Chapter 2

An Electrochemical DNA Sensing System Using Modified Nanoparticle Probes for Detecting Methicillin-Resistant *Staphylococcus aureus*

Hiroaki Sakamoto, Yoshihisa Amano, Takenori Satomura, and Shin-ichiro Suye

Abstract

We have developed a novel, highly sensitive, biosensing system for detecting methicillin-resistant *Staphylococcus aureus* (MRSA). The system employs gold nanoparticles (AuNPs), magnetic nanoparticles (mNPs), and an electrochemical detection method. We have designed and synthesized ferrocene- and single-stranded DNA-conjugated nanoparticles that hybridize to MRSA DNA. Hybridized complexes are easily separated by taking advantage of mNPs. A current response could be obtained through the oxidation of ferrocene on the AuNP surface when a constant potential of +250 mV vs. Ag/AgCl is applied. The enzymatic reaction of L-proline dehydrogenase provides high signal amplification. This sensing system, using a nanoparticle-modified probe, has the ability to detect 10 pM of genomic DNA from MRSA without amplification by the polymerase chain reaction. Current responses are linearly related to the amount of genomic DNA in the range of 10–166 pM. Selectivity is confirmed by demonstrating that this sensing system could distinguish MRSA from *Staphylococcus aureus* (SA) DNA.

Key words DNA biosensor, Nanoparticle, Electrochemical detection, Magnetic separation, Chronoamperometry

1 Introduction

Contamination of food and the environment by harmful microorganisms comes serious problem. Among the contaminating microorganisms, methicillin-resistant *Staphylococcus aureus* (MRSA), is a common causative agent of hospital-acquired infections, and is difficult to treat because of its resistance to β-lactam antibiotics [1]. Therefore, a quick and simple detection system for harmful microorganisms, including MRSA is required to determine appropriate treatment options.

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Conventional culture [2, 3] and real-time polymerase chain reaction (PCR) methods [4, 5] have been used for the detection of harmful microorganisms. Culture methods involve the growth of microorganisms on plates with specialized media that allows propagation of specific bacteria and visual enumeration of colonies. Detection of MRSA by culture method offer high sensitivity. However, the time required for growth and visualization of MRSA colonies precludes rapid detection. Real-time PCR is expensive and requires a relatively large-sized device. Thus, it is unlikely to be practical for routine on-site analyses.

In recent years, for the purpose of on-site analyses, a device has been developed for DNA sensing using an electrochemical technique. This device has advantages such as ease of use and a compact size. However, many of the electrochemical methods that have been reported only detected a synthetic DNA fragment as the target [6–8]. Detecting DNA from actual clinical samples will require amplification of the DNA extracted from the sample or an improved detection system, because amount of DNA in the sample is extremely small.

In the current study, we have constructed a novel DNA biosensing system using two types of nanoparticles; gold nanoparticles (AuNPs) and magnetic nanoparticles (MNPs) [9]. MNPs are modified by DNA probe I conjugation and AuNPs are modified by DNA probe II with ferrocene derivative. In this system, whole target DNA (not DNA fragments) is extracted from MRSA cells. DNA probes I and II are complementary to the *mecA* gene, which is an MRSA biomarker. Both types of nanoparticles are hybridized to genomic DNA, driving conjugate formation and magnetic separation. These samples are measured by an electrochemical analyzer to obtain the oxidation current of ferrocene on AuNPs (Fig. 1). In order to enable measurements without amplification of target DNA by PCR, L-proline dehydrogenase (LPDH) [10] and L-proline are used to amplify the detection current. The catalytic reaction between L-proDH and L-proline produces electrons, which react with the oxidized form of ferrocenecarboxylic acid. As a result, the concentration of the reduced form of ferrocenecarboxylic acid increases and oxidation currents are amplified. Consequently, it is confirmed that L-proDH and L-proline contributed to amplification of the oxidation current (Figs. 2 and 3). This sensing system, using a nanoparticle-modified probe, has the ability to detect 10 pM genomic DNA from MRSA without amplification by PCR. Current responses are linearly related to the amount of genomic DNA in the range of 10–166 pM. Selectivity is confirmed by showing that this sensing system could distinguish MRSA from SA DNA. Importantly, this sensing system allows for quick detection because PCR is not required and requires simple equipment that can be used on-site.

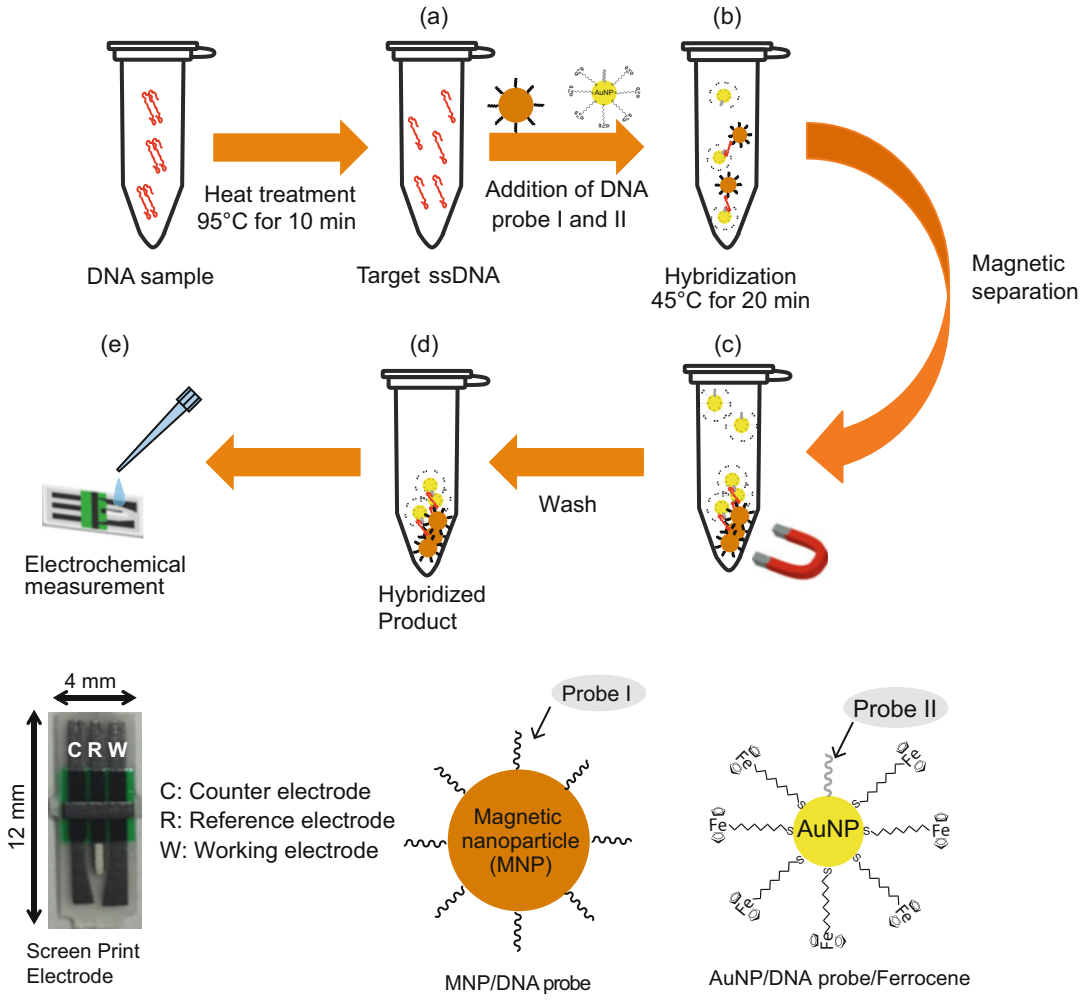

Fig. 1 Schematic illustration of target DNA detection using the DNA sensing system with modified AuNPs and MNPs. (**a**) Genomic DNA was heat-treated at 95 °C for 10 min to obtain ssDNA. (**b**) ssDNA was incubated with 3 μL of modified MNPs and AuNPs at 45 °C for 2 h, and (**c**, **d**) subsequently washed once for 3 min with 50 mM KPB (pH 6.5) followed by magnetic separation of nanoparticles. The separated conjugate was resuspended in 10 μL of 50 mM KPB (pH 6.5). (**e**) Hybridization products are applied to the SPE and restabilized for 100 s. Current responses are measured before the application of a droplet of enzyme and substrate, and again at 100 s following the application of a droplet

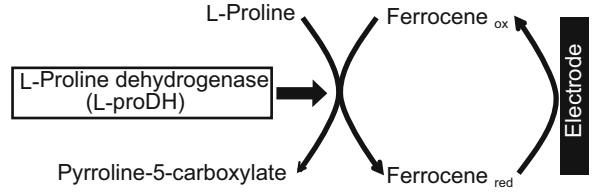

Fig. 2 Schematic diagram of electron transfer. Ferrocene$_{ox}$ is reduced by the catalytic reaction of L-proDH and L-proline. Thus, current responses are amplified because ferrocene$_{red}$ was increased

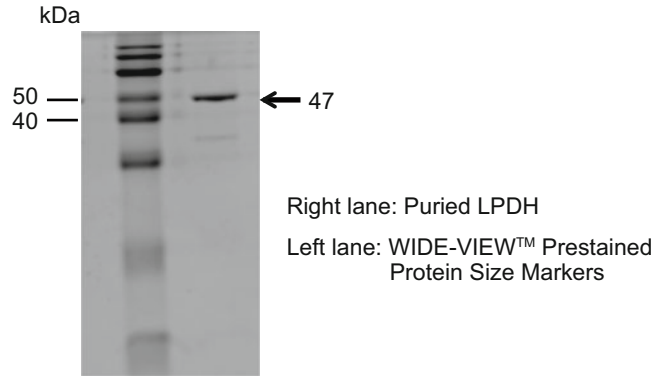

Fig. 3 SDS-PAGE of LPDH

2 Materials

2.1 Chemicals and Apparatus

1. MNP-modified amine groups with an average diameter of 100 nm are purchased from Magnabeat (Chiba, Japan).
2. AuNPs with an average diameter of 15 nm are purchased from British BioCell International (Cardiff, UK).
3. Dried yeast extract, tryptone, sodium chloride, dipotassium hydrogen phosphate, and potassium dihydrogen phosphate are purchased from Nacalai Tesque (Kyoto, Japan).
4. Ferrocene carboxylic acid, L (-)-proline, ethanol, β-mercaptoethanol, WIDE-VIEW Prestained Protein Sizw Maker, ampicillin sodium, and dithiothreitol are obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).
5. *N*-[(4-maleimidomethyl) cyclohexylcarbonyloxy] sulfosuccinimide sodium salt (Sulfo-SMCC) and sulfosuccinimidyl acetate (Sulfo-NHS-acetate) are obtained from Thermo Fisher Scientific Inc. (Rockford, IL, USA).
6. All other chemicals are of analytical grade. Deionized water that is filtered through a Milli-Q water purification system (Millipore Co., Bedford, MA, USA) is used for experiments in this study.
7. DNA probes targeting the MRSA *mecA* gene encoding methicillin resistance are purchased from Hokkaido System Science (Hokkaido, Japan). The sequences of the DNA probes used in this study are as follows:

   Probe I, 5′-SH-$(CH_2)_6$-TCTGGAACTTGTTGAGCAGAGG TTC-3′;
   Probe II, 5′- GCTTTGGTCTTTCTGCATTCCTGG-$(CH_2)_3$-SH.
8. Magnetic separation is conducted by Dynal MPC®-96S (Dynal Biotech, Oslo, Norway).

9. MRSA (ATCC-70060) is obtained from ATCC™.
10. Extraction of genomic DNA is performed using a MonoFas® bacterial genomic DNA extraction kit VII (GL Science, Tokyo, Japan), in accordance with the instruction manual.
11. Chronoamperometry experiments are performed with a Model 800B Electrochemical Analyzer (BAS Inc., Tokyo, Japan) and a screen printed electrode (SP-P DEP Chip (SPE)) (Bio Device Technology, Ishikawa, Japan), which consist of carbon electrodes as the working and counter electrodes and an Ag/AgCl as the reference electrode. All potentials are presented in terms of Ag/AgCl electrode potentials.

2.2 Expression and Purification of LPDH from the Hyperthermophilic *Archaeon* Aeropyrum pernix

1. *Escherichia coli* BL21-Codonplus® (DE3)-RIPL Competent Cells are purchased from Agilent Technologies (Santa Clara, CA, USA).
2. A plasmid vector from our laboratory encoding LPDH from a hyperthermophilic archaea, *A. pernix,* is used [10].
3. Preparation of Luria Broth (LB) agar medium: LB medium (tryptone 1% (w/v), yeast extract 0.5%, NaCl 0.5%, agar 2.0%, pH 7.0) is autoclaved at 121 °C for 15 min. The medium is cooled to about 60 °C and 20 mL poured to a petri dish with 1:1000 volume sterile 0.5% ampicillin sodium aqueous solution.
4. Potassium phosphate buffer (KPB) (10 mM, pH 7.2) + 100 mM NaCl (defined as Buffer A).

   Eighty milliliters of 1 M K_2HPO_4 and 20 mL of 1 M KH_2PO_4 are prepared. 1 M K_2HPO_4 is added to 1 M KH_2PO_4 until pH 7.2. Then, 10 mL of the solution and 5.84 g of NaCl are mixed and diluted to 1000 mL with pure water.
5. KPB (500 mM, pH 6.5)

   Eighty milliliters of 1 M K_2HPO_4 and 20 mL of 1 M KH_2PO_4 a prepared. 1 M K_2HPO_4 is added to 1 M KH_2PO_4 until pH 6.5. The solution is then diluted twofold and stored at 4 °C.

2.3 Synthesis of Modified Nanoparticles

1. Dithiothreitol 1 M (DTT)

   DTT (15.4 mg) is dissolved in 1 mL of ultrapure water at the time of use.
2. Methyl cyanide (MeCN) 5% (v/v)/0.1 M triethylamine Acetate (TEAA)

   Two and one half milliliters of 2 M TEAA and 2.5 mL of 100% MeCN are mixed with 45 mL of ultrapure water.
3. 30% (v/v) MeCN

   15 mL of 100% MeCN is diluted with 35 mL of ultrapure water.

4. Magnet nanoparticle (MNP)
   MNP-modified amino group with and average diameter of 100 nm are purchased from Magnabeat (Chiba, Japan).
5. Au nanoparticle (AuNP)
   AuNP with an average diameter of 15 nm are purchased from British Biocell International (Cardiff, UK).

3 Methods

3.1 Expression and Purification of LPDH from the Hyperthermophilic Archaeon, A. pernix

1. Expression of recombinant *Escherichia coli* by transformation
   Competent cryopreserved cells (*E. coli* BL21-Codonplus® (DE3)-RIPL) and the plasmid solution (pLPDH) are thawed on ice. Next, 2 μL of XL10-Gold® β-mercaptoethanol (Stratagene/Agilent Tehnologies Inc., CA, USA) solution, diluted tenfold with purified water, is added to 100 μL of competent cells, and placed on ice for 10 min with stirring every 2 min. 5 ng of the plasmid is added and the mixture allowed to stand for 30 min on ice. The mixture is then heated to 42 °C for 20 s, followed by a 2 min quenching on ice. 900 μL of Super Optimal broth with catabolite repression medium is then added and the mixture incubated for 1 h with shaking at 180 rpm at 37 °C. After incubation, the mixture is coated on a flat plate covered in LB medium containing ampicillin, and cultured overnight at 37 °C.
2. Culture of recombinant *E. coli*
   Sixty grams of Overnight Express™ Instant TB Medium (Novagen, USA) is dissolved in deionized water and 10 mL of glycerol is added. The TB medium is increased to 1000 mL with deionized water, poured into a 2000 mL baffled Erlenmeyer flask, and autoclaved at 121 °C for 15 min. After cooling, the medium is added to a 0.5% ampicillin sodium aqueous solution (1000:1) and inoculated with recombinant *E. coli*. The medium is incubated at 37 °C with shaking at 130 rpm for 16 h.
3. Harvesting and lysing of bacterial cells
   After completion of the culture, cells are recovered by centrifugation at 5950 × *g*, 4 °C, for 10 min. The cell pellet is washed in 0.85% physiological saline. The cells are then suspended in Buffer A for five times the cell amount and lysed using a sonicator (UD-201, Tomy Seiko, Tokyo, Japan). Conditions are two times for 3 min with the oscillation mode at 100% and an output value of 6 at 0 °C. The disrupted cell suspension is then centrifuged at 10,000 rpm, 4 °C, for 20 min. The supernatant containing LPDH is harvested and used as a crude enzyme solution.

4. Heat treatment

   As LPDH from hyperthermophile *A. pernix* shows high thermostability [10], it is very easy to purify LPDH by heat treatment to remove contaminant protein from cell-free extract. The crude enzyme in Buffer A is heated in a water bath at 80 °C for 10 min, then rapidly cooled on ice. Denatured proteins are removed by centrifugation at 11,600 rpm, 4 °C, for 15 min.

5. Ni-chelating chromatography (*see* **Note 1**)

   A Ni-NTA Superflow column (450 × 300 mm, id) is used. 100 mL of 50 mM Ni aqueous solution, followed by 300 mL of pure water, is applied to the column. The column is then equilibrated with Buffer A. After applying the heat-treated enzyme solution, the column is again washed with Buffer A.

   Enzyme elution is performed with a linear gradient of imidazole from 100 to 500 mM. Fractions are collected using a fraction collector. Enzymatic activity measurements and SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (Running gel: 15% pH 8.8, Stacking gel: 3%, pH 6.8) are performed on each fraction. The active fractions are identified and combined (*see* **Note 2**).

6. Gel filtration chromatography

   A Sephacryl S-300 column (880 × 300 mm, id) is equilibrated with 10 mM KPB (pH 7.0). The enzyme solution containing 50% glycerin is applied to the column with 10 mM KPB (pH 7.0) as the mobile phase. Fractions are collected using a fraction collector. Enzymatic activity measurements and SDS-PAGE are performed on each fraction and the active fractions are identified and combined.

3.2 Synthesis of Modified Nanoparticles for the Probes

1. Production of AuNP/Probe II/ferrocene conjugates

   Commercial thiolated probes are modified with a protecting group to prevent disulfide bond formation between the probe molecules. The protecting group is removed by adding 500 μL of 0.1 M DTT to the thiol probe in the dry state for 30 min at room temperature. Columns (Sep-Pak® Light C18 Cartridges, 13 × 4 mm, id) are equilibrated and washed with 5 mL of 100% MeCN and 5 mL of 2 M TEAA (pH 7.0) using a syringe. Then, 250 μL of deprotected thiol probe solution is added to 750 μL of ultrapure water, and passed through the column using a syringe to allow adsorption of the probe. The column is then washed with 5 mL of the 5% MeCN/0.1 M TEAA solution and 10 mL of ultra-pure water. 10 mL of 30% MeCN is then applied slowly and the deprotected thiolated probe is collected. Finally, the solvent is removed by lyophilization and the probe obtained for use in subsequent experiments.

   AuNPs are conjugated with Probe II by first mixing 1 mL of AuNPs, 4 nmol of Probe II, and 400 μL of 1 mM 11-ferrocenyl-

1-undecanethiol (Dojindo, Kumamoto, Japan) in ethanol, followed by incubation at 25 °C for 48 h under light shielding. Unconjugated Probe II and 11-ferrocenyl-1-undecanethiol molecules are removed by centrifugation at 15,000 × *g* for 30 min followed by washing with 1 mL of TE (Tris-EDTA) buffer (pH 8.0). The sediment obtained is resuspended in 100 μL of TE buffer and stored at 4 °C until further use.

2. Production of MNP/Probe I conjugates

   To conjugate MNP to Probe I, 1 mg of MNPs with modified amine groups is incubated for 2 h with 300 μg of sulfo-sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate in 100 mM phosphate buffered saline (PBS) (pH 7. 2) containing 200 mM NaCl. Subsequently, MNPs are separated magnetically and washed three times in 100 mM PBS (pH 7.2) for 3 min. The washed conjugates are resuspended in 1 mL of 100 mM PBS (pH 7.2) and incubated with 5 nmol of probe I for 8 h. This is followed by washing three times with 100 mM PBS (pH 7.2). The sediment obtained is resuspended in 1 mL of 3 mM sulfo-*N*-hydroxysulfosuccinimide acetate as a blocking buffer. The MNP/probe I conjugate is washed three times with 100 mM PBS (pH 7.2), resuspended in 200 μL of 10 mM PBS (pH 7.4) containing 200 mM NaCl, and stored at 4 °C until further use.

3.3 Electrochemical DNA Sensing System Using Modified Nanoparticle Probe

1. DNA hybridizations

   DNA probes are complementary to the *mecA* gene, which is an MRSA biomarker. Both types of nanoparticles are hybridized to genomic DNA, driving conjugate formation and magnetic separation (*see* **Note 3**). Genomic DNA is heat-treated at 95 °C for 10 min to obtain ssDNA. ssDNA is incubated with 3 μL of modified MNPs and AuNPs at 45 °C for 2 h, and subsequently washed once for 3 min with 50 mM KPB (pH 6.5) followed by magnetic separation of nanoparticles. The separated conjugate is resuspended in 10 μL of 50 mM KPB (pH 6.5) (*see* **Note 3**).

2. Electrochemical measurements

   The hybridized products are measured by an electrochemical analyzer to obtain the oxidation current of ferrocene on AuNPs. Fifteen microliters of KPB (50 mM, pH 6.5) is dropped at +250 mV on a Screen Printed Electrode (SPE: Bio Device Technology, Ishikawa, Japan), then applied potential set at +250 mV vs. Ag/AgCl and left to stabilize the current. Hybridization products are applied to the SPE and restabilized for 100 s. Then, 2 μL each of 100 mM L-proline and 342 M LPDH is added. Current responses are measured before the application of a droplet of enzyme and substrate, and again at 100 s following the application of a droplet (Fig. 4a).

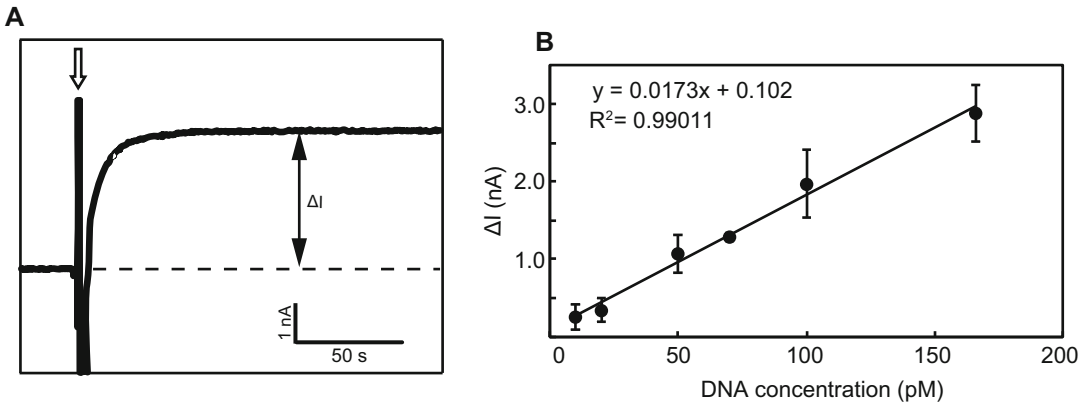

Fig. 4 (**a**) Chronoamperometry of hybridization products. The *arrowed line* indicates when L-proDH and L-proline were injected, after which oxidation currents were immediately observed. (**b**) Quantification of the DNA from MRSA based on current response. The error bars show standard deviations of triplicate experiments (n = 3)

Table 1
Purification of LPDH from recombinant cells

Steps	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Fold purification	Yield (%)
Crude extract	65.1	651	0.100	–	–
Heat treatment	63.2	121	0.524	5.24	97.1
Ni-NTA Superflow	54.0	60.4	0.894	8.94	82.9
Sephacryl S-300	55.2	59.1	0.934	9.34	84.8

4 Notes

1. Purification of LPDH
   Recombinant of LPDH is purified to homogeneity through heat treatment and two column chromatography steps.
2. SDS-PAGESDS
   Using SDS-PAGE, the size of LPDH is estimated to be 47 kDa, indicating the enzyme has a homodimeric structure. The enzyme is of sufficient purity for use in electrochemical measurements.
3. This sensing system using a nanoparticle-modified probe has the ability to detect 10 pM genomic DNA from MRSA without amplification by PCR. Current responses are linearly related to the amount of genomic DNA in the range of 10–166 pM (Fig. 4b). Selectivity is confirmed by showing that this sensing system could distinguish MRSA from SA DNA (Table 1).

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