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Electrochemical detection of *Pseudomonas aeruginosa* 16S rRNA using a biosensor based on immobilized stem–loop structured probe

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ABSTRACT

We have designed an electrochemical DNA biosensor based on stem–loop structured probes for enzymatic detection of *Pseudomonas aeruginosa* 16S ribosomal RNA (rRNA) in composting degradation. The probe modified with a thiol at its 5′ end and a biotin at its 3′ end was immobilized on a gold electrode through self-assembly. The stem–loop structured probes were "closed" when target was absent, then the hybridization of the target induced the conformational changes to "open", along with the biotin at its 3′ end binding with streptavidin-horseradish peroxidase (HRP), and subsequent quantification of the target was detected via electrochemical detecting the enzymatic product in the presence of substrate. Under the optimum experiment conditions, the amperometric current response to HRP-catalyzed reaction was linearly related to the logarithm of the target nucleic acid concentration, ranging from 0.3 and 600 pg/μL, with the detection limit of 0.012 pg/μL. A correlation coefficient of 0.9960 was identified. The 16S rRNA extracted from *P. aeruginosa* was analyzed by this proposed sensor. The results were in agreement with the reference values deduced from UV spectrometric data. The biosensor was indicative of good precision, stability, sensitivity, and selectivity.

1. Introduction

Biosurfactants are amphipathic compounds excreted by microorganisms. Possessing advantages over their chemical counterparts in biodegradability, e.g. lower toxicity, more ecological acceptability and effectiveness at extreme temperature and pH [1–3], they are widely used for emulsification, increasing detergency, wetting and phase dispersion, as well as solubilization [4–6]. Recent researches show that, a large number of biosurfactants are produced in solid organic matter degradation process, particularly in compost [7,8]. These biosurfactants have an active influence in the organic matter degradation process in compost. For example, biosurfactants optimize the water and gas phase distribution in compost medium. They accelerate the forming of liquid membrane on garbage particle surface, and provide biological reaction sites for the degradation, and also, they can enhance the transfer and dispersion of organic matter in the liquid film. At the same time, they promote the formation of mobility biofilm, thereby create a favorable condition for high-efficiency bioremediation of organic pollutants [9]. Rhamnolipid, as a most common biosurfactant, whose characteristics and functions have been deeply studied [10–13], is a glycolipid-type biosurfactant, mainly produced by *Pseudomonas aeruginosa* (*P. aeruginosa*) [14], a Gram-negative bacterium living in soil and aqueous environments [15], as well as in compost system. Therefore, it is important to investigate the population dynamics of *P. aeruginosa* for controlling the composting process.

The stem–loop structured DNA probes are superior to linear probes in several aspects for the detection of nucleic acid [16]. The greatest advantage is the superior mismatched discrimination. They also have great potential for real-time monitoring of analyzes [17]. Stem–loop structure based assays are fast, simple and inexpensive, and capable of real-time monitoring of nucleic acid reactions both *in vivo* and *in vitro* [18]. Since the first stem–loop structured oligonucleotide probes called molecular beacon (MB) reported in 1996 [19], there have been strong interests in its applications and further development in recent years. Most of early works showed that molecular beacons have been mainly used in a homogeneous liquid solution [20–23], and the vast majority of MBs utilized a novel implementation of fluorescence energy transfer. With the development of the research, there are also sev-

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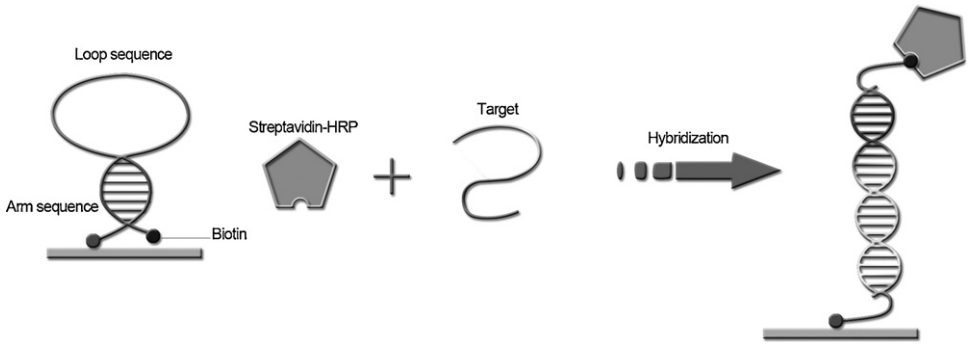

Fig. 1. Working principle of the stem–loop structured biosensor. Before hybridization, the probe is closed, when target nucleic acid was captured by the probe, the conformational of the stem–loop structure has changed, triggering the attached biotin group on the electrode surface, binding the streptavidin-horseradish peroxidase (HRP), which produced a electrochemical signal.

eral reports on surface-immobilized MBs for the design of DNA biosensor. Bockisch et al. reached a conclusion based on a large number of references: If utilizing the fluorescence energy transfer, the observed fluorescence enhancement of immobilized MBs would be much less than the MBs in solution, so immobilized MBs do not provide the sensitivity desirable for DNA biosensors [16]. Therefore, the electrochemical DNA biosensors based on stem–loop structured utilized enzymatic amplification have been developed [16,24].

Quantifying the 16S ribosomal RNA (rRNA) gene can reveal the abundance of microorganism in the environment. Sequence analysis of the 16S rRNA gene has been widely used to identify bacterial species and perform taxonomic studies [25–30]. The first use of the rRNA gene as a target for DNA probes to detect and classify microorganisms was described in 1986 [31]. Although *P. aeruginosa* can be cultured easily, it is difficult to get detecting when it is present in low numbers [32].

In this paper, an electrochemical DNA biosensor based on immobilizing the stem–loop structured oligonucleotide probe on a gold electrode for *P. aeruginosa* detection was developed. The stem–loop structured oligonucleotide probe is specific to *P. aeruginosa* 16S rRNA genes, streptavidin-horseradish peroxidase (HRP) label [33] was used as the conformational switch. The probe contained a stem of complementary 6 bp segments and a loop recognition sequence complementary to a 21 bp segments of the 16S rRNA of *P. aeruginosa*. A stem length of 6 bp provided the optimal background-to-signal ratio [16]. The probe modified with a biotin at its 3′ end and a thiol at its 5′ end was immobilized onto the gold electrode surface through self-assembly in the presence of mercaptopropionic acid (MPA). When the target nucleic acid was captured by the probe, the conformation of the stem–loop structure changed to trigger the specific interaction between the biotin and streptavidin-horseradish peroxidase (HRP) on the electrode surface. Therefore, the quantity of target nucleic acid was detected via electrochemical detecting the enzymatic product (Fig. 1). Total RNA isolated from *P. aeruginosa,* was applied to the biosensor detection after fragmentation.

2. Materials and methods

2.1. Chemicals and microorganism

P. aeruginosa was purchased from China Centre for Type Culture Collection in lyophilized form (the storage number is CCTCC AB93066). Albumin bovine serum (BSA) and streptavidin labeled horseradish peroxidase (HRP) were purchased from Dingguo Biotechnology Co., Ltd. (Beijing, China). Tris (hydroxymethyl) aminomethane (Tris) and 3-mercaptopropionic acid (MPA) were supplied by Sigma. Lysozyme, RNAprep pure Cell/Bacteria Kit and 5× loading buffer were provided by Tiangen Biotech Co., Ltd. (Beijing, China). Super green II was purchased from Fanbo Biochemicals Co., Ltd. (Beijing, China). All chemicals used in this study were of analytical reagent grade. All other solutions were prepared with ultrapure (>18 MΩ) water from a Millipore Milli-Q water purification system. Tris–HCl buffer (0.1 M Tris adjusted to pH 8.0 with 0.1 M HCl) and Tris–EDTA buffer (TE, 10 mM Tris–HCl and 1 mM EDTA, pH 8.0) were used in this work. The buffer of 0.01 M sodium phosphate-buffered saline (PBS) was used as incubating and washing buffer of pH 7.4.

2.2. Oligonucleotides and instrumentation

Based on *P. aeruginosa* 16S rRNA gene sequences available in GenBank, a sequence alignment has been applied through the Blast procedure to design the stem–loop structured probe whose loop sequence mismatched the 16S rRNA gene sequences of other bacterial strains in more than two positions. The oligonucleotides were synthesized by Sangon (Shanghai, China). The sequences of the oligonucleotides are shown in Table 1. A single mismatched sequence was applied to test the selectivity of the DNA sensor.

Electrochemical measurements were executed on CHI660B electrochemistry system (Chenhua Instrument, Shanghai, China) with a three-electrode system consisting of a gold working electrode (AiDa Technology Development Co., Ltd, Tianjin, China) with a diameter of 2 mm, a Ag/AgCl reference electrode and a platinum wire auxiliary electrode in a 25 mL beaker. Gel images were captured on a Gel Doc 2000 imaging system (Bio-Rad Laboratories, USA). A Sigma 1–14 Microcentrifuge (Sigma, Germany) and a Model CS501-SP thermostat (Huida Instrument, Chongqing, China) were used in the assay. An Eppendorf BioPhotometer was used to determine the concentration of the extracted RNA. All the work was accomplished at room temperature (25 °C) unless otherwise mentioned.

2.3. Preparation of RNA sample

P. aeruginosa was cultured on basic culture medium (adding beef extract 5.0 g, peptone 10.0 g, agar 20.0 g and NaCl 10.0 g into 1.0 L distilled water, adjusting pH to 7.0, followed by biocidal treatment) at 37 °C for about 36 h. The medium was solid, meaning *P. aeruginosa* was grown on plates. Total RNA was isolated from *P. aeruginosa* using the RNAprep pure Cell/Bacteria Kit according to the manufacturer's instructions (Tiangen Biotech Co., Ltd.). The presence of 16S rRNA was confirmed by gel electrophoresis in 1% agarose and TAE running buffer. Isolated RNA was fragmented by addition of a special buffer with the volume ratio of 4:1. The buffer was made with DEPC-treated distilled water, and consisted of 200 mM Tris, 500 mM potassium acetate, 150 mM magnesium acetate (pH 8.1) [34]. The mixture was incubated in a water bath (95 °C) for 10 min, and was subsequently cooled on ice and diluted to desired assay concentrations in SSC buffer (75 mM sodium citrate with 750 mM NaCl, pH 7.0) [16].

2.4. Sensor fabrication

The gold electrode surface was first mechanically polished to a mirror with 300 and 50 nm alumina slurry successively, and then was ultrasonic cleaning with ultra

Table 1
Sequences of the probe and its targets.

Name	Sequence
P1 (probe)	5′-(-C_6Thiol)-ggccgtGATAACGTCCGGAAACGGGCGacggcc-(-3′C6)(biotin)-3′
T1 (P1 complement)	5′-CGCCCGTTTCCGGACGTTATC-3′
T1M1 (single mismatch of P1)	5′-CGCCCCTTTCCGGACGTTATC-3′

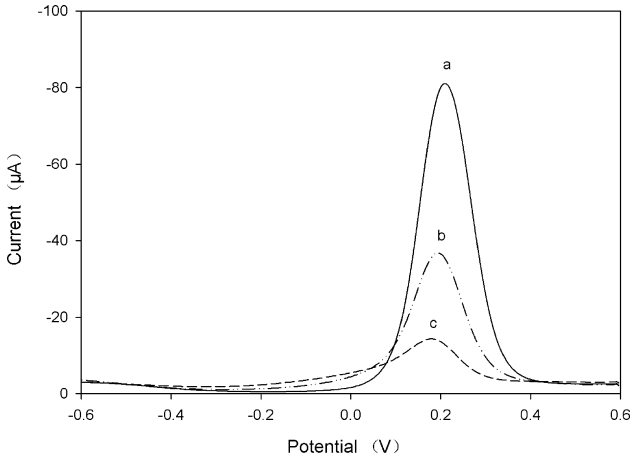

Fig. 2. SWVs of different electrodes in a 0.1 M KCl solution containing 5.0 mM ferricyanide. (a) Bare electrode; (b) gold electrode modified with P1 and MPA; (c) the modified gold electrode incubated 2 h in the presence of T1 at 37 °C.

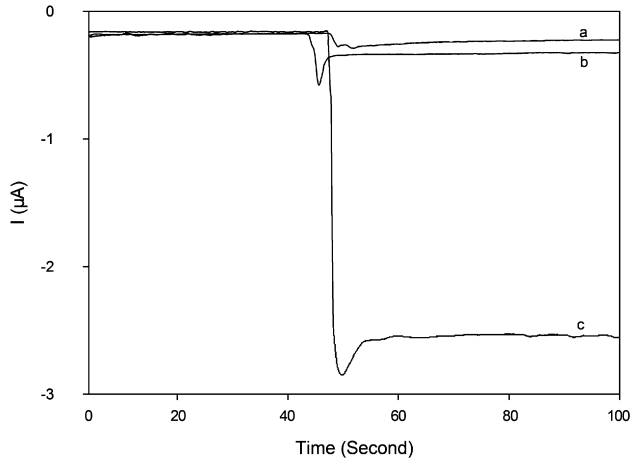

Fig. 3. Chronoamperometric curves of the different electrodes in the successive addition of H_2O_2, amperometric measurements were performed by applying a working potential of −0.15 V under unceasing stirring until transient currents were allowed to decay to a steady-state value. (a) Bare gold electrode; (b) T1 probe and MPA modified gold electrode,the electrode was incubated 2 h in the presence of T1M1 (single mismatch of P1); (c) T1 probe and MPA modified gold electrode, the electrode was incubated 2 h in the presence of T1 (P1 complement).

pure water for 3 min. The cleaned electrode was electrochemically pretreated in 0.5 M H_2SO_4 by cyclic scans from −0.5 to +1.0 V at 100 mV/s until the shape of cyclic curves no longer changed. The self-assembly was carried out by adding 10 μL P1 (10 ng/μL) solution on the gold surface of the electrode, which was then kept at 4 °C for 10 h. After adsorption, the electrode was thoroughly rinsed with PBS solution to remove any nonbonding materials. It is mentioned that the stem–loop structured oligonucleotide should be treated subsequently at 75 °C and ice-cold water bath for 30 and 10 min, respectively, and the procedure should be repeated again to avoid the forming of dipolymer [24].

2.5. Hybridization and detection

Hybridization of the target nucleic acid was carried out with adding 10 μL full complement (T1), or a single mismatch (T1M1) onto the electrodes. Then the electrode was incubated at 37 °C for 2 h in a humid chamber, followed by rinsing with PBS solution. It was then soaked in a tube containing 1% BSA (dissolved in PBS) for 1 h to block the active sites of the electrodes. After rinsing by PBS solution, 20 μL of 1:100 diluted streptavidin-HRP was dropped on the surface of the electrodes and was incubated at 4 °C for 30 min. The electrochemical detection was carried out by a CHI660B electrochemistry system (Chenhua Instrument, Shanghai, China). Electrochemical redox current catalyzed by HRP was measured amperometrically by addition of 0.1 M H_2O_2 into PBS (pH 7.4) containing 1 mM hydroquinone at a working potential of −0.15 V, under unceasing stirring. The sharply increase of the reduction current suggested that the streptavidin-HRP have been captured on the electrode. The prepared RNA samples were applied to the biosensor detection following the same procedure used for synthetic oligonucleotides. The concentration of the isolated RNA was also measured by UV spectrometry on Eppendorf BioPhotometer for comparison.

3. Results and discussion

3.1. Electrochemical performance of the biosensor

Theoretically, the thiolated oligonucleotides are immobilized on gold surface specialized through thiol-gold bonding. Previous studies have demonstrated that using blocking agents would reduce nonspecific adsorption and maintain original structure in the biomolecules self-assembly process [35–37]. Herein, MPA was used as the blocking agent. P1 probe and MPA with the molar ratio of 1:5 were self-assembled on the electrode surface, which exhibited the relatively better performance [24].

The morphological features of the modified electrode were investigated by square wave voltammetry (SWV). As shown in Fig. 2, an obvious peak is observed (curve a) at +0.209 V of the bare gold electrode. And the peak current decreased markedly (curve b) when the gold electrode was modified with P1 and MPA. This phenomenon implied that the electrode surface had been blocked by the immobilized biomolecules which impeded the pathway of electron transfer between the probe-coated electrode and ferricyanide in solution [37,38]. Early study showed that the addition of target nucleic acid made an increase of conductivity for the sensor containing single-stranded probe oriented perpendicular to the gold electrode surface [39]. But in our work, the peak current decreased again after hybridization with target nucleic acid (curve c). This was probably due to the electrostatic repulsion between the negatively charged ferricyanide ions and the polyanionic phosphate backbone of DNA layer [37].

The selectivity of the biosensor was investigated by analyzing total complementary, and single mismatch oligonucleotide under the controlled hybridization and electrochemical condition set. Fig. 3 demonstrated typical chronoamperometric curves of biosensors for successive additions of the same amounts of H_2O_2 under optimum experimental conditions [40]. Amperometric responses of the biosensor to 0.6 ng/μL T1M1 (curve b) and 0.6 ng/μL T1 (curve c) under the optimum experimental conditions were compared, with that of the bare gold electrode also evaluated (curve a). For P1–T1, the current increment was 2.2 μA upon adding 0.1 M H_2O_2 to the detection medium, whereas about 0.2 μA was detected for P1–T1M1. The results illustrated that the biosensor exhibited a rapid and sensitive response in single mismatch discrimination.

3.2. Optimization of self-assembly and hybridization

Previous researchers have demonstrated that the adsorption kinetics of self-assembly process on an electrode surface could be often described as a two-step process [41]. First, there is a fast growth of the film thickness to 80–90% of the final value, typically within a few minutes, and then a slower reorganization process was conducted to approach an equilibrium coverage density in approximately 10–20 h. We studied the effect of self-assembly time of P1 (10 ng/μL) upon the electron transfer characteristics detected by SWV in a 5.0 mM ferricyanide solution containing 0.1 M KCl (Fig. 4). It was easy to find the peak current response dropped fast in the first 2 h, but the follow-up testing demonstrated that the probe coverage did not form a stable and ordered monolayer, instead produced unstable amperometric response to target DNA. The current continued to decrease with increasing assembly time and showed a leveling off after 10 h, indicating that the coverage of probe had reached saturation. To achieve a highly stable and ordered oligonucleotide monolayer, self-assembly time of 10 h was

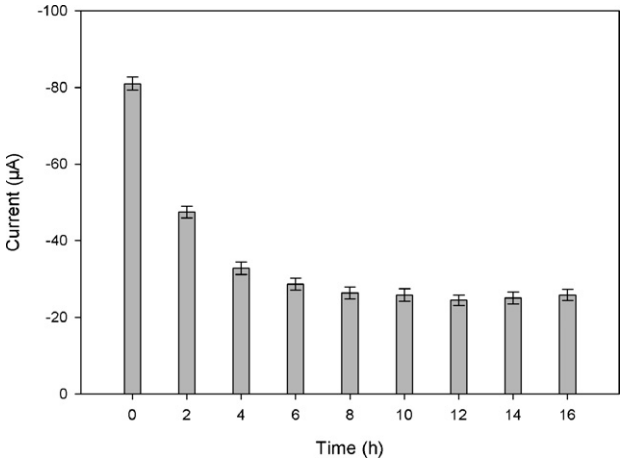

Fig. 4. The dependence of peak current of SWVs on self-assembly time of P1 (10 ng/μL) measured in a 0.1 M KCl solution containing 5.0 mM ferricyanide. The vertical bars designate the standard deviations for the means of three replicative tests.

selected throughout the experiment. The peak current response increased with the concentration increment of P1 between 0 and 100 ng/μL, and then it showed a very slight increase thereafter, we used 10 ng/μL in subsequent experiment because the further increases of P1 led to no increment of the signal.

Hybridization time was also investigated via SWV method. The effects of hybridization time vs. the SWV peak current change before and after hybridization are displayed in Fig. 5. Hybridization efficiency increased with the enhancement of the peak current change value. The hybridization amount rises fast between 30 and 90 min, and finally shows a leveling off after 120 min. Therefore, 120 min was selected as the optimal hybridization time for the whole experiment to ensure high efficiency. Some studies discovered the optimal temperature for hybridization is generally 5–25 °C under the melting temperature (*Tm*) of the probes [37], and the *Tm* of P1 in this work was about 75 °C, so the optimal temperature for hybridization in our experiment should be below 50 °C. However, relatively higher temperature causing dissociation of P1–T1 also reduced the efficiency of hybridization [24], and most early works [24,37] on DNA hybridization demonstrated that the optimal temperature for hybridization was generally about 37 °C. Thus, the optimal hybridization temperature was chosen as 37 °C.

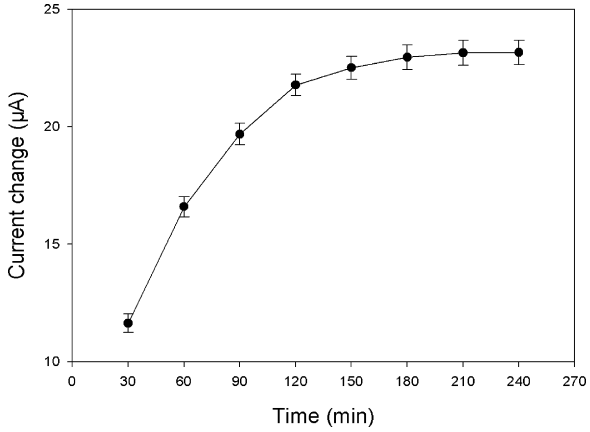

Fig. 5. The effects of hybridization time on the SWV peak current change before and after hybridization. The applied concentration of T1 was 0.6 ng/μL. The vertical bars designate the standard deviations for the means of three replicative tests.

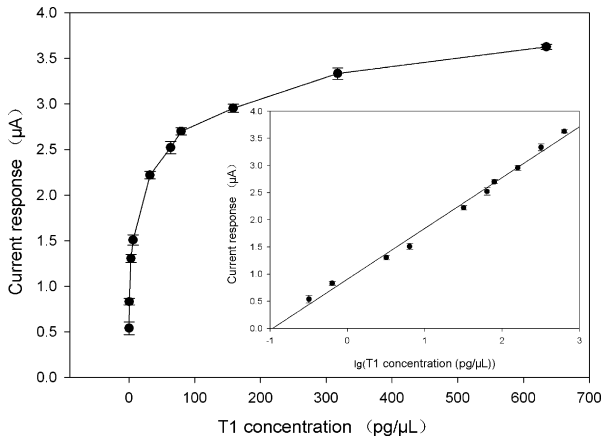

Fig. 6. Calibration curves of the sensor response to T1 under the optimal experimental conditions. Inset: linear regression of current response vs. the logarithm of T1 concentration. The vertical bars designate the standard deviations for the means of three replicative tests.

3.3. Calibration of the biosensor for the detection of synthesized oligonucleotides

Both the cyclic voltammetry and amperometry can show the HRP-catalyzed reduction current increased with the increment of the concentration of nucleic acid, but generally speaking, the amperometry produces much more precise changes at low nucleic acid concentrations than voltammetry. Thus, the amperometry was used for the nucleic acid quantification. The insert figure in Fig. 6 shows the amperometric current response to HRP-catalyzed reaction was linearly related to the logarithm of the target nucleic acid concentration. Under the optimum experiment conditions, a linear relationship between 0.3 and 600 pg/μL was obtained. The corresponding regression equation is

$$I = (0.9092 \pm 0.0504) + (0.9343 \pm 0.0295) \times \lg C$$

where I is the current response (μA), and C is the target nucleic acid concentration (pg/μL). A correlation coefficient of 0.9960 was identified. Each of the calibrations was performed three times. The average of the relative standard deviations (RSD) was 6.8%, with the detection limit of 0.012 pg/μL. The high sensitivity obtained was comparable to, or even exceed recently reported values obtained by DNA sensors [16,24,37].

3.4. Detection of P. aeruginosa 16S rRNA

16S ribosomal RNA is a macromolecule with a complex secondary structure. So we employ thermal denaturation and catalytic fragmentation (the details showed in Section 2.3) to reduce the potentially inhibitory effects of the secondary structure on the detection sensitivity. After that, only fragmentation of the target allowed to be detected. Then hybridization and electrochemical detection were carried out, which followed the same steps as detecting T1. Catalytic reduction current was detected at −0.15 V. The results were in good agreement with the reference values deduced from UV spectrometric data shown in Table 2. These data displayed that the average concentration recovered by biosensor were a little less than the concentration calibrated by UV spectrometry. It could be implied that even fragmented 16S ribosomal RNA poses some sterical constraints for efficient hybridization to immobilized stem–loop structures, resulting in a reduced detection sensitivity of 16S RNA [16].

Table 2
Detection results of 16S rRNA samples from *P. aeruginosa* by the biosensor and UV spectrometry.

Concentration calibrated by UV spectrometry (pg/μL)	Average concentration recovered by biosensor* (pg/μL)	Recovery (%)
5.41	4.95 ± 0.02	91.49
27.80	31.24 ± 0.05	112.37
99.21	95.19 ± 0.07	95.95
130.40	127.52 ± 0.06	97.79

* Average values and standard deviations of three replicates.

4. Conclusions

The proposed study reported an enzyme-amplified amperometric DNA electrochemical biosensor based on conformational change of stem–loop probe for recognition of target sequence of *P. aeruginosa* 16S rRNA at low levels. We used hybridized oligonucleotides to the sensor at first, under the optimum experiment conditions, the amperometric current response to HRP-catalyzed reaction was linearly related to the logarithm of the target nucleic acid concentration between 0.3 and 600 pg/μL, with a correlation coefficient of 0.9960. The detection limit was 0.012 pg/μL, exhibiting a very high sensitivity. Total RNA was isolated from *P. aeruginosa* using the RNAprep pure Cell/Bacteria Kit. Thermal denaturation and catalytic fragmentation were employed to obtain the target 16S rRNA segments for biosensor detection. The experimental parameters were optimized to maximize the sensitivity and speed up the assay time. The proposed biosensor was indicative of good sensitivity, precision, and selectivity. The detection results of 16S rRNA samples from *P. aeruginosa* were in good agreement with those of electrophoresis and UV spectrometry. Through this method, it will be very convenient to investigate the population dynamics of *P. aeruginosa* in the composting process. The selectivity of the biosensor by detected single mismatch oligonucleotide under the controlled hybridization and electrochemical condition set was investigated, but the specificity of this biosensor when challenged with total RNA from another bacterial species in actual tests are not analyzed, we will do it in the further experiments. Future studies are needed for the reusability of the biosensor.

Acknowledgements

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References

[1] Costa SG, Nitschke M, Haddad R, Wberlin MN, Contiero J. Production of *Pseudomonas aeruginosa* LBI rhamnolipids following growth on Brazilian native oils. Process Biochem 2006;41:483–8.
[2] Thanomsub B, Pumeechockchai W, Limtrakul A, Arunrattiyakorn P, Petchleelaha W, Nitoda T. Chemical structures and biological activities of rhamnolipids produced by *Pseudomonas aeruginosa* B189 isolated from milk factory waste. Bioresour Technol 2006;98:1149–53.
[3] Thavasi R, Subramanyam Nambaru VR, Jayalakshmi S, Balasubramanian T, Banat IM. Biosurfactant production by *Azotobacter chroococcum* isolated from the marine environment. Mar Biotechnol 2009;11:551–6.
[4] Desai JD, Banat IM. Microbial production of surfactants and their commercial potential. Microbiol Mol Biol Rev 1997;61:47–64.
[5] Ilori MO, Amobi CJ, Odocha AC. Factors affecting biosurfactant production by oil degrading *Aeromonas* spp. isolated from a tropical environment. Chemosphere 2005;61:985–92.
[6] Nitschke M, Pastore GM. Production and properties of a surfactant obtained from *Bacillus subtilis* grown on cassava wastewater. Bioresour Technol 2006;97:336–41.
[7] Zhang BY, Huang GH, Chen B. Production of biosurfactants in batch reactor for food waste composting. In: Almorza D, Brebbia CA, Sales D, editors. Waste management and the environment. UK: Wessex Institute of Technology; 2003. pp. 131–40.
[8] Vishwanathan SA, Bagley ST. Isolation and characterization of solvent-utilizing thermophilic bacterial. In: 101st General Meeting of the American Society for Microbiology. 2001. p. 641.
[9] Tang L, Zeng G, Shen G, Li Y, Zhang Y, Huang G, et al. Simultaneous amperometric determination of lignin peroxidase and manganese peroxidase activities in compost bioremediation using artificial neural networks. Anal Chim Acta 2006;579:109–16.
[10] Banat IM, Makkar RS, Cameotra SS. Potential commercial applications of microbial surfactants. Appl Microbiol Biotechnol 2000;53:495–508.
[11] Dean SM, Jin Y, Cha DK, Wilson SV, Radosevich M. Phenanthrene degradation in soils co-inoculated with phenanthrene-degrading and biosurfactant-producing bacteria. J Environ Qual 2001;30:1126–33.
[12] Rahman KSM, Banat IM, Thahira J, Thayumanavanb T, Lakshmanaperumalsamy P. Bioremediation of gasoline contaminated soil by a bacterial consortium amended with poultry litter, coir pith and rhamnolipid biosurfactant. Bioresour Technol 2002;81:25–32.
[13] Prabhu Y, Phale PS. Biodegradation of phenanthrene by *Pseudomonas* sp. strain PP2: novel metabolic pathway, role of biosurfactant and cell surface hydrophobicity in hydrocarbon assimilation. Appl Microbiol Biotechnol 2003;61:342–51.
[14] Shulga A, Karpenko E, Vildanova-Martsishin R. Biosurfactant-enhanced remediation of oil-contaminated environments. Adsorpt Sci Technol 2000;18:171–6.
[15] Sotirova A, Spasova D, Vasileva-Tonkova E, Galabova D. Effects of rhamnolipid-biosurfactant on cell surface of *Pseudomonas aeruginosa*. Microbiol Res 2009;164:297–303.
[16] Bockisch B, Grunwald T, Spillner E, Bredehorst R. Immobilized stem–loop structured probes as conformational switches for enzymatic detection of microbial 16S rRNA. Nucleic Acids Res 2005;33:e101, doi:10.1093/nar/gni101.
[17] Summerer D, Marx A. A molecular beacon for quantitative monitoring of the DNA polymerase reaction in real-time. Angew Chem Int Ed 2002;41:3620–2.
[18] Goel G, Kumar A, Puniya AK, Chen W, Singh K. Molecular beacon: a multitask probe. J Appl Microbiol 2005;99:435–42.
[19] Tyagi S, Kramer FR. Molecular beacons: probes that fluoresce upon hybridization. Nat Biotechnol 1996;14:303–8.
[20] Kostrikis LG, Tyagi S, Mhlanga MM, Ho DD, Kramer FR. Spectral genotyping of human alleles. Science 1998;279:1228–9.
[21] Li JJ, Fang X, Schuster S, Tan W. Molecular beacons: a novel approach to detect protein–DNA interactions. Angew Chem Int Ed 2000;39:1049–52.
[22] Matsuo T. In situ visualization of messenger RNA for basic fibroblast growth factor in living cells. Biochim Biophys Acta 1998;1379:178–84.
[23] Li J, Geyer R, Tan W. Using molecular beacons as a sensitive fluorescence assay for enzymatic cleavage of single-stranded DNA. Nucleic Acids Res 2000;28(e52):1–5.
[24] Mao X, Jiang J, Xu X, Chu X, Luo Y, Shen G, et al. Enzymatic amplification detection of DNA based on Molecular Beacon biosensors. Biosens Bioelectron 2008;23:1555–61.
[25] Choi BK, Wyss C, Gobel UB. Phylogenetic analysis of pathogen-related oral spirochetes. J Clin Microbiol 1996;34:1922–5.
[26] Schmalenberger A, Schwieger F, Tebbe CC. Effect of primers hybridizing to different evolutionarily conserved regions of the smallsubunit rRNA gene in PCR-based microbial community analyses and genetic profiling. Appl Environ Microbiol 2001;67:3557–63.
[27] Clarridge III JE. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clin Microbiol Rev 2004;17:840–62.
[28] Munson MA, Banerjee A, Watson TF, Wade WG. Molecular analysis of the microflora associated with dental caries. J Clin Microbiol 2004;42:3023–9.
[29] Petti CA, Polage CR, Schreckenberger P. The role of 16S rRNA gene sequencing in identification of microorganisms misidentified by conventional methods. J Clin Microbiol 2005;43:6123–5.
[30] Chakravorty S, Helb D, Burday M, Connell N, Alland D. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. J Microbiol Methods 2007;69:330–9.
[31] Kohne D, Hogan J, Jonas V, Dean E, Adams T. Novel approach for rapid and sensitive detection of microorganisms: DNA probes to RNA. Washington DC: American Society for Microbiology; 1986, pp.110–112.
[32] London CJ, Griffith LP. Characterisation of Pseudomonads isolated from diseased fleece. Appl Environ Microbial 1984;47:993–7.
[33] Tang L, Zeng G, Shen G, Li Y, Zhang Y, Huang D. Rapid detection of picloram in agricultural field samples using a disposable immunomembrane-based electrochemical sensor. Environ Sci Technol 2008;42:1207–12.

[34] Small J, Call DR, Brockman FJ, Straub TM, Chandler DP. Direct detection of 16S rRNA in soil extracts by using oligonucleotide microarrays. Appl Environ Microbiol 2001;67:4708–16.
[35] Peña SR, Raina S, Goodrich GP, Fedoroff NV, Keating CD. Hybridization and enzymatic extension of Au nanoparticle-bound oligonucleotides. J Am Chem Soc 2002;124:7314–23.
[36] Du H, Strohsahl CM, Camera J, Miller BL, Krauss TD. Sensitivity and specificity of metal surface-immobilized "Molecular Beacon" biosensors. J Am Chem Soc 2005;127:7932–40.
[37] Tang L, Zeng G, Shen G, Li Y, Liu C, Li Z, et al. Sensitive detection of *lip* genes by electrochemical DNA sensor and its application in polymerase chain reaction amplicons from *Phanerochaete chrysosporium*. Biosens Bioelectron 2009;24:1474–9.
[38] Xie H, Zhang C, Gao Z. Amperometric detection of nucleic acid at femtomolar levels with a nucleic acid/electrochemical activator bilayer on gold electrode. Anal Chem 2004;76:1611–7.
[39] Hianik T, Gajdo V, Krivanek R, Oretskaya T, Metelev V, Volkov E, et al. Amperometric detection of DNA hybridization on a gold surface depends on the orientation of oligonucleotide chains. Bioelectrochemistry 2001;53: 199–204.
[40] Caruana DJ, Heller A. Enzyme-amplified amperometric detection of hybridization and of a single base pair mutation in an 18-base oligonucleotide on a 7-μm-diameter microelectrode. J Am Chem Soc 1999;121: 769–74.
[41] Chen D, Li J. Interfacial design and functionlization on metal electrodes through self-assembled monolayer. Surf Sci Rep 2006;61:445–63.