1110 ppm), and measured immediately in a plate reader without shaking. The time-response curves showed a continuous induction of bioluminescence signals when cells were exposed to different blood levels. The curves increased monotonically and reached a maximum after 3 h with values of 2.2×10^5 RLU in LB and 8.5×10^4 RLU in human urine in response to 124 ppm of blood.

By comparing the dose−response curves to heme (in synthetic urine) and blood (in LB and human urine) using transfer functions at 180 min after induction (Figure 4d), we can estimate the relationships between heme concentrations in synthetic urine and the blood level in real tested urine samples. We obtained that 1 μM heme is equivalent to about 1000 ppm of blood. Figure 4d shows that the developed biosensor (V_2) can detect and distinguish between various levels of lysed blood cells in urine with an approximated operating range of 10−100 ppm of blood.

Next, we built a setup allowing for an accurate and rapid detection of heme using a commercial optical unit and the bacterial sensor (Figure 5a and Supporting Information, Section 5). We applied our whole-cell biosensor to measure the heme concentrations dissolved in synthetic urine (Figure 5b and Figure S10 of the Supporting Information). The system output represented by the number of pulses generated per time in hertz units is proportional to the number of photons generated per time by cells. In agreement with the plate-reader results, the V_1 whole-cell biosensor (Figure 5b, left) showed a very high background (leakiness) signal when no heme was added (around 400 Hz at 180 min). This result indicates that it is challenging to use the prototype (V_1) circuit to discriminate between the different heme concentrations. In contrast, the V_2 whole-cell biosensor (Figure 5b, right) demonstrated a low background OFF signal (around 10 Hz at 180 min) and can discriminate between multiple heme concentrations. With a high heme level (0.5 μM), the V_2 whole-cell biosensor reached a maximum response (420 Hz) of 42-fold after 3 h.

Pairwise t tests (Figure 5c) between the V_2 circuit responses to different inducer concentrations at 90 and 105 min indicated that different inducer concentrations could be distinguished at 105 min or later with statistical significance. The exception is between responses to 0.5 and 1 μM heme, for which the sensor shows no significant difference. This result is not surprising given the bacterial sensor's operating range, previously determined in plate reader measurements (0.01−0.16 μM).

Finally, we tested the V_2 whole-cell bacterial biosensor using two samples of lysed human blood within the sensor operating range (20 and 120 ppm) dissolved in human urine. Blank samples (the urine samples without blood) (Figure 5d) and blood solution without cells (plasma) were also tested (Figure S11 of the Supporting Information). The measured signals started to increase after 60 min and reached a maximum after 3 h. Using the blank sample, the signal from V_2 reached the maximal value of 50 Hz. In the presence of a low level of blood (20 ppm), the V_2 biosensor reached a signal of 100 Hz. The high blood level in urine (120 ppm) resulted in a maximum signal of 325 Hz after 3 h, corresponding to an ON-to-OFF ratio of 6.5-fold.

In this study, we developed a whole-bacterial biosensor that can detect heme in urine. The biosensor comprises genetically engineered bacteria and a single-photon avalanche photodiode. The genetically engineered bacteria cells include two modifications. First, we included a promoter that is sensitive to heme and an outer-membrane transporter. Second, we engineered the *luxCDABE* reporter by splitting it into two subcircuits. This strategy allowed us to reduce the OFF-state (leaky expression) and increase the biosensor sensitivity when it operates at low input levels (Tables S3 and S4 of the

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Supporting Information summarize the biosensors parameters). Optimizing the ON-to-OFF ratio by increasing the ON-state level or reducing the OFF-state level improves the biosensor reliability to have a clear signal detection in the presence of background noise.

Here, we tested three biological circuits for heme detection. The first circuit (V_1) carrying a heme-sensitive promoter that drives the entire *luxCDABE* genes displayed an excellent maximum signal (9.2×10^5 RLU/OD_{600}) but also a high leaky expression (1.4×10^5 RLU/OD_{600}), resulting in a narrow dynamic range (ON/OFF ≈ 6.5-fold). The second circuit (V_2) carried a heme-sensitive promoter that drives the *luxCDE* genes and a constitutive expression of *luxAB* genes. The V_2 circuit demonstrated a much higher dynamic range (ON/OFF ≈ 320-fold at 110 min), owing to the tight basal control (10^3 RLU/OD_{600}), while maintaining a sufficient maximum output level (3.2×10^5 RLU/OD_{600}). The V_2 circuit also has a narrower operating range (0.01−0.3 μM) and a highly digital dose−response (Hill coefficient = 3). While this binary characteristic is ideal for primary screening, it is not well-suited for discriminating between small changes in input levels. To engineer a more desirable system with a more analog dose−response that enables accurate distinction between different heme concentrations, we created a third design (V_3). This design carries a heme-sensitive promoter that drives the *luxCDE* genes and AHL-LuxR inducible promoter P_{lux} that drives *luxAB* genes. The sensor displayed a wide operating range (0.01−0.63 μM) and a sufficient dynamic range (ON/OFF ≈ 40-fold at 110 min), owing to the low basal control (2×10^3 RLU/OD_{600}), while maintaining a maximum output level 8×10^4 RLU/OD_{600}). Since the V_2 design demonstrated a higher fold-change response, we decided to integrate it with the optoelectronic unit. We showed that our whole-cell biosensor based on the V_2 design could successfully discriminate between heme concentrations from 0.01 to 0.16 μM in synthetic urine and between lysed blood levels in human urine within the operating range from 10 to 100 ppm of blood. Considering that 1 mL of blood in healthy adults contains about 5×10^9 red cells, our biosensor can detect three ranges: low blood levels with less than 5×10^4 RBC/mL, medium blood levels with a range between 5×10^4 and 5×10^5 RBC/mL, and high blood levels with higher than 5×10^5 RBC/mL. These concentrations are comparable to the lowest levels that most dipsticks from different companies could detect (from 2×10^4 to 1×10^6 RCBs cells per 1 mL).[21] In summary, bacterial biosensors can offer a new platform to reliably detect target substances (*e.g.*, heme) compared to other conventional methods. However, future efforts will also focus on reducing bacterial biosensor operational complexity and cost.

METHODS

Chemicals. All the chemicals were of the highest analytical grade. A stock solution of Hemin (ferric chloride heme) (Sigma) was prepared by dissolving hemin powder in 1 M NaOH (Sigma) to a concentration of 25 mM, diluting with double distilled water to a final concentration of 100 μM, and sterilizing with a 0.2 μm poly(ether sulfone) (PES) filter. Samples of human blood were supplied by the Magen David Adom (MADA) organization. Whole blood was centrifuged for 10 min at 1100 g. The supernatant plasma was removed, and precipitated RBCs were washed with PBS. An additional cycle of the centrifuge was implemented in similar conditions, and the RBCs were lysed by DDW for a final solution of 40% RBC in lysed blood. Sigmatrix urine diluent (Sigma) was used as a nonbiological diluent that mimics human urine. This product contains calcium chloride, magnesium chloride, potassium chloride, sodium chloride, sodium phosphate, sodium sulfate, urea, and creatinine with sodium azide.

Bacterial Strains and Genes Origins. The *Escherichia coli* (*E. coli*) 10β strain was used to construct all plasmids, and the *E. coli* MG1655 wild type strain was used in the kinetics experiments (see Table S1 of the Supporting Information for the genotype). Plasmid pBR-2TTS-pLux, which harbors the *Photorhabdus luminescens luxCDABE* genes (GenBank accession number M90093) downstream of a multiple cloning site, and pACYC-*luxCDE* were kindly supplied by the S. Belkin lab and served as the source of the *P. luminescens lux* genes. Each functional unit (*luxAB* and *luxCDE*) was isolated by PCR amplification, using primers that introduced an Acc65I/*Kpn*I restriction site before each unit and a *Bam*HI/*Xma*I restriction site after each unit.

Plasmid Construction. All the plasmids in this work were constructed using basic molecular cloning techniques[22] (Figure S1 and Tables S2 and S6 of the Supporting Information for plasmids maps, combinations, and genetic parts). Modifications of the plasmids were carried out by restriction digests, performed using New England Biolabs (Beverly, MA) restriction endonucleases and Thermo Scientific FastDigest restriction enzymes. For ligation, T4 DNA ligase and *Taq* polymerase were used. PCR was performed with a Bio-Rad S1000 thermal cycler with dual 48/48 fast reaction modules. Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). The manipulation of different parts (*i.e.*, replacing promoters or genes) was carried out using the same restriction sites. Plasmids were transformed into *E. coli* using a standard heat shock protocol[22] or a MicroPulser electroporator.[23] Colony PCR screening was carried out using forward and reverse primer pairs. Positive clones were sequence-verified. Plasmids were isolated with Qiagen QIAprep spin miniprep kits (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The Macrogen Sequencing Service carried out DNA sequencing (Macrogen Europe, The Netherlands).

Bacterial Culture. *Escherichia coli* strains were stored as glycerol stocks at −80 °C and then grown at 37 °C in a Shel Laboratories SSI5 shaking incubator at 250 rpm in 5 mL of Lysogeny broth (LB-Miller) medium (Fisher), supplemented with carbenicillin (50 μg mL^{-1}), kanamycin (30 μg mL^{-1}), or chloramphenicol (25 μg mL^{-1}). Overnight cultures were diluted 20-fold into 5 mL of fresh LB medium with antibiotics and regrown with shaking at 37 °C and 250 rpm for 2 h (OD_{600} ≈ 0.3).

Plate Reader for Detection of Heme/Blood. Culture aliquots (100 μL) were transferred into wells of 96-well plates, mixed with various concentrations of heme/blood diluted in 100 μL of LB/synthetic urine/human urine or free of heme/blood as a toxicant-free control (LB/synthetic/human urine only). Luminescence and green fluorescent protein (GFP) signals were read at 10 min intervals using a Synergy H_1 monochromator-based multimode microplate reader with shaking (500 rpm) (see Figure S2 of the Supporting Information for GFP reporter results). The luminescence signal was measured at 490 nm. GFP fluorescence was quantified by excitation at a wavelength of 488 nm and emission at a wavelength of 510 nm. All experiments were repeated three times. Luminescence and GFP values were

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measured in arbitrary relative luminescence units (RLUs) and normalized by the optical density of the culture measured at 600 nm.

SPAD Application for Detection of Heme/Blood. To measure bioluminescence, we used a compact and lightweight commercial multi-pixel photon counter (MPPC) from the C13852 series, produced by Hamamatsu (Figures S6 and S7 of the Supporting Information). This detector is also known as a silicon photomultiplier (SiPM) composed of SPADs. These SPADs have a high internal gain, which enables single-photon detection. The module also consists of an amplifier, a comparator unit, a high-voltage power supply, and a temperature control unit. The photosensitive area of a sensor has a size of 1.3 × 1.3 mm^2.

The Hamamatsu SiPM is well-suited to single-photon counting and ultralow light applications. The module operates with an external power supply ($\pm 5V$) The MPPC was integrated to power supplies, signal connectors, and control lights to form a convenient hand-held bioelectronic device. A full sensor characterization is provided in Section 5 of the Supporting Information. To process the output signal from the sensor, we implemented the connection to a PC using an Arduino Mega 2560 card (Figure S8 of the Supporting Information). The signal is transferred from the sensor through an Arduino USB port to the PC. The data is sampled and processed using a basic Python script.

A culture aliquot (100 μL) was transferred into a biochamber with a diameter of 1 cm, mixed with various concentrations of heme or lysed blood diluted in 100 μL of synthetic/human urine, and placed underneath the sensor (Figure S9 of the Supporting Information). Since the number of photons affects the number of pulses generated per time, the frequency of the output signal indicates the light level generated by the *Escherichia coli* cells as a result of exposure to heme or blood solutions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.0c00640.

> Tables of genotype of bacterial strains, plasmid combinations used, summary for plate reader experiments, summary for SPAD experiments, parameters used for fitting of transfer function, and genetic parts, figures of plasmid maps, GFP reporter system, V_1 performance in minimal media, time-response of V_3, dose−responses of second redesigned circuit, MPPC optical module, MPPC common measurement setup, flowchart of heme detection by the bioelectronic device, bioelectronic device, and time-responses of the whole-cell biosensor, and discussions of biological sensing unit, optoelectronic unit, and additional data for whole-cell bacterial biosensors (PDF)

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Author Contributions

N.B. and R.D. designed the study. N.B. and M.H. performed experiments and collected data. I.O. designed and built the optoelectronic circuit. All authors analyzed the data, discussed results, and wrote the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

SPAD, single-photon avalanche photodiode; LCP, low-copy-number plasmid; MCP, medium-copy-number plasmid; HCP, high-copy-number plasmid

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