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Detection of inorganic ions and organic molecules with cell-free biosensing systems

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ABSTRACT

Efforts to use genetically modified cells to detect inorganic ions and organic molecules have been hindered by biosafety concerns, limitation of cell membrane barriers, cytotoxicity problems, and unfriendly operations. To address those challenges, cell-free biosensing systems were established to detect arsenic, mercury, acyl-homoserine lactone, and benzoic acid in this study. The cell-free system mimics biological transcription and translation activities in an open environment without living cells. The most bioactive cell-free system was obtained by screening different *Escherichia coli* strain extracts and Mg^{2+} concentrations. It was found that the sensitivity of cell-free systems could reach nanomolar levels with the short response time. The selectivity experiments showed that cell-free biosensing of inorganic ions had no signal interference. Cell-free biosensing systems, with their wide range of molecule detection, short response time and high specificity, could potentially serve as powerful biosensing platforms for environmental and medical applications.

1. Introduction

Biosensors have been widely used in environmental remediation, food safety, and disease diagnosis (Wang et al., 2016; Cui et al., 2018; Verma et al., 2010). They are more sensitive and more specific than traditional methods such as chemical sensors (Kaur et al., 2015). Synthetic biologists have genetically engineered microbes to detect various ions or molecules (Kim et al., 2018; Jester et al., 2018; Schulte et al., 2017). However, some issues remain to be addressed. The hurdle of field applicability has not been overcome because most biosensors use living, genetically modified microorganisms (GMOs) (Weissbrod et al., 2017; Kick et al., 2018). The application of these GMO biosensors is often not possible due to biosafety concerns, as it must be prevented that GMOs are released into the environment. Moreover, the diffusion of the analyte across the cell membrane is a limiting step, which results in a slower response time of the biosensor (Yagi, 2007). Some analytes might be toxic to the living cells. Another disadvantage of whole-cell biosensors is that they are often not very user-friendly, as they have a limited shelf life and their application outside the laboratory is complicated and requires special equipment. To address these limitations of whole-cell biosensors, cell-free synthesis system as a platform for biosensing has been developed.

Cell-free biosynthesis systems perform biological transcription and translation activities in an open environment, which present unique features different from cell systems. The cell-free system uses DNA or mRNA as the template and expresses proteins by adding reaction substrates, energy substance, inorganic salts, and cofactors (Fig. 1) (Lu, 2017). It has no biosafety problem compared with the cell system because there is no limitation of the cell membrane and no cell growth. The detected substances can be directly added into cell-free systems, and the system has high toxicity tolerance to toxic substances (Karig, 2017). Also, cell-free systems can be lyophilized and stably stored at room temperature for up to one year, a vital necessity for point-of-care applications (Pardee et al., 2014). The biosensing genetic circuits previously engineered *in vivo* can be transplanted to cell-free systems to successfully detect Zika virus in rhesus macaques and acyl-homoserine lactone (AHL) from *Pseudomonas aeruginosa* in human clinical samples (Pardee et al., 2016; Wen et al., 2017). Although cell-free systems have immense potentiality on biosensing, they still have some challenges, such as the effective design of genetic circuits and the specificity of cell-free biosensing. The fundamental kinetics of a genetic circuit in cell-free systems differs from that in living cells (Karzbrun et al., 2011). As a

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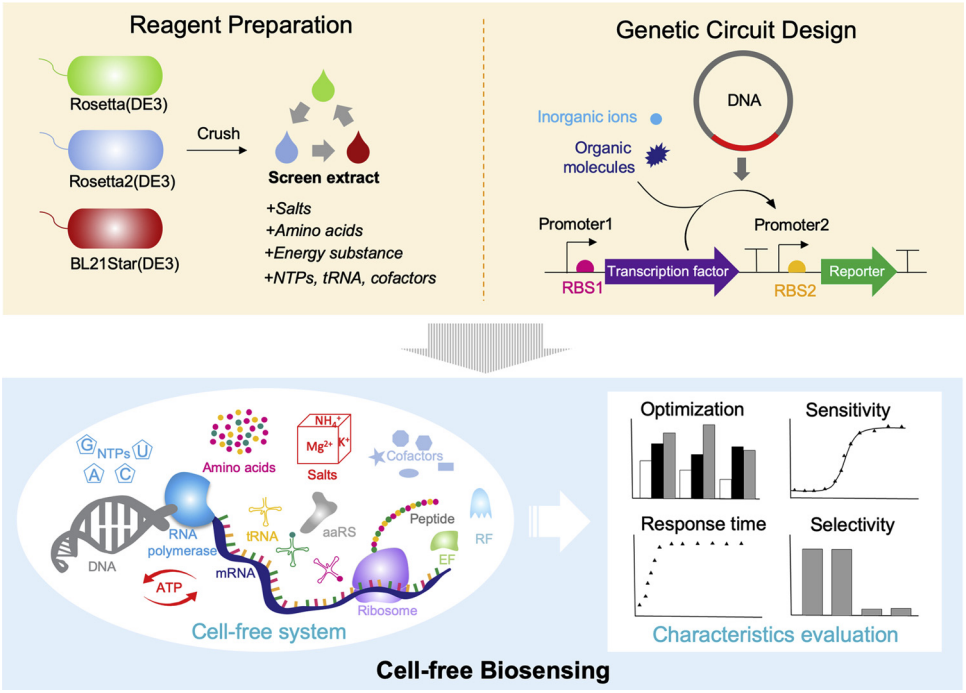

Fig. 1. The workflow for engineering cell-free biosensing systems.

result, the performance of the genetic circuit in cell-free systems and living cells is often qualitatively similar, yet quantitatively different (Chappell et al., 2013). Biosensing elements must also have good specificity. Cell-free systems and cell systems are different in component and environment (Garamella et al., 2016), which might cause the specificity of biosensing elements previously designed in the cell system to lose in cell-free systems. Therefore, the biosensing genetic circuits of cell-free systems need to be developed independently of the cell system. To explore these challenges of cell-free biosensing, five different biosensors were constructed to detect inorganic ions and organic molecules in cell-free systems.

Inorganic ions and organic molecules are the most common substances in the world that need to be detected. Some inorganic ions are extremely harmful to the environment and the human body (Ayangbenro and Babalola, 2017; M et al., 2014; Giovanella et al., 2016). Rapid detection of inorganic ions in the environment can prevent some diseases. Arsenic and mercury, two typical toxic inorganic ions, were selected and detected in this study. For organic molecules, AHLs and benzoic acid were selected. AHL was a type of quorum sensing signal molecule that regulates the expression of many physiological properties (Waters and Bassler, 2005; Dong et al., 2001). It was present in the sputum, urine, and blood of many patients with cystic fibrosis (Wen et al., 2017). Therefore, it is essential to establish an effective method for detecting AHLs. Two AHLs, 3OC12-HSL and pC-HSL (Fig. 2A), were tested in this study. However, the most troubling of all is that biosensing systems can only use well-characterized transcription factor ligands to identify biomarkers. However, many biomarkers do not have corresponding transcription factor ligands, which significantly limits the application of biosensing systems. Researchers suggested that metabolic enzymes were added to biosensing systems to expand the range of detection (Voyvodic et al., 2018). The metabolic enzyme can convert an undetectable molecule into a ligand molecule of an existing transcription factor. Benzoic acid (Fig. 2A) could be obtained by one-step enzymatic reaction of 64 biomarkers and detected by biosensor elements, which could effectively expand the range of cell-free biosensing. Detecting inorganic ions and organic molecules by cell-free systems could help to monitor the environment and diagnose diseases, it also can help to understand the mechanisms of cell-free systems better.

In this study, five genetic circuits that could response the five substances (arsenic, mercury, 3OC12-HSL, pC-HSL, and benzoic acid) were constructed in cell-free systems by standard synthetic biology modules, using the green fluorescent protein (GFP) as the reporter protein. The most suitable cell-free system was obtained by screening three different *E. coli* extracts and Mg^{2+} concentrations. Moreover, the sensitivity, response time, and selectivity of five cell-free biosensors were explored (Fig. 1). The research purpose is to expand the application of cell-free biosensing systems and guide researchers to design better genetic elements in cell-free systems.

2. Materials and methods

2.1. Genetic circuit construction

Plasmid construction and DNA manipulations were performed following standard molecular biology techniques. The mercury (J23115-RBS32-merR-B0015-P_{merT}) and benzoic acid (BenR-B0015-P_{Ben}) sensor fragments were synthesized by GENEWIZ. The arsenic (J23101-RBS32-arsR-B0015-P_{arsR}) sensor, LuxR gene, and RpaR gene were purchased from Addgene (Addgene#78636, Addgene#785142, and Addgene#785148) (Wang et al., 2014; Scott and Hasty, 2016). Wild-type GFP and sfGFP (super-folder green fluorescent protein) genes are kept in the laboratory. Plasmid pSB3K3 (p15A ori, Kan ^{r}) was used to carry the five biosensors (Addgene#78636) (Shetty et al., 2008). The sequences of plasmids were verified by TianYi Biotechnology. Plasmid DNA purification was carried out using the Qiagen Plasmid Maxi Kit. The information of all plasmids can be found in the supplemental materials.

2.2. Extract preparation

Extracts were prepared as described by Wen et al. with several modifications (Wen et al., 2017). An overnight culture of *E. coli* was used to inoculate 200 mL of 2xYT-P (1.6% wt Trytone, 1% wt Yeast Extract, 0.5% wt NaCl, 22 mM KH_2PO_4 and 40 mM K_2HPO_4) media in 1 L flasks at a dilution of 1:20. The culture was added to 4 L fermenter

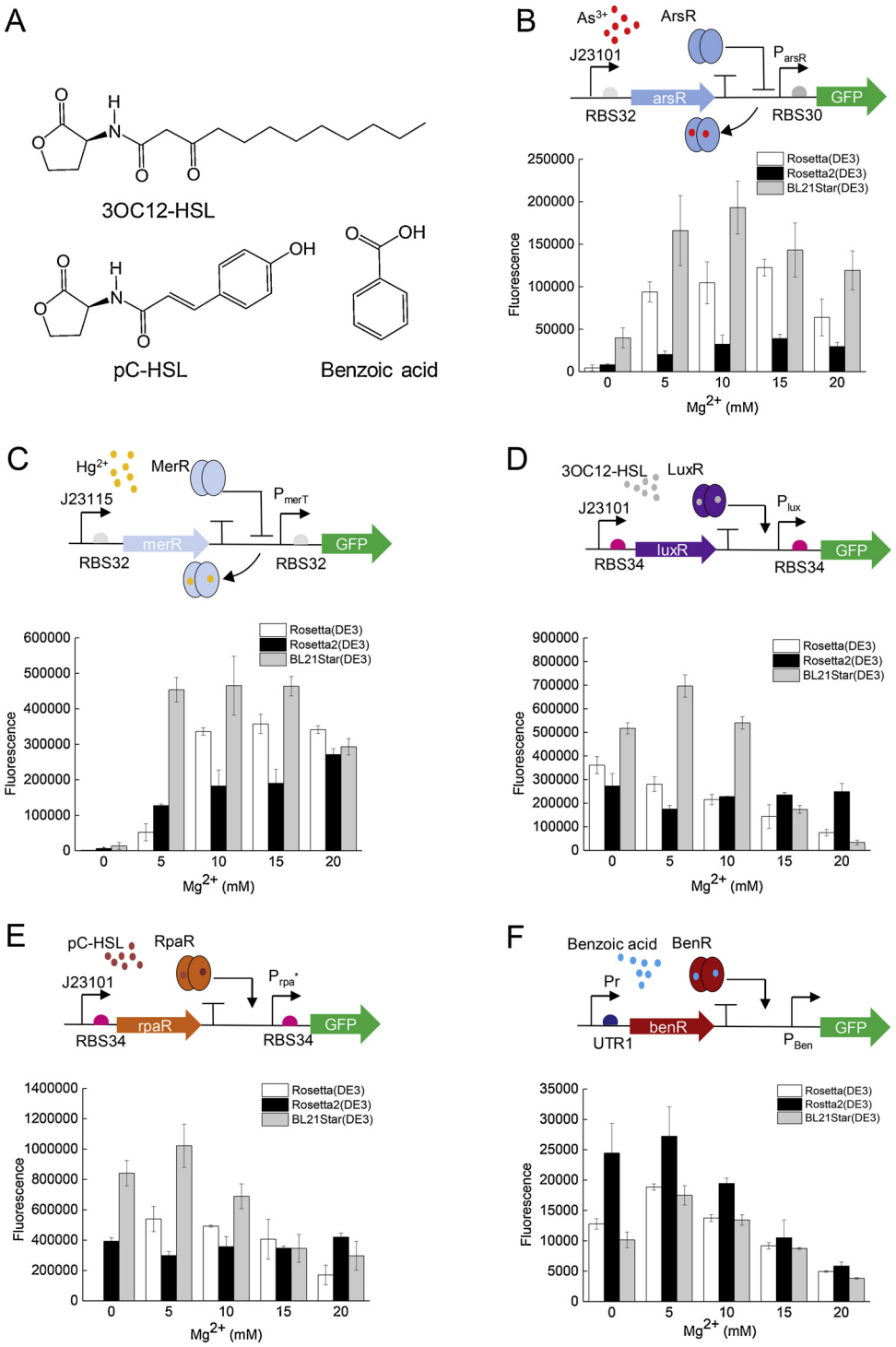

Fig. 2. The design and characterization of five cell-free biosensors. (A) The structure diagrams of AHLs and benzoic acid. (B) Arsenic (1 μM). (C) Mercury (1 μM). (D) 3OC12-HSL (10 μM). (E) pC-HSL (10 μM). (F) Benzoic acid (1 mM). Error bars, s.d. (n = 3).

with the cultivation at 37 °C and 500 rpm for 3.5 h. The culture was spun down at 10,000 × g at 4 °C for 10 min. Cell pellets were washed twice with 100 mL ice-cold S30A buffer (14 mM Mg-glutamate, 60 mM K-glutamate, 50 mM Tris, pH 7.7) and centrifuged afterward at 10,000 × g at 4 °C for 10 min. Cell pellets were then resuspended in 30 mL ice-cold S30A buffer and transferred to pre-weighed 50 mL Falcon conical tubes where they were centrifuged at 10,000 × g at 4 °C for 10 min, removing the supernatant after each. Finally, the tubes were reweighed and flash frozen in liquid nitrogen before storing at −80 °C.

Next day, cell pellets were thawed on ice and re-suspended in 1 mL S30A buffer per gram cell pellet. Cell suspensions were lysed via twice pass through a high-pressure crusher at 15000–20000 psi and then centrifuged at 12,000 × g at 4 °C for 10 min to separate cellular cytoplasm. After centrifugation, the supernatant was carefully transferred to new tubes, and 3 μL of 1 M DTT was added per 1 ml of lysate. The sample was incubated at 37 °C with 120 rpm shaking for 80 min to digest remaining mRNA with endogenous nucleases. Subsequently, the extract was centrifuged at 12,000 × g at 4 °C for 10 min, and the supernatant was transferred to 6–8 kDa MWCO dialysis bag and dialyzed in 1 L of ice-cold S30B buffer (14 mM Mg-glutamate, 60 mM K-

glutamate, ~5 mM Tris, pH 8.2) at 4 °C for 3 h. The extract was re-centrifuged at 12,000 × g at 4 °C for 10 min. The supernatant (cell-free extract) was collected and aliquoted into new tubes and flash frozen in liquid nitrogen before storing at −80 °C.

2.3. Cell-free reactions

The cell-free reaction system was prepared as described previously with several modifications (Lu et al., 2013). The reaction mixture includes: 175 mM potassium glutamate, 10 mM ammonium glutamate, 2.7 mM potassium oxalate, 33 mM phosphoenolpyruvate (PEP), 50 mM HEPES (pH 8), 1.5 mM ATP and GTP, 0.9 mM UTP and CTP, 0.26 mM Coenzyme A (CoA), 0.2 mg/mL tRNA, 0.33 mM NAD, 0.75 mM cAMP, 1 mM spermidine, 0.06 mM folinic acid, 2 mM of each of the 19 amino acids, 0.33 mM NAD, 2.5% PEG8000 and 30% (volume) of *E. coli* extract. The Mg-glutamate level was depended on the kind of biosensors. The plasmid concentration in the cell-free system was 5 nM except for the benzoic acid cell-free biosensor. The plasmid concentration of the benzoic acid cell-free biosensor was 15 nM. Cell-free reactions (20 μL) were incubated at 30℃ for 16 h.

2.4. Gene expression assay

Fluorescence outputs representing gene expression levels were measured by a microplate reader (infinite M200PRO, TECAN). When the cell-free reaction finished, 10 μL sample was taken into a 96-well black plate (CORNING) for measuring green fluorescence (485 nm for excitation, 520 nm for emission, Gain 100).

2.5. Mathematical model and data analysis

To compare the performance of biosensors, the biosensing behavior of five cell-free biosensors were modeled. The resulting data (Fig. 3A–E) were fitted to a Hill function model (Wang, 2015).

$$f([I]) = k(\alpha + [I]^n/(K_M^n + [I]^n))$$

Where [I] is the concentration of the inducer. K_M and n are the Hill constant and coefficient, respectively, relating to the promoter-regulator/inducer interaction. k is the maximum expression level due to induction, and α is a constant relating to the basal level of the promoter due to leaky expression. f([I]) is the level of expression relative to basal expression.

3. Results and discussion

3.1. Construction of cell-free biosensing systems

Five cell-free biosensors for detecting inorganic ions (arsenic and mercury) and organic molecules (3OC12-HSL, pC-HSL, and benzoic acid) were constructed by using standardized biological modules. The synthetic biosensor circuit comprised the regulatory proteins and the cognate promoters fused to a GFP reporter (Fig. 1). For inorganic ions, ArsR and MerR are transcriptional repressors which bind to their corresponding promoters and prohibit the transcription process. When arsenic and mercury ions were presented in cell-free systems, they would bind with ArsR and MerR. The resulting complexes will fall off from their corresponding promoters, enabling the transcription process to proceed. The biosensing mechanism of organic molecules was different from inorganic ions. LuxR, RpaR, and BenR are transcriptional activators. The complexes of these proteins and their cognate organic molecules would activate transcriptional reaction, resulting in the high expression level of GFP.

To rationally allocate the resources of cell-free systems, promoters and ribosome binding sites (RBSs) with different strength were used to regulate the transcription and translation of genes. As shown in Fig. 2, for arsenic, 3OC12-HSL, and pC-HSL, the promoters of their transcription factors were J23101 with strong strength (Kelly et al., 2009). For mercury, the promoter of the transcription factor was J23115. Three different RBSs (RBS30, RBS32, and RBS34) were chosen for arsenic, mercury, 3OC12-HSL, and pC-HSL, which are from the Registry of Standard Biological Parts. As shown in Fig. 2, RBS34 has strong ribosome binding strength, which was in front of the *luxR* and *rpaR* genes aiming to enhance the production of transcription factors LuxR and RpaR. P_{merT} is a strong promoter, and P_{arsR} is a weak promoter (Baojun et al., 2013). In order to increase fluorescence output and balance expression of the regulatory and fluorescent protein. The weak RBS32 was placed behind promoter P_{merT}, and strong RBS30 was placed behind promoter P_{arsR}. For benzoic acid, the transcription factor peptide chain was longer than the others. When the promoter and RBS of the regulatory protein were J23101 and RBS34, the expression of the fluorescent protein was the same as the blank (see Fig. S6). So the strongest endogenous promoter Pr combined with the most powerful RBS named UTR1 were selected to express the transcription factor (Shin and Noireaux, 2010). The promoter P_{Ben} of sfGFP used native RBS from *Pseudomonas putida.* In all cases, the terminators for the gene transcription were B0015 (Galdzicki et al., 2011).

The optimization in the design of genetic elements was not enough, and it was also required to optimize the cell-free reaction system. It is well known that cell extracts affect protein expression in cell-free systems (Shin and Noireaux, 2010). Three different commercial *E. coli* strains, BL21Star(DE3), Rosetta(DE3) and Rosetta2(DE3) were selected to improve the cell-free reaction systems. BL21Star(DE3) contains *rne131* gene mutant, which could enhance the stability of mRNA, thereby improving protein expression. The Rosetta(DE3) strain contains the pRARE plasmid and provides tRNAs with six rare codons of AUA, AGG, AGA, CUA, CCC, and GGA. The Rosetta2 (DE3) strain provides tRNAs with seven rare codons of AUA, AGG, AGA, CUA, CCC, GGA, and CGG. Besides the cell extracts, Mg^{2+} is involved in protein synthesis and energy metabolism, because it was an activator of many enzymes (Pasternak et al., 2010). So it had a significant effect on cell-free systems (Shin and Noireaux, 2012). The most suitable Mg^{2+} concentration for different protein expression was different. The Mg^{2+} concentration in cell-free systems must be well explored to maximize the fluorescence signal.

The cell-free synthesis reaction was carried out with different cell extracts and magnesium glutamate concentrations to improve biosensing ability. The most suitable cell-free system was screened by evaluating the fluorescence signal. Fig. 2B-C showed cell-free biosensors for detecting arsenic and mercury. The results indicated that when cell extract was BL21Star(DE3) and Mg^{2+} concentration was 10 mM, arsenic and mercury biosensors obtained the maximum fluorescence signal. For cell-free 3OC12-HSL and pC-HSL biosensors, the biosensing mechanism was different from inorganic ions. When AHLs existed in the cell-free system, they bound to the transcription factors and activated the transcriptional reactions. As shown in Fig. 2D-E, when cell extract was BL21Star(DE3), and Mg^{2+} concentration was 5 mM, 3OC12-HSL and pC-HSL biosensors got the maximum output signal. For cell-free benzoic acid biosensor, as shown in Fig. 2F. When the cell extract was Rosetta2(DE3) and the Mg^{2+} concentration was 5 mM, the output fluorescence reached the maximum intensity. The regulatory protein BenR of benzoic acid did not have rare codon CGG. The reason why Rosetta2(DE3) cell extract was the most suitable for benzoic acid biosensor was not clear, and further exploration was needed. By designing the biosensing genetic circuits and optimizing the cell-free systems, five cell-free biosensing systems have been successfully established.

3.2. The sensitivity of biosensors

Based on the optimized cell-free biosensing systems, the sensitivity of each biosensor was further explored. As shown in Fig. 3, the response output of almost all biosensors increased with the increase of the substance concentration. When the levels of detected substances reached

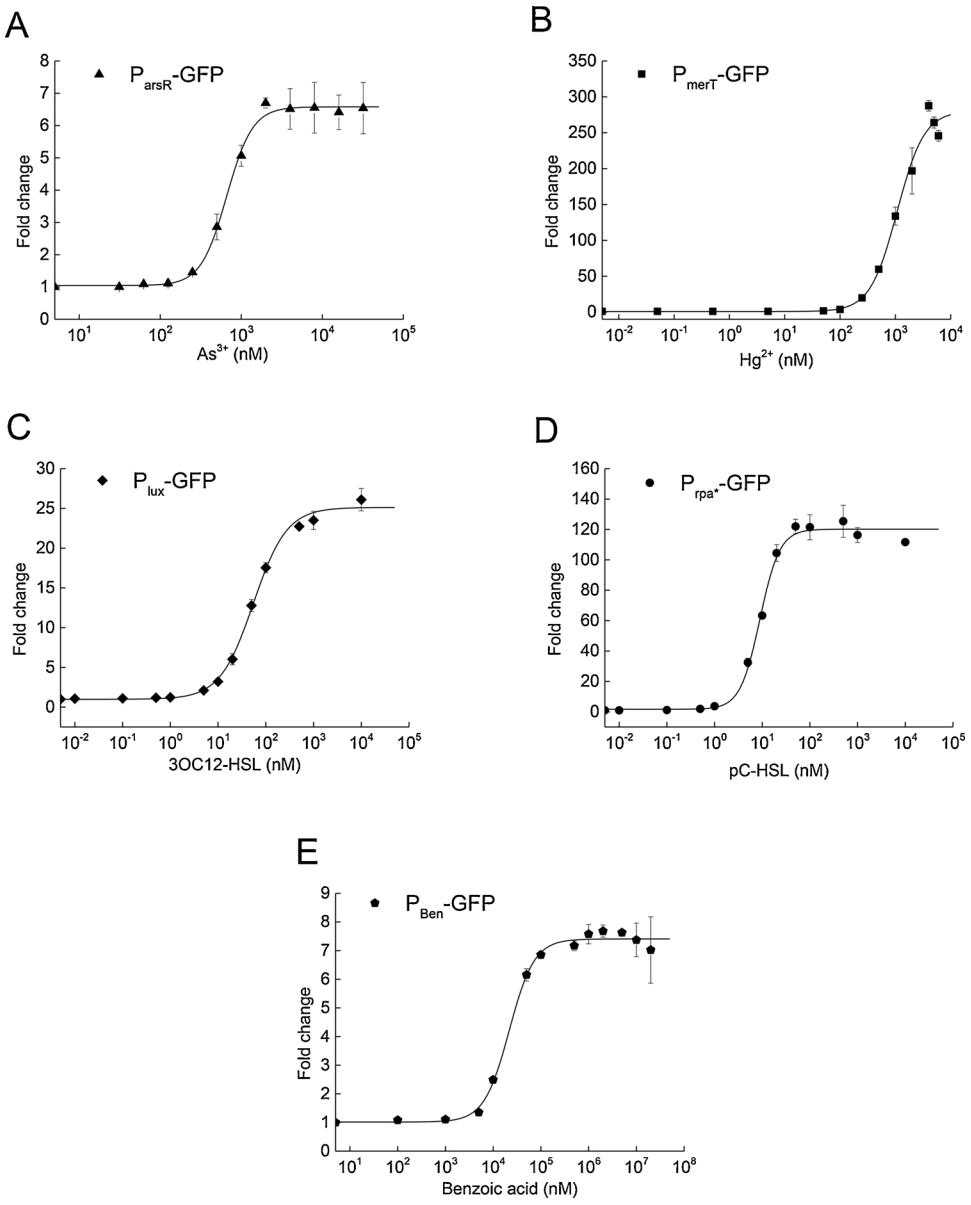

Fig. 3. The response concentration curve of five cell-free biosensors. (A) Arsenic (0, 0.0312, 0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32 μM As^{3+}). (B) Mercury (0, 0.00005, 0.0005, 0.005, 0.05, 0.1, 0.25, 0.5, 1, 2, 4, 5, 6 μM Hg^{2+}). (C) 3OC12-HSL (0, 0.01, 0.1, 0.5, 1, 5, 10, 20, 50, 100, 500, 1000, 10,000 nM). (D) pC-HSL (0, 0.01, 0.1, 0.5, 1, 5, 10, 20, 50, 100, 500, 1000, 10,000 nM). (E) Benzoic acid (0, 0.1, 1, 5, 10, 50, 100, 500, 1000, 2000, 5000, 10,000, 20,000 μM). Error bars, s.d. (n = 3).

Table 1
The best fits for characterized dose responses of biosensors.

Sensor reporter	Target	k	α	n	K_M (nM)	R^2
P_{arsR}-GFP	Arsenic	5.533	0.189	2.722	661.005	0.9972
P_{merT}-GFP	Mercury	279.500	0.003	1.780	1078.074	0.9893
P_{lux}-GFP	3OC12-HSL	24.131	0.041	1.240	54.927	0.9978
P_{rpa*}-GFP	pC-HCL	118.503	0.014	2.100	8.929	0.9950
P_{Ben}-GFP	Benzoic acid	6.390	0.159	1.658	21702.843	0.9962

certain values, the output fluorescence kept constant. The maximum concentration of target molecule that cell-free biosensor can detect has been explored (see Fig. S7 and Table S1). The response curves of arsenic and mercury biosensors were shown in Fig. 3A-B. When the concentrations of arsenic and mercury were higher than 0.25 μM, the fluorescence value increased significantly. It was more sensitive than the cell-based biosensing system (Baojun et al., 2013). This is due to the advantage of cell-free systems. Without the limitation of transmembrane transportation, the substances could be directly added into cell-free systems and used for biosensing. To characterize these five cell-free biosensors, a Hill function model for the environment responsive promoter steady state input-output response was applied in this study (Baojun et al., 2013), the functions model derived from a steady state assumption (Wang, 2015). The characterization data were fitted to the model of the biosensor, and the fitted model parameters were shown in Table 1. The mercury biosensor had a smaller background (0.189 for arsenic and 0.003 for mercury) and a higher dynamic range (5.533 for arsenic and 279.5 for mercury) than the arsenic biosensor, which showed that cell-free mercury biosensor had better biosensing abilities.

The response curve trends of organic molecules were similar to inorganic ions. Fig. 3C-D showed the response curves of the 3OC12-HSL and pC-HSL molecules, which were similar. However, from the data in Table 1, the pC-HSL biosensor had lower detection limit (8.929 nM for pC-HSL and 54.927 nM for 3OC12-HSL) and higher Hill coefficient (2.1

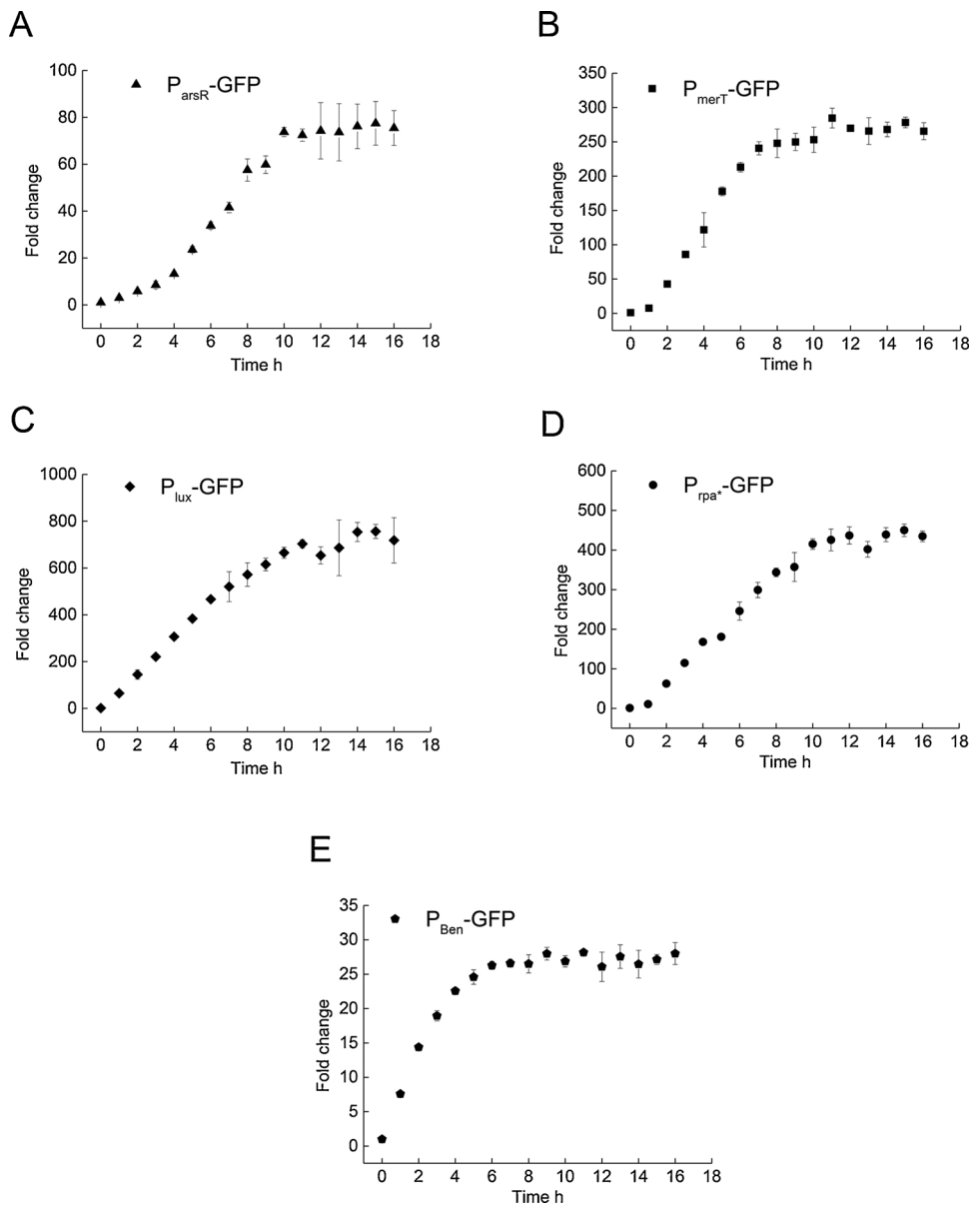

Fig. 4. The response time of five cell-free biosensors. (A) Arsenic (1 μM). (B) Mercury (1 μM). (C) 3OC12-HSL (10 μM). (D) pC-HSL (10 μM). (E) Benzoic acid (1 mM). Error bars, s.d. (n = 3).

for pC-HSL and 1.24 for 3OC12-HSL) than the 3OC12-HSL biosensor. The detection limit was lower, and the cell-free biosensor was more sensitive. The Hill coefficient was related to the slope of the curve. Larger Hill coefficient means faster biosensing response. Fig. 3E showed the response curve of benzoic acid. The fluorescence signal had significant change when the concentration of benzoic acid was over 10 μM in the cell-free system. The benzoic acid biosensor was less sensitive than other cell-free biosensors. One possible reason for the low sensitivity of the benzoic acid biosensor was that the peptide chain of regulatory protein BenR was longer compared with other biosensors (see supplemental materials), which was probably difficult to be synthesized in the cell-free system. From the modeling data in Table 1, the benzoic acid biosensor also had a high background level and a low dynamic range.

Through the exploration of the sensitivities of five biosensors, the detection limit of cell-free biosensing systems could reach micromolar levels, and AHLs even could reach nanomolar levels. In cell-free systems, the detected substances could be added without the limitation of the cell membrane barriers and directly used for biosensing, which therefore made cell-free biosensing systems have lower detection limits and background than cell systems. In the future studies, to enhance the sensitivity, stronger promoters and RBSs need to be designed, and better cell-free systems using less energy and few resources to maximize the protein production need to be further developed.

3.3. The response time of biosensors

Response time is an essential parameter for actual biosensor applications. Short response time allows for quickly getting the detection results and making better decisions. Cell-based biosensors require maintaining cell growth and replication. The substances need to cross the cell membrane so that they can be detected. These factors apparently delay the detection time. Cell-free systems do not need to preserve cellular activity, and there is no self-replication process. The substances can directly react with biosensor elements in the cell-free environment to generate a signal without delay.

Arsenic and mercury were extremely harmful to the environment and health, and therefore their rapid detection could avoid greater

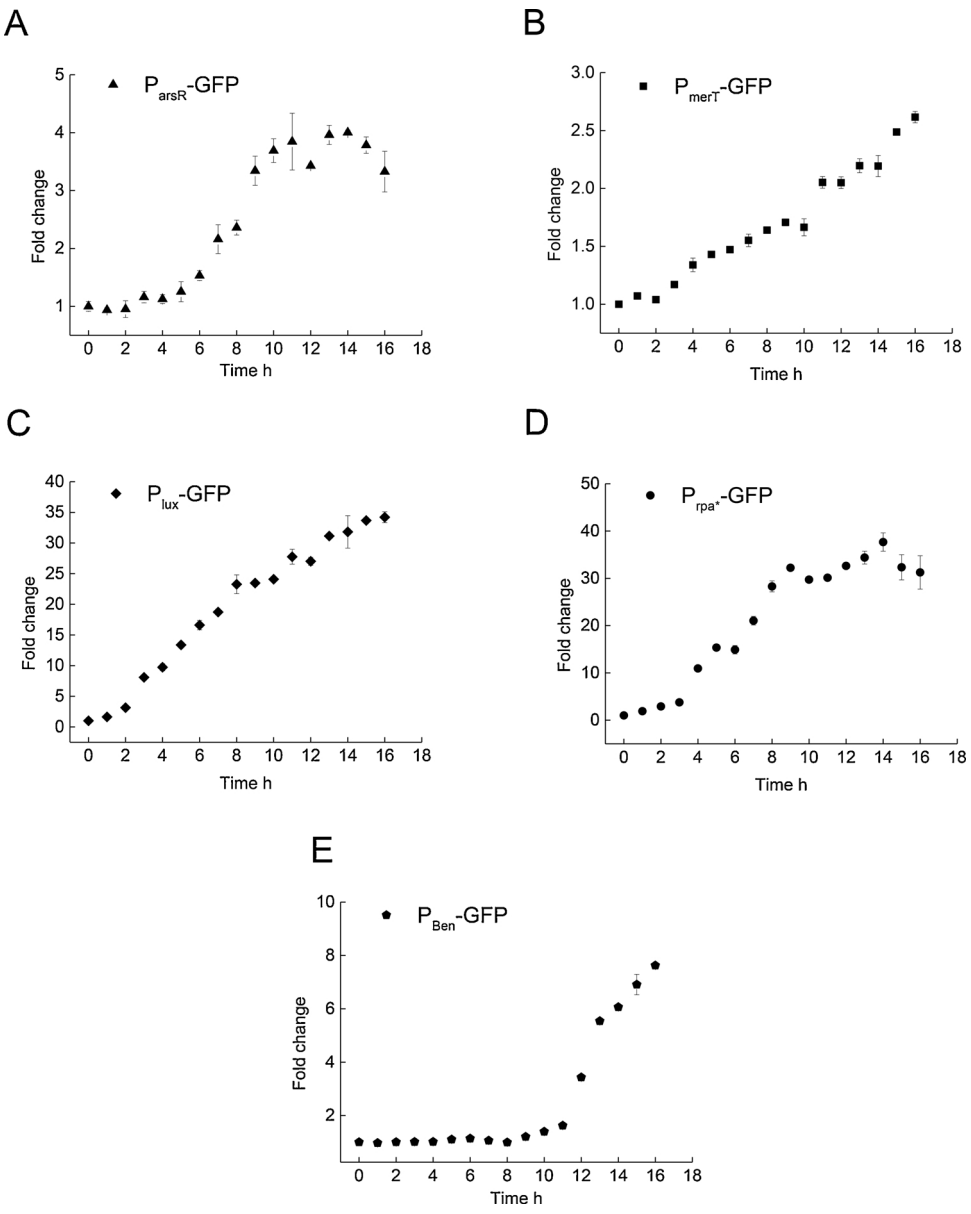

Fig. 5. The response time of five cell biosensors. (A) Arsenic (1 μM). (B) Mercury (1 μM). (C) 3OC12-HSL (10 μM). (D) pC-HSL (10 μM). (E) Benzoic acid (1 mM). Error bars, s.d. (n = 3).

harm. Fig. 4A-B showed the response time of cell-free arsenic and mercury biosensors. The cell-free arsenic biosensor had a slow fluorescence increase in the first 4 h, and the signal began to increase faster after 4 h. After 10 h, the fluorescence remained constant, and the cell-free synthesis reaction terminated. For cell-free mercury biosensor, the fluorescence increase rate was stable in the first 7 h, and then the reaction stopped after 7 h. By comparing these two cell-free inorganic ion biosensors, the cell-free arsenic biosensor had a low-speed reaction period in the early stage, while the mercury maintained a high-speed reaction. A possible explanation was that the regulatory protein ArsR of arsenic ion might have very solid binding strength to the promoter. Fig. 5A-B showed the response time of cell-based arsenic and mercury biosensors. The response time curve of cell-based arsenic biosensor was the same as the cell-free arsenic biosensor, which indicated that arsenic crossed the cell membrane easily. The fluorescence of cell-based mercury biosensor increased for 16 h, and the rate was slow. Cell-based mercury biosensor had a long response time, which may infer that mercury crossing the cell membrane was difficult compared to arsenic. Cell-free inorganic ion biosensors showed faster respond time compared to cell-based inorganic ion biosensors.

Many important disease biomarkers and environmental pollutants are organic molecules, whose rapid detection is obviously of great practical importance. Fig. 4C–D showed the response time of the quorum sensing molecules 3OC12-HSL and pC-HSL. The fluorescence signals of these two biosensors increased linearly and remained stable after 11 h. Fig. 5C–D showed the response time of cell-based 3OC12-HSL and pC-HSL biosensors. The rate of increasing in fluorescence was slow in first 2 h. It was likely that 3OC12-HSL and pC-HSL was crossing the cell membrane. After 9 h, the fluorescence of cell-based pC-HSL biosensor was stable, and cell-based 3OC12-HSL biosensor was still increasing slowly. By comparing the response time of cell-free biosensors and cell biosensors, the response time of cell-based 3OC12-HSL biosensor was longer than that of its cell-free counterpart. But the time when pC-HSL cell biosensor response peaked was shorter than that of cell-free pC-HSL biosensor. A possible reason was that cell produced pC-HSL molecule, which speeded up the synthesis of GFP. The response time of benzoic acid was shown in Fig. 4E. The signal increase of cell-free benzoic acid biosensor was faster than other biosensors. The

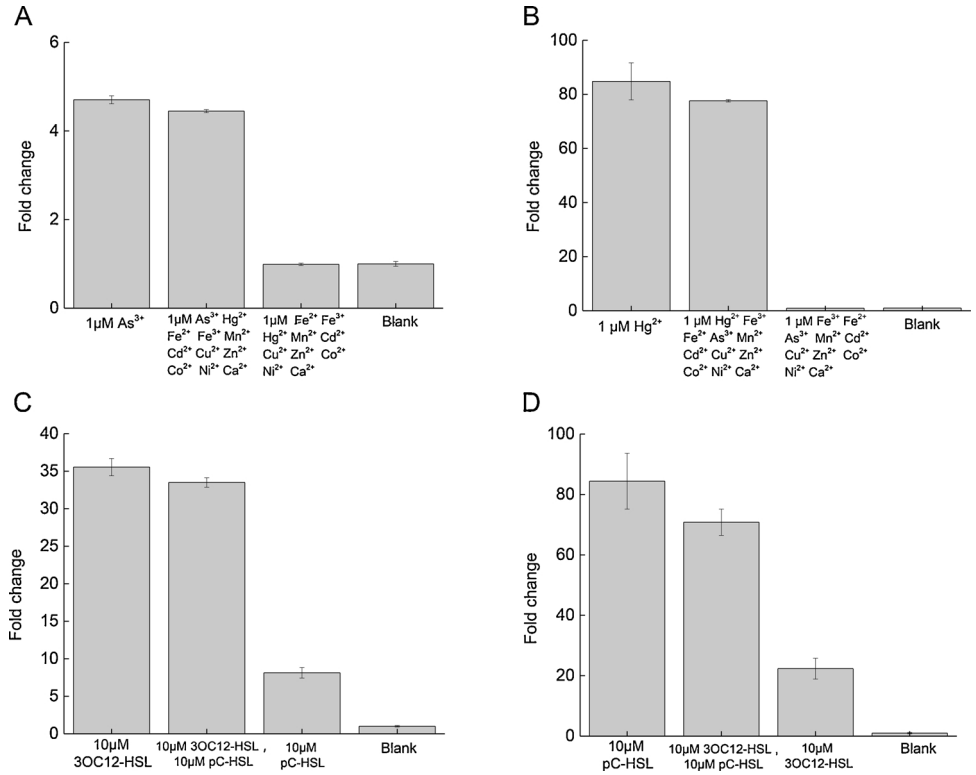

Fig. 6. The specificity of five cell-free biosensors. (A) Arsenic. (B) Mercury. (C) 3OC12-HSL. (D) pC-HSL. Error bars, s.d. (n = 3).

benzoic acid biosensor reached a steady state after 6 h. The needed plasmid concentration of cell-free benzoic acid biosensor was higher than those of the other cell-free biosensors, and the promoter and RBS of the regulatory protein BenR were stronger than others. As a result, the protein synthesis rate in the cell-free benzoic acid biosensing system was faster, and the reaction response time was shorter. The experimental design and the results also demonstrated guidance for shortening the time of cell-free protein synthesis. The response time of cell-based benzoic acid biosensor was shown in Fig. 5E. Cell-based benzoic acid biosensor did not increase fluorescence in first 10 h. It had a longer time delay compared to the cell-free system, which indicated that benzoic acid crossing cell membrane with a long time.

Compared to cell systems, cell-free biosensing systems presented a shorter response time. That was because the cell-free system has an open environment. The substances can directly be detected, skipping the process of transmembrane transport. Moreover, all energy and reaction substrates only served the target reaction in cell-free systems, not like complicated cell systems. The green fluorescence signal of five cell-free biosensors could be clearly seen in the UV light with naked eyes after 3 -h reaction. However, compared with other chemical sensors and physical sensors, three hours were still too long. Shortening the detection time could increase the practical value of cell-free diagnosis, which was a problem that needed to be solved in the cell-free system. Reducing the response time of cell-free biosensing systems needed the engineering of biomolecular machinery to further improve the transcription and translation activities in cell-free systems.

3.4. The selectivity of biosensors

Many factors need to be considered when biosensors are applied to real life. The most important factor was the selectivity of biosensors. The samples to be tested usually are mixtures, and other substances in the samples might cause signal interference, which made the results inaccurate. Therefore, the selectivity of five cell-free biosensors should be explored. To investigate the selectivity of arsenic ion and mercury ion, the water solution containing various inorganic ions was prepared. As shown in Fig. 6A-D, the cell-free biosensors of arsenic ion and mercury ion were very specific. Other inorganic ions present in the system could not generate fluorescent signal interference. This result was consistent with the previous studies using cell systems (Baojun et al., 2013). The selectivity of quorum sensing signal molecules mainly examined the interaction between 3OC12-HSL and pC-HSL, as shown in Fig. 6C-D. There were signal interferences between two molecules, but the synergistic effect was not produced when two molecules were mixed.

The development of highly specific biosensors was critical. In general, cell-free systems do not have many metabolic pathways as cell systems, and therefore the signal interference in cell-free biosensing systems should be small. The selectivity of cell-free biosensors primarily depended on the biosensor elements, which needed further development. By exploring the sensitivity, the response time, and the selectivity of five biosensors, it was clearly known that the cell-free system could serve as a robust biosensing platform.

4. Conclusions

The cell-free system could be a powerful biosensing platform because of its open nature and easy genetic operation. By optimizing the design of genetic elements and cell-free systems, arsenic, mercury, 3OC12-HSL, pC-HSL, and benzoic acid biosensors were successfully achieved in cell-free systems. The detection limits could reach the nanomolar level, and the response time was much shorter than cells. Moreover, cell-free arsenic and mercury biosensors presented good selectivity. However, cell-free biosensing systems still had many challenges to be addressed for practical applications. Lower detection limit, faster response time, and better selectivity need to be further developed. The system stability, the cost, and in situ detection are also significant concerns. This study not only proved the feasibility and practicality of cell-free biosensing systems but also offered a direction on how to design better systems. With the development of cell-free

synthetic technology, cell-free biosensing systems would explicitly present their values for improving the environment and human health.

Conflicts of interest

There are no conflicts of interest to declare. This article does not contain any studies with human participants or animals.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jbiotec.2019.05.011.

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