Electro-microchip DNA-biosensor for bacteria detection

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This paper presents a bacteria biosensor based on DNA hybridization detection with an electro-microchip transducer. *Acinetobacter baumannii* was chosen as DNA sample source, because the occurrence of bacteremia caused by *Acinetobacter baumannii* is high in hospitals worldwide. Our strategy is based on DNA hybridization of PCR amplified bacteria DNA with biotin labelled primers and detection enhancement using gold-streptavidin nanoparticles and Ag^+-hydroquinone solution. Gold nanoparticles catalyze silver ions reduction by hydroquinone. The gradually precipitated silver metal between the two electrodes of the electro-microchip allows electrons to pass. The detection limit for *Acinetobacter baumannii* genomic DNA sample is 0.825 ng mL^{-1} (1.2 fM). Probe specificity was investigated by screening various species of bacteria, various strains of a single species and various species of a single genus. The proposed DNA hybridization method is easy, convenient, and rapid. Moreover, it has potential applications in detection of bacteria causing infections and clinical diagnosis.

1. Introduction

Bacterium detection can be achieved by hybridization of a bacterium DNA sample with a specific oligonucleotide probe. The DNA sample, extracted from a bacterium, can be amplified by polymerase chain reaction (PCR). The probe is designed to from the intergenic transcribed spacer (ITS) region of the bacterium by sequence alignment, and it is complementary to the specific sequence of a DNA sample. So, the probe has a high specificity for the DNA sample of a bacterium and can be used for specific bacterium detection.

DNA hybridization is commonly employed in hospitals for diagnosing the infectious bacteria, including *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Chryseobacterium indologenes*, *Pseudomonas fluorescens* and other infectious microorganisms. For example, bacteremia caused by *Acinetobacter baumannii* is difficult to treat, and the mortality rate is high. It is necessary to develop a new DNA hybridization method for rapid and precise bacterial detection.

In conventional methods, chemiluminescence staining or fluorescent staining are commonly used to detect the DNA hybridization reaction.[1,2] In recent years, colloid metal nanoparticles have been applied in various domains. Gold nanoparticles, silver nanoparticles, and platinum nanoparticles are most commonly used. In order to enhance the detection sensitivity of colloid metal nanoparticles, many researchers have increased the diameter size of nanoparticles to increase the detection signal. Many detection methods have been developed to detect nanoparticle tags after DNA hybridization, including electrochemical techniques,[3–7] absorption spectrometry, surface enhanced Raman spectroscopy (SERS),[8–11] and surface plasma resonance (SPR).[12,13] Although these methods can improve the sensitivity of gold nanoparticle-based methods to detect nanomolar or even picomolar concentrations of DNA sample, they generally need sophisticated processes and expensive instruments.

The immunogold labeling technique is commonly used for nanoparticle marking.[14,15] Due to the high electron density of nanoparticles, colloidal gold-labeled proteins appear dark brown under an electron microscope, producing distinct images of labeling. This image response can be further enhanced using silver precipitation on the surface of the nanoparticles, called immunogold silver staining (IGSS).[16,17] Recently, the IGSS technique was found to increase the sensitivity of sequence-specific DNA detection.[18] IGSS can greatly enhance the performance of traditional electron and optical microscopes for analysis and observation.

Mirkin *et al.* demonstrated that nanoparticles can be used to label DNA molecules to produce a detection signal, which can increase the detection sensitivity when silver ions are reduced by hydroquinone on the nanoparticles surface.[18–20] Li *et al.* used the specific binding property between biotin and streptavidin, combined with nanoparticles and a chemiluminescence detection system, to determine DNA concentration.[21] Wang *et al.* used a novel method for detecting DNA hybridization, based on the precipitation of gold or silver on AuNPs tags and an electrochemical stripping detection.[22–24] The signal amplification and the low detection limit (nanomolar and picomolar levels) were achieved by precipitation of gold and silver onto the colloidal gold label. In this study, the DNA sample labelled with biotin was hybridized with a specific oligonucleotide probe which was immobilized on an electro micro-chip under a hybridization reaction. Then, AuNPs-streptavidin was used to react with the biotin target. After the silver precipitation occurred on the AuNPs, the variation in impedance value was measured in the electro micro-chip by LCR meter. Finally, the specificity tests

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using different DNA samples in the electro-microchip are discussed. These methods have been recently applied to immunoassay.[25–27] The principle is using silver precipitation on the surface of the nanoparticles, which is obtained from the silver ions, to enhance the amplified signal. The silver metal covering the nanoparticles has a high absorption for light, enhancing the detection signals based on color and electron.

The aim of this study was to develop a new detection method of DNA hybridization that used an electro-microchip to detect the DNA hybridization reaction signal. The DNA probe was first immobilized on a glass slide. Surface chemistry modification with 3-glycidoxypropyl-trimethoxysilane (3-GPS) was used to create the epoxy-silane group to increase bonding with the amine group on the glass slide surface.[28] The DNA sample labelled with a biotin molecule was hybridized to a specific DNA probe in the hybridization reaction. The DNA samples labelled with biotin and AuNPs-streptavidin were then conjugated. The AuNPs-streptavidin was then used to react with the biotin-labeled DNA and used as a label of the DNA samples and as a catalyst for silver precipitation. The silver enhancement reaction was used to magnify the detection signals, and the variation of the impedance value was measured with a commercial LCR meter for DNA hybridization analysis. The detectable concentration limit of the DNA samples was 0.825 ng mL^{-1}. The probe specificity tests for different DNA samples in the electro-microchip include: (1) the specificity tests for various other species of bacteria, and (2) the performance test among various strains of a single species and various species of a single genus.

2. Materials and methods

2.1 Reagents

The DNA probe and primer were purchased from MDBio Inc., Taiwan. Trisodium citrate dehydrate, sodium dodecyl sulfate, N-laurylsarcosine, sodium chloride, AuNPs-streptavidin, silver enhancement solutions A and B, and blocking reagent were purchased from Sigma-Aldrich. *Taq* DNA polymerase was obtained from Fermentas. The 3-GPS and toluene were obtained from Acros.

Four bacteria species were used in this study: *Acinetobacter baumannii* (*A. baumannii*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Chryseobacterium indologenes* (*C. indologenes*), *and Pseudomonas fluorescens* (*P. fluorescens*), were obtained from National Cheng Kung University Medical Center (Tainan, Taiwan). Most of these species were isolated from blood or sputum. All species were subcultured on tryptic soy agar, incubated at 35 °C for 18 to 20 h, and then used for DNA extraction. The probe sequences for each bacterium are shown in Table 1.

Table 1 The probe sequence and PCR product for each bacterium

Microorganism	Probe code	Sequence (5′–3′)	PCR product size
A. baumannii	Abau2	CGG TAA TTA GTG TGA TCT GAC GAttttttttt	1200 bp
P. aeruginosa	Paer2R	GGA TCA CGC AGC ACA CCA ACA ACA ATttttttttt	1100 bp
C. indologenes	Cind5R	TAC TAA TTT CTA GTG GGT TTT GTAttttttttt	1200 bp
P. fluorescens	Pflu2	GGA AGC AGC CCG AAA TTG GGTttttttttttt	1100 bp

2.2 DNA extraction and amplification

The boiling method was used to extract the DNA from the bacteria.[29] Shortly, one colony of a pure culture was suspended in 50 μL of sterilized water and heated at 100 °C for 15 min. After centrifugation in a microcentrifuge (6000 × g for 3 min), the supernatant was stored at −20 °C for further use.

The bacterium-specific universal primers 2F (5′ TTG TAC ACA CCG CCC GTC 3′) and 10R (5′ TTC GCC TTT CCC TCA CGG TA 3′) were used to amplify a DNA fragment that encompassed a small fragment of the 16S rRNA gene region, the ITS, and a small fragment of the 23S rRNA gene region. The primers used to amplify the bacterial DNA were labeled with a biotin molecule at the 5′ end (commercially synthesized). PCR was performed with 5 μL (1 to 5 ng) of DNA template in a total reaction volume of 50 μL consisting of 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.8 mM deoxyribonucleoside triphosphates (0.2 mM each), 1 μM (each) primer, 1 U of *Taq* DNA polymerase, and 50 μL of a mineral oil overlay. An OmniGen thermal cycler (Hybaid Limited, Middlesex, UK) was used for PCR.[30] The PCR product size for each bacterium is shown in Table 1.

2.3 DNA hybridization model

For this proposed method, the surface of the glass slide was modified by 3-GPS solution. The modified glass slide was more hydrophobic, on which the DNA probes could be immobilized. The specific DNA probe immobilized on the glass slide was used to hybridize DNA sample labeled with biotin. Then, AuNPs-streptavidin was added to the reaction chamber to react with the biotin-labeled DNA that was specifically bound to the probe immobilized on the chip. In the silver enhancement process, the conjugation of DNA sample and AuNPs-streptavidin will catalyze the silver ions in the silver enhancement solution to reduce to the silver metals. Then, the surface of the AuNPs would be covered with the reduced silver metals. The proposed model is shown in Fig. 1. After the DNA hybridization, an impedance change was measured with a commercial LCR meter. The impedance data are then analyzed using a personal computer.

In the silver enhancement process, the silver enhancement solution is a mixture of the hydroquinone solution and Ag+ solution at a ratio of 1 : 1. The silver ions in the silver enhancement solution were reduced by the catalysis of AuNPs and a large number of silver particles precipitated around the AuNPs.[18] The silver precipitate constructed a "bridge" between the two electrodes of the electro-microchip, allowing the electrons to pass, and thus decreased the impedance between the two electrodes.

2.4 Electro-microchip fabrication and design

The proposed electro-microchip consisted of two components: the DNA hybridization well and thin-film parallel electrodes. A 3.0 mm-thick layer of poly-dimethylsiloxane (PDMS) with

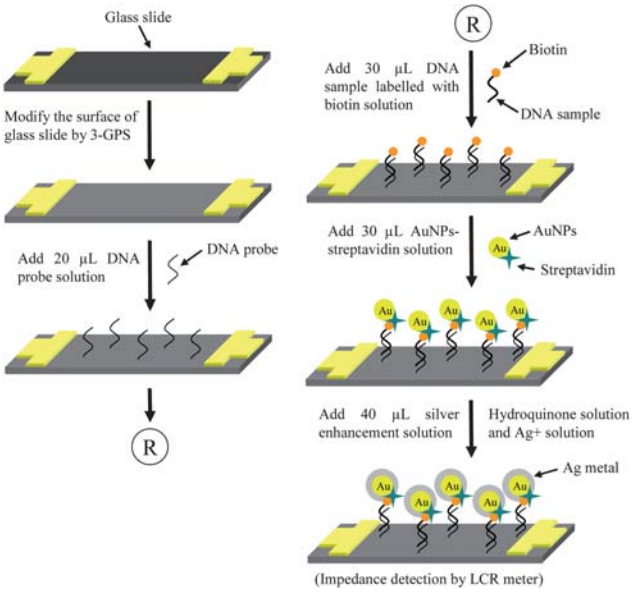

Fig. 1 Principle of the proposed method for DNA hybridization and signal amplification with silver enhancement. AuNPs-streptavidin conjugated with the DNA samples act as catalysts to reduce silver ions to silver metal.

2.5 mm-diameter holes was bonded to the glass surface with patterned parallel electrodes to form the fixed-volume (40 μL) wells for DNA hybridization. The electrodes were made of a 200 nm gold layer with a 20 nm chromium under-layer. The gap between the parallel electrodes was 20 μm and the electrode length was 1500 μm.

The thin-film parallel electrodes were fabricated using micro-electro-mechanical system (MEMS) technology. Gold and chromium thin films were thermally evaporated onto glass slides. Photolithography was used to transfer the electrode pattern to the thin films. The parallel electrodes were formed after chemical wet etching, as shown in Fig. 2.

2.5 Surface modification of glass slide

The 3-GPS was used to modify the functional group of the glass surface to epoxy-silane so that the specific probe (including the functional group of the amino) could be fixed to the glass slide.

One percent 3-GPS was prepared by mixing 1 mL of 3-GPS solution with 99 mL of toluene in a beaker. After soaking in this solution for 30 min, the substrates were rinsed several times by fresh toluene to remove any unbound 3-GPS molecules. Finally, the modified substrates were dried with N_2 and stored in an oven (110 °C) for 40 min.

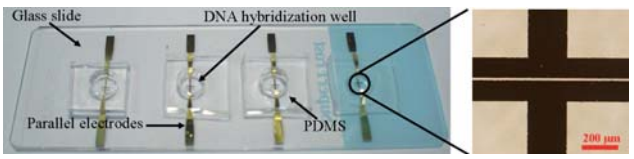

Fig. 2 Pictures of the DNA hybridization electro-microchip which includes electro-microchip with a gap of 20 μm and a PDMS reaction well.

2.6 DNA hybridization procedures

DNA probe grafting and sample hybridization are presented on Fig. 1. After the glass slide was cleaned and modified, 20 μL of DNA probe solution (5 μM) was pipetted into the reaction wells on the electro-microchip, and incubated at 37 °C overnight to immobilize the oligonucleotide probes on the glass slide. After the glass slide was washed with buffer solution (0.25 × SSC and 0.1% SDS) three times to remove the unbound probes, 30 μL of the target DNA sample (mixed with DNA hybridization solution) was added and the mixture was incubated at 50 °C for 1.5 h. After washing with buffer solution three times to remove the unbound DNA sample, 30 μL of AuNPs-streptavidin solution was added and the mixture was incubated at 37 °C for 2 h. The glass slide was washed with buffer solution three times to remove the unbound AuNPs-streptavidin, rinsed with distilled water three times and dried with nitrogen gas. The silver enhancement solution was added into the reaction wells and the impedance in the wells were measured.

The detection procedure was as follows: 40 μL of silver enhancement solution was added into the reaction wells; the solution was removed and refilled every 3 min (repeat 7 times). This procedure could prevent the natural formation of silver metals in the presence of silver enhancement solution.[20] Before impedance detection, the reaction well was washed 3 times with distilled water and dried with nitrogen gas to ensure complete removal of reaction solution inside the well. The LCR meter was used to detect the change of impedance in the reaction area between two parallel electrodes, with data automatically recorded by the computer.

2.7 Impedance measurement system

The measurement system includes: (i) an LCR meter, which is the main instrument for measuring the variation of the impedance, (ii) the Lab-VIEW® program for automatically acquiring the data, and (iii) an electro-microchip. The LCR meter is set to measure the impedance modulus at 100 Hz and 0.5 V. The GPIB protocol is used to link the computer and the LCR meter, and the Lab-VIEW® program is used to acquire the measurement data.

3. Results and discussion

3.1 Contact angle measurement

The clean glass slides were used as the unmodified substrate. The water contact angle of the unmodified substrate is 34.72°, and that of the 3-GPS modified substrate is 59.83°, as shown in Fig. 3. The results show that the 3-GPS modification effectively made the glass slide more hydrophobic. The 3-GPS could be used to modify the functional group of the glass surface to epoxy-silane. After the modification of the glass surface, the specific probe (including the functional group of the amino) could be fixed to the glass slide to process the hybridization experiments.

3.2 Silver precipitation on nanoparticles

In order to verify the applicability of the proposed DNA hybridization method, the AuNPs-streptavidin must be demonstrated to catalyze silver ions to silver metals. The relationship

Fig. 3 Water contact angles for cleaned glass slide. (a) 34.72° before 3-GPS modification and (b) 59.83° after 3-GPS modification.

between the various concentrations of AuNPs-streptavidin (from 10^{-2} to 10^{-10} μM) and the impedance value has been discussed. The silver ions were reduced to silver metals by the catalysis of AuNPs-streptavidin. The "silver bridge" between parallel electrodes was filled gradually. Then the decreased impedance was measured by LCR meter. The measurements are based on detecting the difference of the impedance. The higher the sample concentration is, the more reduced the silver metal will be, which indicates a lower impedance. The measurements were carried out every 3 min. The impedance difference of the various sample concentrations can be clearly observed in 15 min, as shown in Fig. 4. Even though the reaction well was washed every 3 min, silver ions in the silver enhancement solution of the negative control were reduced to silver metal in 18 min, which led to the impedance decrease. The impedance was infinite at the beginning of the experiment since the silver enhancement solution did not precipitate. The change of the impedance can be observed clearly from 15 min. However, at 18 min, because of the reduced silver metal, the impedance also dropped to the measurable region even in the negative control. Since a shortest detection time is desirable, the experiment data at 15 min was the optimal choice for analysis.

When the concentrations of AuNPs-streptavidin were lowered to 10^{-4} μM, the impedance value was increased and the concentration effect was observed, as shown in Fig. 4a. When the concentrations were lowered to 10^{-7} μM, the profile of the impedance values was similar to Fig. 4a, and the concentration effect was also observed in 15 min after silver enhancement reaction, as shown in Fig. 4b. When the concentrations were under 10^{-7} μM, the profile of the impedance values and the concentration effect was not changed obviously in 15 min (data not shown). The detectable concentration limit of AuNPs-streptavidin is 10^{-7} μM, and the relative standard deviation (RSD) is 12% ($n = 3$). Because the impedance values were quite large (excess 100 kΩ) in the lower concentration of AuNPs-streptavidin, the suitable concentration of AuNPs-streptavidin was 10^{-3} μM (RSD is 23%, $n = 3$) in the DNA hybridization method when considering the accuracy, reliability, and convenience of the experiment.

In this experiment, it was found that AuNPs-streptavidin acted as an ideal catalyst for amplifying the signal in silver enhancement reaction. Even when the concentrations of AuNPs-streptavidin were lowered to the micro range, silver ions could still be reduced to silver metals in the presence of the silver enhancement solution. Evidently, AuNPs-streptavidin, like gold nanoparticles, still retained their catalytic ability to enhance silver precipitation.

3.3 Detectable concentration limit for DNA sample

To obtain the detection limit of DNA sample, serial dilutions of the DNA sample (*Acinetobacter baumannii*) ranging from 27.5 to

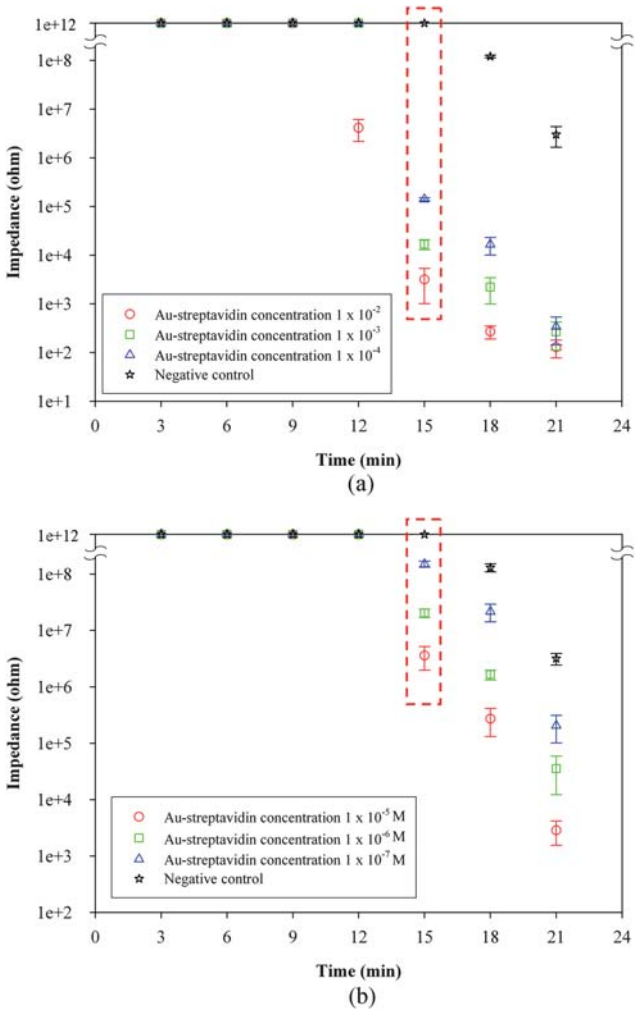

Fig. 4 Impedance value for various concentrations of AuNPs-streptavidin. (a) AuNPs-streptavidin concentrations from 1×10^{-2} to 1×10^{-4} μM, (b) AuNPs-streptavidin concentrations from 1×10^{-5} to 1×10^{-7} μg mL^{-1}.

8.25×10^{-7} μg mL^{-1} were analyzed by the chip under the condition of 5 μM of probe and 10^{-3} μM of AuNPs-streptavidin. Instead of DNA sample, distilled water was used as the negative control. Since silver metal formed through the reduction of silver ions under catalysis of AuNPs, the silver metal gradually filled the space between the electrodes and thus the impedance value in the well gradually decreased. The impedance change provided a meaningful measurement for the quantitative analysis of our proposed hybridization analysis. The results in Fig. 5 show the concentration effect of DNA sample at 15 min. When the concentrations of DNA sample were from 8.25×10^{-5} to 8.25×10^{-7} μg mL^{-1}, a linear dose effect did not exist (data are not shown). The detection limit of the DNA sample was 8.25×10^{-4} μg mL^{-1} in 15 min (RSD is 13%, $n = 3$). The results show that the DNA hybridization reactions can be measured with electro-signal detection methods. The relationship between the concentration of DNA sample and the impedance signal was established. The detection limit of DNA sample was 0.825 ng mL^{-1} (1.2 fM) using 5 μM of oligonucleotide probe and 10^{-3} μM of AuNPs-streptavidin.

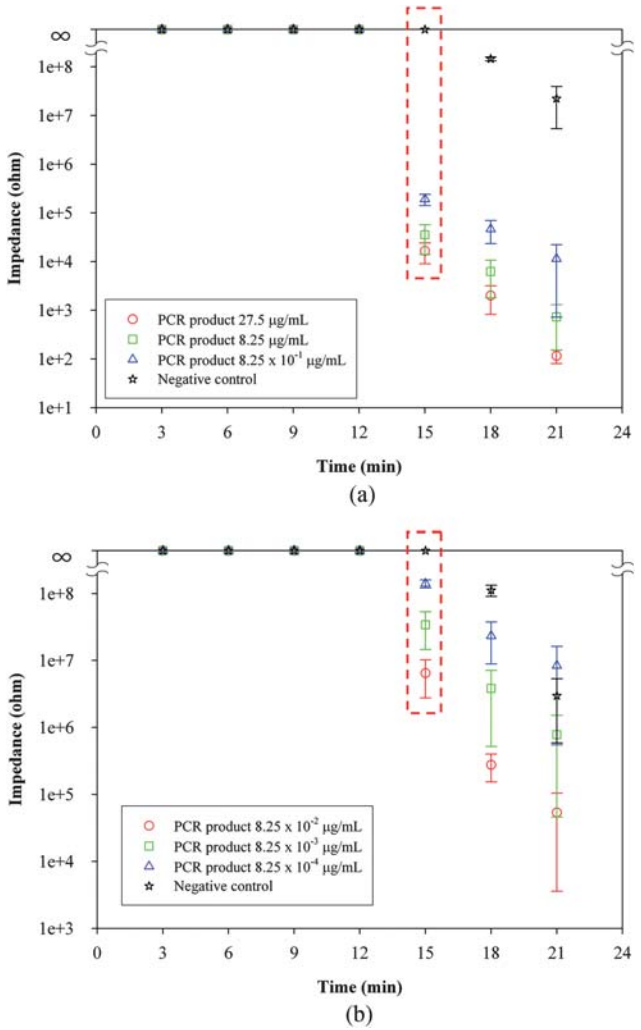

Fig. 5 Impedance value for various concentrations of DNA samples. (a) DNA concentrations from 27.5 to 8.25 × 10^{-1} μg mL^{-1}, (b) DNA concentrations from 8.25 × 10^{-2} μg mL^{-1} to 8.25 × 10^{-4} μg mL^{-1}.

3.4 Biosensor specificity

To verify the application of the electro-microchip, impedance changes were measured for four species of bacteria: *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Chryseobacterium indologenes*, and *Pseudomonas fluorescens*. The impedance values were quite large (over 100 kΩ) in low concentrations of DNA samples ranging from 8.25 × 10^{-1} to 8.25 × 10^{-7} μg mL^{-1}. Therefore, the suitable concentration of DNA samples for specificity testing was 8.25 μg mL^{-1} using 5 μM of probe and 10^{-3} μM of AuNPs-streptavidin. The results showed that the various DNA samples only reacted with their own specific probes in 15 min, as shown in Table 2. Therefore, if a specific probe was used, the hybridization reaction was specific.

Table 2 Impedance value and RSD value of specific probe test for various species of bacteria. The various DNA samples only reacted with its own specific probe

	Impedance value of specific probe for various species of bacteria (kΩ)			
	A. baumannii	*P. aeruginosa*	*C. indologenes*	*P. fluorescens*
A. baumannii	13.9	∞	∞	∞
P. aeruginosa	∞	15.1	∞	∞
C. indologenes	∞	∞	13.5	∞
P. fluorescens	∞	∞	∞	53.1
RSD% ($n = 3$)	84%	90%	88%	46%

Table 3 Impedance value and RSD value of the *A. baumannii* for various strains of a single species and for various species of a single genus

	A. baumannii (kΩ)	RSD% ($n = 3$)
A. baumannii (BCRC 10591T)	13.9	84%
A. baumannii (BCRC 15884)	102	69%
A. baumannii (LMG 984)	391	97%
A. genomic species 3	∞	—
A. genomic species 6	∞	—
A. calcoaceticus	∞	—
A. haemolyticus	∞	—

Table 4 Impedance value and RSD value of the *P. aeruginosa* for various strains of a single species and for various species of a single genus

	P. aeruginosa (kΩ)	RSD% ($n = 3$)
P. aeruginosa (BCRC 10944T)	15.1	90%
P. aeruginosa (ATCC 27853)	175.6	58%
P. aeruginosa (LMG 984)	∞	—
P. mendocina	∞	—
P. stutzeri	∞	—
P. pseudoalcaligenes	∞	—
P. putida	∞	—

3.5 Specificity of various strains of a single species and various species of a single genus

To verify the feasibility of the DNA hybridization microchip, we used DNA samples of various strains of a single species and various species of a single genus to conduct impedance analysis. DNA samples from three strains of *Acinetobacter baumannii* (BCRC 10591^T, BCRC 15884, and LMG 984) and from two strains of *Pseudomonas aeruginosa* (BCRC 10944^T and ATCC 27853) were used. In addition, DNA samples from the genera o *Acinetobacter* (*Acinetobacter* genomic species 3, *Acinetobacter* genomic species 6, *Acinetobacter calcoaceticus*, *Acinetobacter haemolyticus*) and *Pseudomonas* (*Pseudomonas alcaligenes*, *Pseudomonas mendocina*, *Pseudomonas stutzeri*, *Pseudomonas pseudoalcaligenes*, and *Pseudomonas putida*) were also tested. Again, the results demonstrated that the hybridization reaction was species-specific, not strain-specific and cross-hybridization was not caused by other species in the same genus (Table 3 and Table 4).

4. Conclusion

A new method for DNA hybridization analysis was developed. The method was based on an electro-microchip that detected DNA hybridization using AuNPs-streptavidin conjugate as a catalyst for silver precipitation in the presence of silver ions and hydroquinone. The detection limit of DNA sample was

0.825 ng mL^{-1} (1.2 fM) using 5 μM of probe and 10^{-3} μM of AuNPs-streptavidin. The hybridization reaction in the electro-microchip was species-specific if an appropriate probe was used. The proposed method is simple, fast (within 15 min), convenient, and low-cost. In addition, only a small amount of the reagent is required (as little as 30 μL DNA sample per well). This approach has many potential uses in the area of clinical diagnosis.

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