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High sensitivity detection of 16s rRNA using peptide nucleic acid probes and a surface plasmon resonance biosensor

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ABSTRACT

A signal enhancing method allowing highly sensitive detection of *E. coli* 16s rRNA was developed using peptide nucleic acid (PNA) as a capture probe and a surface plasmon resonance (SPR) sensor as a detector. 16s rRNA has been used as a genetic marker for identification of organisms, and can be analyzed directly without PCR amplification due to the relatively high number of copies. PNA has a neutral backbone structure, therefore hybridization with 16s rRNA results in the ionic condition being changed from neutral to negative. A cationic Au nanoparticle was synthesized and used for signal amplification by ionic interaction with 16s rRNA hybridized on the PNA probe-immobilized SPR sensor chip. This method resulted in a detection limit of *E. coli* rRNA of $58.2 \pm 1.37\,\mathrm{pg\,mL^{-1}}$. Using this analytical method, *Staphylococcus aureus* was detected without purification of rRNA.

1. Introduction

The 16s rRNAs are commonly used genetic markers for identification of organisms that can be analyzed directly without PCR amplification due to a relatively high number of copies [1]. There have been several reports on the direct detection of 16s rRNA using oligonucleotide probes. Guschin et al. and Bavykin et al., for example, used gel element microarrays to directly and specifically detect fragmented RNA from simple model microbial communities [2,3]. Small et al. recently described an oligonucleotide array for the direct detection of 16s rRNA [4]. However, significant sensitivity limitation compared to PCR-based assays was observed. The 16s rRNA does not readily hybridize to oligonucleotide probes because its secondary structure prevents probes from interacting with complementary rRNA. To overcome these constraints, Small et al. used biotinylated oligonucleotides near the position of the capture probe in the hybridization buffer, which appeared to relax structural interference and increase hybridization efficiency [4]. However, the detection sensitivity was $14\,\mu\mathrm{g\,mL^{-1}}$ of total RNA representing 7.5×10^6 cells, which is still unsatisfactory. Recently 16s rRNA has been analyzed directly using an surface plasmon resonance (SPR) sensor system without labeling, but the lower detection limit was $2\,\mu\mathrm{g\,mL^{-1}}$ of 16s rRNA [5,6].

In this report, a signal amplification method for high sensitivity detection of 16s rRNA was developed using peptide nucleic acid (PNA) probes with an SPR biosensor system. Since first designed in 1991 by Nielsen et al. [7], PNA has been used for molecular diagnostics [8,9], antisense [10],

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and the other sensing technologies [11] due to its superior chemical and biological stability as well as its hybridization efficiency. PNA has a non-ionic backbone structure composed of *N*-(2-aminoethyl)-glycine units without pentose sugar moieties or phosphate groups [7–11]. Changes in surface property upon hybridization of DNA or RNA, from neutral to negative, allow selective electrostatic adsorption of positively charged materials such as fluorescent polymers [12], conjugated polymers [13], photoactive polymers [14], conductive polymers [15] and surface enhanced Raman scattering (SERS)-active Ag nanoparticle clusters [16]. Kerman et al. designed ferrocene-conjugated chitosan nanoparticles (Chi-Fc) that can be used as an electroactive indicator of PNA and DNA hybridization [17]. Nanoparticles modified with positively charged chitosan attached to the negatively charged phosphate backbone of DNA and gave rise to a high electrochemical oxidation signal from ferrocene. In this study, we used a surface plasmon resonance biosensor to detect the amount of hybridization with similar signal amplification strategy. The SPR biosensor is sensitive to changes in the thickness or refractive index of biomaterials at the interface between a thin Au film and an ambient medium and thus able to characterize biomolecular interactions in real time without labeling [18]. Signal enhancement methods for protein interactions [19] and small molecule detection [20] in an SPR biosensor by surface characterized Au nanoparticles have been reported. If Au nanoparticles are adhered onto the SPR sensor surface, the SPR angle is shifted remarkably through strong optical coupling between the gold film and nanoparticles [18,21,22]. Herein, hydrophilic cationic Au nanoparticles were prepared and designed to specifically interact with 16s rRNA to amplify the hybridization reaction between PNAs and *E. coli* 16s rRNA. The analysis using the SPR sensor enables to measure the amounts of binding at each analytical step, while other reports adopting the same analytical strategy such as fluorescence [12] and SERS [13] is possible only to detect end points. It is expected that the analytical method described in this report is convenient and sensitive in the detection of nucleic acids without requiring a second hybridization and additional labeling of samples [22].

2. Experimental

2.1. Materials

1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS) and 1.0 M ethanolamine (pH 8.5) were obtained from Biacore AB (Uppsala, Sweden). 11-Mercaptoundecanoic acid (MUA), H_2SO_4, and H_2O_2 were purchased from Aldrich (Milwaukee, WI, USA). A gold chip for an SPR sensor, consisting of a 2 nm chromium adhesion layer and 45 nm of gold deposited on a 25 mm-diameter round glass, was purchased from K-MAC (Daejeon, Korea). MW 500 K and 10 K amino dextran were obtained from Invitrogen Corporation (Carlsbad, CA). Biotinylated PNAs were synthesized by Panagene (Deajon, Korea). PNA sequences were designed based on the sequence data of bacterial 16s rRNA from GenBank:

E. coli specific	Biotin~5′-actgctgcctcccgtag-3′
Staphylococcus aureus specific	Biotin~5′-gccctttgtattgtcca-3′
Non-specific	Biotin~5′-gacccttttgtactatcc-3′

NHS-biotin and neutravidin was purchased from PIERCE (Rockford, IL). *E. coli* rRNA was obtained from Roche (Mannheim, Germany). Au colloid (20 nm), biotin–dextran (MW 10,000) and all other reagents were purchased from Sigma (St. Louis, MO).

2.2. Apparatus

SPR measurements were performed using an AutoLab ESPRIT instrument (Eco Chemie, Utrecht, Netherlands), which adopts cuvette type and has two channels. The size distribution and surface charge of the Au nanoparticles were measured using an ELS-Z photometer (Otsuka Electronics, Japan).

2.3. Procedures

2.3.1. Preparation of cationic Au nanoparticles

Au colloids (1 mL) were centrifuged at 12,000 rpm for 10 min then resuspended in 0.5 mL of 1 mM NaOH. An equal volume of ethanol containing 2 mM MUA was added to the resuspended solution. After 12 h, the solution was washed with 1 mM NaOH twice. PBST (0.01 M phosphate buffer with 0.138 M NaCl, 0.0027 M KCl, 0.05% Tween 20; 0.2 mL) was added to the solution, followed by addition of 10 K amino dextran to 0.2 mg mL^{-1}. For immobilization of amino dextran on the surface of MUA self-assembled Au nanoparticles, 50 μL of 0.4 M EDC, coupling reagent, was added to the solution. After 30 min of incubation at room temperature, 50 μL of 1 M ethanolamine and 0.1 M NHS were added respectively to change the unreacted carboxyl group to hydroxyl group then incubated for 15 min at room temperature. After this blocking reaction, particles were washed with 30 mM Tris–HCl (pH 7.4).

The size distribution and surface charge of the Au nanoparticles at initial, MUA self-assembled and amino dextran-immobilized conditions were measured at room temperature using the ELS-Z photometer by the principle of dynamic light scattering (DLS) and electrophoretic light scattering (ELS).

2.3.2. Preparation of a biotin-functionalized gold surface

A biotin-functionalized gold surface was prepared by a modified method of Ryu et al. [23]. The bare gold chip was cleaned with piranha (H_2SO_4/H_2O_2, 3/1, v/v) solution at 65 °C for 15 min. The gold chip was immersed in 10 mM ethanolic MUA at room temperature for 20–24 h to form a MUA self-assembled monolayer. The chip was washed with ethanol and water, sonicated for 5 min in ethanol, and dried under a stream of nitrogen. The terminal carboxylic acid group of MUA was activated by immersing the chip in a solution of 0.1 M EDC and 0.025 M NHS in DW for 15 min. Next, the NHS-activated gold chip was immersed in a 0.1 mg mL^{-1} 500 K amino dextran in 10 mM phosphate buffer for an hour then rinsed with distilled water. The amino dextran-

Table 1 – Various methods to prepare samples for the analysis of *Staphylococcus aureus* cells.

Methods	Cell suspension buffer	Cell lysis after boiling
I	DW[a]	No
II	DW	Sonication[c]
III	DW	Homogenization[d]
IV	LB[b]	No
V	LB	Sonication
VI	LB	Homogenization

[a] Deionized RNase free water.
[b] Lysis buffer, pH 4.0 and 25 mM Tris–HCl containing 25 mM EDTA and 1% SDS.
[c] Using BRANSON 8210 sonicator, 3 min.
[d] Using eppendorf tube fixed homogenizer (RNase free), 1 min.

Table 2 – Particle size and zeta potential analyses.

	Particle diameter (nm)	Zeta potential (mV)
Initial Au nanoparticles	27.4 ± 1.5	−37.2 ± 4.9
MUA self-assembled nanoparticles	28.5 ± 3.4	−21.8 ± 4.6
Amino dextran-coated	133.8 ± 2.0	26.4 ± 2.7

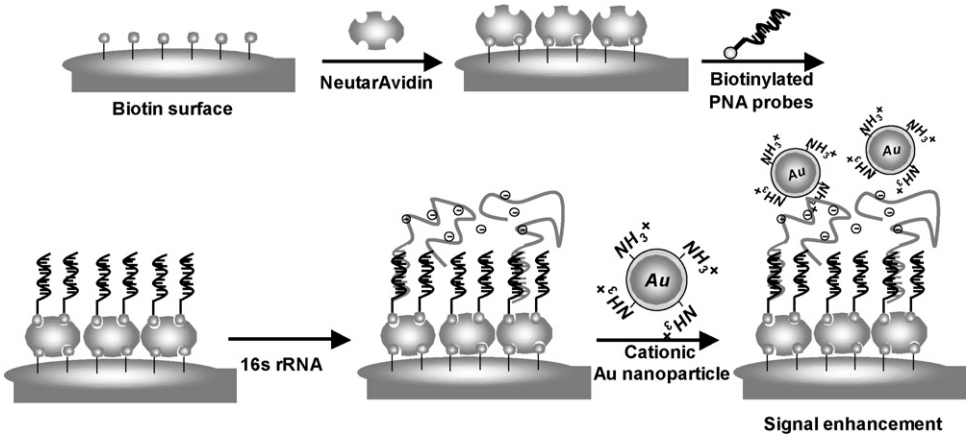
immobilized substrate was immersed in 1 M ethanolamine for 10 min to substitute unreacted carboxyl groups with hydroxyl groups. To create the biotin-functionalized substrate, unreacted part of the amine groups of amino dextran was reacted with 0.1 mg mL^{-1} NHS-biotin in PBS (0.01 M phosphate buffer with 0.138 M NaCl and 0.0027 M KCl) for 30 min. The chip was rinsed with distilled water twice and dried with argon gas.

2.3.3. *Analysis of 16s rRNA using an SPR sensor*

The overall analysis procedure was described in Scheme 1. The biotin-functionalized gold chip was attached to a half-cylinder prism with refractive-index matching oil (nD = 1.517) and inserted into the AutoLab SPR instrument. The volume of loaded sample was 50 μL. After each step, the chip was washed and equilibrated with a 10 mM HEPES buffer containing 0.15 M NaCl and 0.05% Tween 20 (pH 7.4). For all reaction steps except rRNA hybridization, mixing rate and mixing volume were 16.7 μL s^{-1} and 10 μL, respectively. *E. coli*-specific and non-specific biotinylated PNA probes (10 μg mL^{-1} in PBS) were immobilized on the neutravidin surface for 10 min. To block unreacted biotin-binding sites of neutravidin, the SPR chip was reacted with 0.1 mg mL^{-1} biotin–dextran in PBS. Before hybridization, *E. coli* rRNA in a hybridization buffer (pH 7.7) consisting of 20 mM sodium phosphate, 300 mM sodium chloride and 1 mM EDTA was denatured at 95 °C for 5 min and reacted with the PNA probes on the SPR chip for 1 h. After the hybridization reaction, the equilibrium buffer was changed to 30 mM Tris buffer (pH 7.4), allowed to equilibrate and the cationic Au nanoparticle in the Tris buffer (1.0 of O.D. at 520 nm) reacted for 15 min, followed by re-equilibration of the same Tris buffer.

2.3.4. *Analysis of S. aureus 16s rRNA*

S. aureus ATCC10537 strain was obtained from American Type Culture Collection (ATCC, Rockvile, MD, USA). The cells were grown and maintained in Nutrient broth (per liter, 3 g of Bacto Beef Extract, 5 g of Bacto Peptone [Difco Laboratories, Detroit, Mich.]; pH 7.2). For culture preparation, 3 mL of Nutrient broth in 5 mL test tube was inoculated with *S. aureus* cells (8×10^6 CFU mL^{-1}). The culture solution was incubated at 37 °C, 180 rpm on a rotary type shaker (Green science SI-S) and grown until an OD_{600} of 1.0–1.1 (approximately 2×10^9 CFU mL^{-1}). Cells were harvested by centrifugation at 5000 rpm in an Eppendorf centrifuge for 15 min. After the supernatant was decanted, the pellet was resuspended in sterilized deionized water (DW) or a lysis buffer (25 mM Tris–HCl, 25 mM EDTA, 1% SDS, and adjust to pH 4.0). The suspended solution was boiled at 100 °C for 20 min and then lysed by sonication or homogenization. The sample preparation methods were listed in Table 1. The cell lysate (80 μL) was mixed with 20 μL of 5× hybridization buffer (100 mM Na-phosphate, 1.5 M sodium chloride, and 5 mM EDTA, pH 7.7). Subsequent procedure was same to the analysis method described in Section 2.3.3 besides using



Scheme 1 – Schematic illustration of 16s rRNA hybridization with PNA probes and its signal amplification by an ionic interaction with cationic Au nanoparticles.

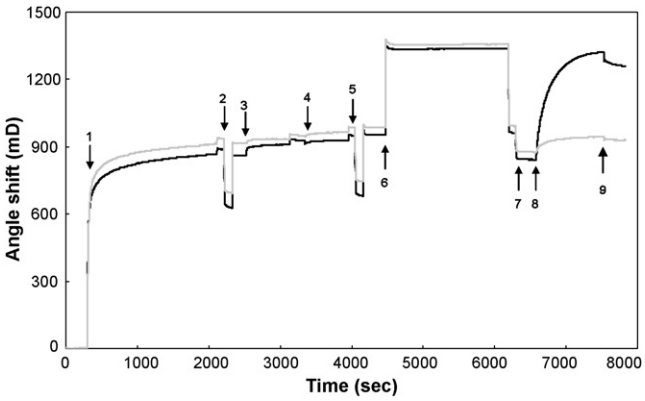


Fig. 1 – SPR sensorgram representing the analysis of 400 ng mL^{-1} *E. coli* rRNA with signal amplification. Black and gray lines correspond to specific and non-specific PNA probes, respectively. (1) Neutravidin, (2) 10 mM NaOH, (3) specific or non-specific PNA probes, (4) biotin–dextran, (5) 10 mM NaOH, (6) 400 ng mL^{-1} *E. coli* rRNA, (7) running buffer change (30 mM Tris, pH 7.4), (8) cationic Au nanoparticles, and (9) 30 mM Tris buffer, pH 7.4.

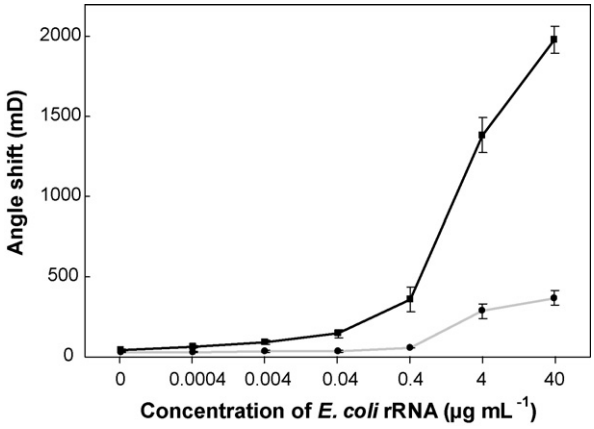


Fig. 2 – Signal-amplified SPR angle shift at various concentrations of *E. coli* rRNA. The data points were obtained in duplicate and error bars represent the standard deviations of duplicate runs. (■) Specific probe; (●) non-specific probe.

S. aureus-specific biotinylated PNA probe instead of *E. coli*-specific one.

3. Results and discussion

The cationic Au nanoparticles were characterized by a DLS method. Particle diameters and zeta potentials were measured three times repeatedly, and the average values and standard deviations were listed in Table 2. The initial Au particles show anionic charge due to citrate coated onto bare Au surface, and the MUA self-assembled also show an anionic charge due to the carboxylic acid residue of MUA. Amino dextran modified particle shows cationic charge by the amine group and larger size than the other particles in previous two steps by capping the amino dextran.

A biotin-functionalized SPR chip was prepared via MUA self-assembled on a bare gold surface, covalent bonding of amino dextrans on the MUA surface, and biotinylation of amino dextrans. Amino dextran was used to minimize non-specific binding at subsequent steps as a similar approach to Ryu et al. [23]. Scheme 1 illustrates our procedure for an SPR-based hybridization combined with signal amplification using cationic Au nanoparticles. The specific and non-specific PNA probes sequence against *E. coli* 16s rRNA and *S. aureus* 16s rRNA were designed based on the sequence data of GenBank and immobilized on the SPR sensor chip surface by the biotin–neutravidin interaction. The analysis procedure consisted of mainly three steps: hybridizing of rRNA samples with the PNA probes immobilized on a gold chip; exchanging equilibrium buffer to a low-salt buffer (30 mM Tris buffer, pH 7.4); and binding the cationic Au nanoparticles at the same Tris buffer condition. To achieve specific signal amplification, careful consideration to ionic conditions should be given. The analytical procedure was designed for cationic nanoparticles to be specifically interacted with rRNA. To minimize non-specific interactions, a hydrophilic biotinylated sensor surface was prepared. Amino dextran was immobilized on the carboxylated surface using an amine coupling method followed by reaction with NHS-biotin [23]. Neutravidin was immobilized on the hydrophilic biotinylated sensor surface, and then the biotinylated PNA probes were immobilized on its unreacted biotin-binding sites. Neutravidin instead of avidin or streptavidin was used because it has a neutral isoelectric point and therefore does not interact with the cationic nanoparticle at neutral pH conditions [24]. Fig. 1 is a sensorgram representing the analytical procedure of Scheme 1. The angle shift caused by neutravidin immobilization was 900 mD (millidegree), corresponding to 7.5 ng per 1 mm^2 surface area (1 mD of AutoLab SPR system corresponds to 8 RU of Biacore instruments [25], and 1 RU is equivalent to 1 pg protein per 1 mm^2 surface area). The angle shifts by immobilization of the specific and non-specific biotinylated PNA probes were 61.3 and 42.2 mD, respectively, and those by hybridization of 0.4 μg mL^{-1} *E. coli* rRNA were 12.5 and 7.2 mD, respectively. After hybridization, the equilibrium buffer was changed from 10 mM HEPES buffer containing 0.15 M NaCl and 0.05% Tween 20 (pH 7.4) to 30 mM Tris buffer (pH 7.4). The change of the equilibrium buffers-induced negative shifts of SPR angles which were 113 and 111 mD for the specific and non-specific probes, respectively. The negative shifts of the SPR angles were caused by decrease in refractive index of the buffers by salt reduction. After equilibrium with the Tris buffer, the cationic Au nanoparticles were poured onto the sensor chip. As shown in Fig. 1, the shift of the SPR angle was 432 mD on the specific probe and 46 mD on the non-specific probe. The ratio of the SPR angle shifts by the ionic interaction on the specific to the non-specific PNA channel is 9.4-fold, indicating that signal amplification is achieved successfully.

Based on the signal amplification method, *E. coli* rRNA of varying concentrations was assayed. Fig. 2 describes angle shifts via signal amplification depending on the concentration of *E. coli* rRNA. We observed an increase of SPR angle shift with increasing the concentration of *E. coli* rRNA. SPR

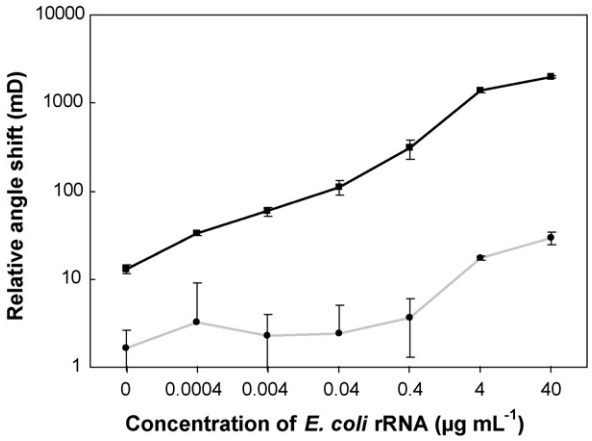


Fig. 3 – Comparison of the *E. coli* rRNA analyses with and without signal amplification. Relative SPR angle shifts were the signal-amplified angle shift for the specific probe substracted by that of the non-specific probe. The data points were obtained in duplicate and error bars represent the standard deviations of duplicate runs. (■) Signal amplification; (●) direct 16s rRNA hybridization.

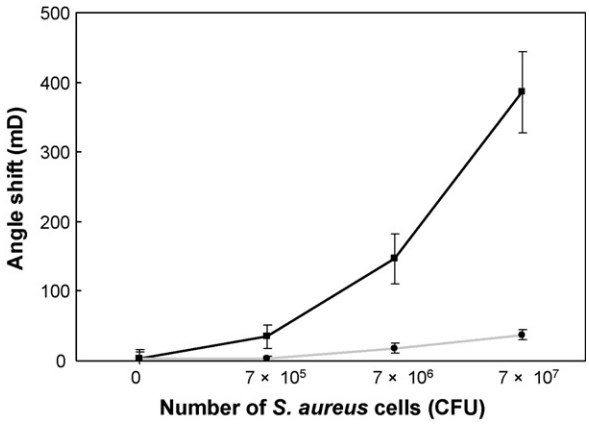


Fig. 5 – Signal-amplified SPR angle shift at various concentrations of *S. aureus*. The data points were obtained in duplicate and error bars represent the standard deviations of duplicate runs. (■) Specific probe; (●) non-specific probe.

angle shifts were also found for the non-specific probe at high rRNA concentrations. This may be due to non-specific binding of rRNA to the non-specific PNA probe at high concentrations. Nevertheless, the angle shift due to signal amplification for the specific probe was high enough to quantify rRNA. In Fig. 3, the relative angle shifts (the signal-amplified angle shift of the specific probe subtracted by that of the non-specific probe) were plotted against the rRNA concentrations. The relative angle shifts via direct hybridization of rRNA before the signal amplification were also plotted. As shown in Fig. 3, while 400 ng mL^{-1} rRNA was detectable via direct hybridization, 400 pg mL^{-1} rRNA was detectable via the signal amplification methods. The lower detection limit was defined as the smallest concentration or amount of an analyte observed and calculated as the blank signal plus or minus 3 standard deviations; 320 ± 16.76 ng mL^{-1} for direct hybridization and 58.2 ± 1.37 pg mL^{-1} when signal enhancement was employed. These results represent a dramatic signal enhancement, resulting in approximately 5500-fold greater signal sensitivity. Comparing with the other reports for the direct detection of 16s rRNA [4–6], the detection sensitivity in this study is very high.

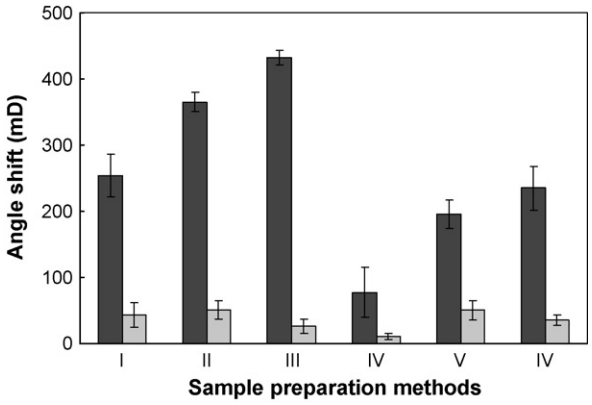


Fig. 4 – Optimization of the sample preparation conditions for the detection of *S. aureus*. The cell lysis conditions are described in Table 1.

To investigate if this signal amplification strategy is applicable in real pathogenic bacteria, *S. aureus* was tested. Cultured *S. aureus* cells were centrifuged and re-suspended with DW or LB (lysis buffer). The suspended cell solution was boiled and then lysed using a sonicator or homogenizer. Various methods for preparing cell lysate were tested to optimize the detection sensitivity as described in Table 1. To investigate the cells to be disrupted effectively, the cell lysates were spread on agar plates composed of the nutrient broth containing 1.8% of Agar powder. As a result, no colony was found in the all cases (data not shown).

The cell lysate was mixed with the hybridization buffer without further purification of rRNA. The mixed sample was allowed to be hybridized on PNA probes and further analyzed with the signal enhancement. As shown in Fig. 4, the Method III was the most efficient showing the highest SPR angle shift for the specific probe with relatively low signal for the non-specific probe. As a whole, DW was more efficient than LB as a cell suspension solution, and homogenization was more efficient than sonication.

Using the Method III, *S. aureus* with varying cell concentrations was assayed. Fig. 5 describes angle shifts via signal amplification depending on the concentration of *S. aureus* cells. We can find that 7×10^5 CFU *S. aureus* is detectable. Although the detection sensitivity is unsatisfactory comparing to other methods such as PCR and immunoassay, the detection method developed in this study has advantages such as no requirement of PCR process via the direct detection of ribosomal RNA, use of cell lysate without any purification process, and versatile design of capture probes targeting the 16s rRNAs. The detection of *S. aureus* cells was possible in 3 h, and therefore the detection method developed in this study has potential to the rapid analysis of bacterial cells.

4. Conclusions

By utilizing the neutral backbone property of PNA, a simple signal enhancing method for high sensitivity detection of 16s rRNA on an SPR sensor surface was developed. A cationic Au nanoparticle was synthesized and used for signal amplification by ionic interaction with 16s rRNA hybridized on the PNA probe-immobilized SPR sensor chip. The lower detection limit of *E. coli* rRNA was $58.2 \pm 1.37\,\mathrm{pg\,mL^{-1}}$ and decreased about 5500-fold comparing to that without signal amplification. When the detection method was applied to the analysis of *S. aureus*, 7×10^5 CFU *S. aureus* cells were detectable. We expect that this signal enhancing method will be applied for highly sensitive identification of bacteria without PCR and labeling.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aca.2008.10.001.

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