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Innovative antimicrobial susceptibility testing method using surface plasmon resonance

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ABSTRACT

Utilizing the ultra sensitivity of surface plasmon resonance (SPR) biosensor to examine drug resistance of bacteria was studied in this research. Susceptible and resistant strains of *Escherichia coli* JM109 to ampicillin and those of *Staphylococcus epidermidis* to tetracycline, served as a blind test, were examined. The bacteria adhered on the Au thin film was treated by the injection of antibiotic flow. The optical property change of the bacteria responded to antibiotics were recorded through SPR mechanism. As a result, the susceptible strain of *E. coli* generally revealed more than three times of SPR angle shift when compared to the resistant one; the susceptible strain of *S. epidermidis* revealed irregular SPR angle shift while the resistant strain kept the SPR angle almost unchanged. The new SPR method took less than 2 h of antibiotic treatment time to complete the antimicrobial susceptibility test. Different from conventional applications of SPR, specific antibodies is not required in this method. As compared to the conventional assays, Kirby-Bauer disk diffusion and variations of broth microdilution usually take 1 day to weeks to issue the report. Using this SPR assay can greatly reduce the waiting period for laboratory tests, and can therefore benefit patients who need proper antibiotic treatments to control bacterial infections. The sensitivity of the SPR biosensor built for the application is around 1.4×10^{-4} on the refractive index.

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1. Introduction

Infectious diseases are a leading cause of morbidity and mortality in hospitalized patients. This fact places a tremendous burden on the clinical microbiology laboratory to rapidly diagnose the agent responsible for patient's infection and to effectively provide therapeutic guidance for eradication of the organism. In agriculture, rapid diagnosis of drug resistance of bacteria also has enormous impact on agriculture economy. Besides, laboratories are asked not only to perform these tasks with efficiency, but also in a cost-effective manner in an era of increasing emphasis on reduction of laboratory expenses (Levinson and Jawetz, 1989). Two common methodologies for antimicrobial susceptibility testing in a clinical laboratory are Kirby-Bauer disk diffusion and variations of broth microdilution (Poupard et al., 1994). These methods usually take from 1 day to weeks to complete the experiments and issue the reports. Such a waiting period is not short for patients and clinical doctors who urgently need the information to adjust the therapeutic strategy. The long incubation period is inevitable for the conventional methods since these detection methods are based on the doubling time of the bacteria being tested. Different from the conventional methods, we report here a novel way to differentiate a susceptible strain of bacteria from a resistant one by using surface plasmon resonance (SPR) technique. This technique detects the refractive index change of tested bacteria subject to antibiotics in real time. The testing method is then independent of the cell doubling time. A much shorter time to obtain the test result is expected. Besides, gene information of bacteria is not required in this testing method.

Surface plasmon was first predicted by Ritchie in 1957 (Ritchie, 1957), verified by Powell and Swan using electron excitation in 1959 (Powell and Swan, 1959), and demonstrated by Otto using optical excitation in 1968 (Otto, 1968). For the surface plasmon excited by light, the dispersion characteristic of a surface plasma wave

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does not allow the light coupled into the surface plasma wave in general: Along the metal–dielectric interface, the spatially varied phase of an optical wave propagating in space is always smaller than that of the surface wave with the same optical frequency. A method to work out this difficulty is to use a prism, by which the spatially varied phase of the propagating optical wave can be increased by the refractive index of the prism. Thus in the implementation of the SPR system, a gold thin film is coated on a prism with the refractive index larger than the tested dielectric material. A p-polarized light beam is projected onto the metal layer through the prism to excite the electromagnetic wave in a surface plasmon mode on the interface between the metal layer and the dielectric material.

For the past two decades, surface plasmon excited by light has been widely applied to the study of biomaterial processes, including biosensors, immunodiagnostics, and kinetic analysis of antibody–antigen interaction (Davies, 1996; Rich and Myszka, 2005). The main application of surface plasmon resonance, SPR, on the biomedical science is to analyze the binding dynamics between antibody and antigen and to sense specific antigen through the chemical binding of corresponding antibody (Davies, 1996; Rich and Myszka, 2005; Safsten et al., 2006; Misono and Kumar, 2005). Since the characteristics of surface plasmon modes depend on the refractive index of the dielectric material within about 100 nm of the dielectric–metal interface, change of the refractive index of the dielectric material alters the coupling efficiency and the optical phase of the reflected light, which is the uncoupled part of the incident light. Therefore, a SPR system does not require fluorescence labeling and provides real-time information with very high sensitivity (Chien and Chen, 2004). Only a very small amount of samples is needed for the detection of the refractive index change through a SPR method. The most updated sensitivity of SPR is reported as 1 pM using nanoparticle amplification (Li et al., 2006).

Most of the biomedical applications of SPR focus on the detection and identification of biomolecules. Recently SPR biosensing techniques start to be applied to the detection and identification of cells, bacteria, or viruses based on affinity and specificity of proteins or aptamers to their cognate targets (Takemoto et al., 1996; Critchley and Dimmock, 2004; Fratamico et al., 1998; Misono and Kumar, 2005). The capture of the desired biomolecules or bacteria is all achieved through antibodies or aptamers pre-coated on the metal thin film, where the SPR occurs. The enormous applications of SPR on biomedical science using antibody–antigen affinity can be found in Jiri Homola's (Homola et al., 1999), and Rebecca L. Rich and David G. Myszka's Surveys (Rich and Myszka, 2005). Although dynamic analysis is required for measuring affinity of cognate molecules, most of the applications of SPR so far can be considered as stationary measurement. It is only used on detection or recognition of cognate targets or development of antibodies. Until recently, using SPR biosensor on detecting whole cells was explored. These efforts include an observation of cell/substrate contacts (Giebel et al., 1999), a reaction of cells to toxicity (Choi et al., 2005), and detection of cholesterol concentration in cell membranes (Ziblat et al., 2006). Different from the studies mentioned above, to our best knowledge, this paper is the first effort to detect the dynamical change of bacteria to antibiotics by surface plasmon resonance biosensor. This is also the first effort to apply SPR biosensor on the detection of cell activities to stimulants. While current antimicrobial susceptible testing methods, namely Kirby-Bauer disk diffusion and variations of broth microdilution, execute antimicrobial test based on the detection of cell duplication ability under the influence of antibiotics, the SPR biosensing method directly detect chemical or physical change of bacteria subject to antibiotics. This research then focused on exploring new application of SPR biosensing technique on antimicrobial susceptibility test. It is expected to take much shorter time than the conventional methods do.

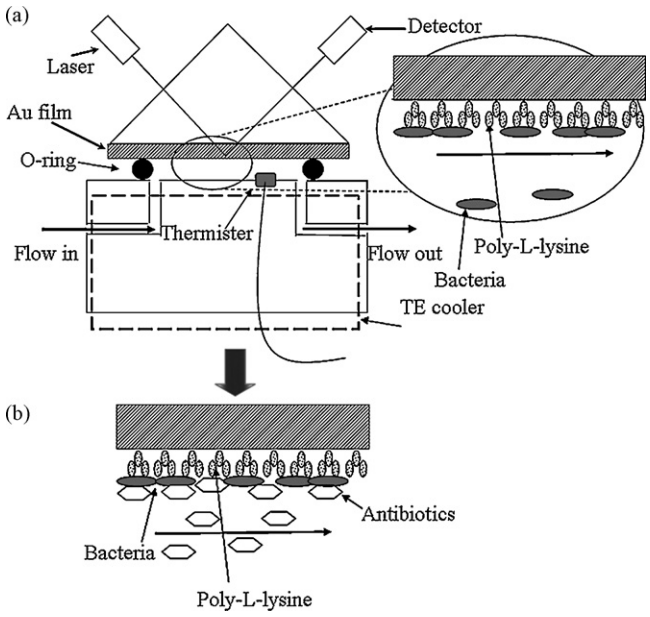

Fig. 1. (a) Schematic illustration of the SPR device and (b) procedures of the experiment.

Due to the easy access of *Escherichia coli* JM109 and *Staphylococcus epidermidis* on hand, the strains of the bacteria were chosen for the antimicrobial susceptibility test in this study. Antibiotics are classified into several categories depending on their mechanisms on the interruption of cell activities, namely cell wall synthesis, cell membrane synthesis, protein synthesis, folic acid biosynthesis, DNA gyrase, and RNA polymerase. Since the surface plasmon resonance is highly sensitive to the change of the refractive index of cells near the cell–metal interface, ampicillin as the antibiotic inhibiting the synthesis of cell walls was used for the examination of *E. coli* JM109. This is designed for the measurement of direct effect of antibiotics on cells. Different from ampicillin, tetracycline works as an inhibitor of protein synthesis. The influence of tetracycline on cell walls and cell membranes is then indirect. Therefore, *S. epidermidis* used as another type of bacteria susceptible/resistant to tetracycline was used as a blind test for the measurement of indirect effect of antibiotics on cells.

2. Materials and methods

2.1. Surface plasmon resonance system

The experimental setup for the antimicrobial test of the bacteria is schematically illustrated in Fig. 1(a). The setup is the combination of two parts: one is the platform of the biosensor for the excitation of surface plasmon polaritons and the other is a fluidic cell chamber. This SPR biosensor platform was implemented based on the Kretschmann-Raether configuration. A Helium–Neon laser of wavelength 632.8 nm was used as the light source to excite surface plasmon polaritons. A prism made of SF11 provides extra spatial phase on the electromagnetic field from the laser to match the dispersion relation of surface plasmon polaritons bound on the surface of Au thin film. The reflection, from Au thin film, of the laser beam was measured as the uncoupled beam and served as the indicator of the coupling or excitation efficiency. The excitation efficiency was determined by the incident angle of the laser beam, as well as by the refractive index of any material contacting the Au thin film.

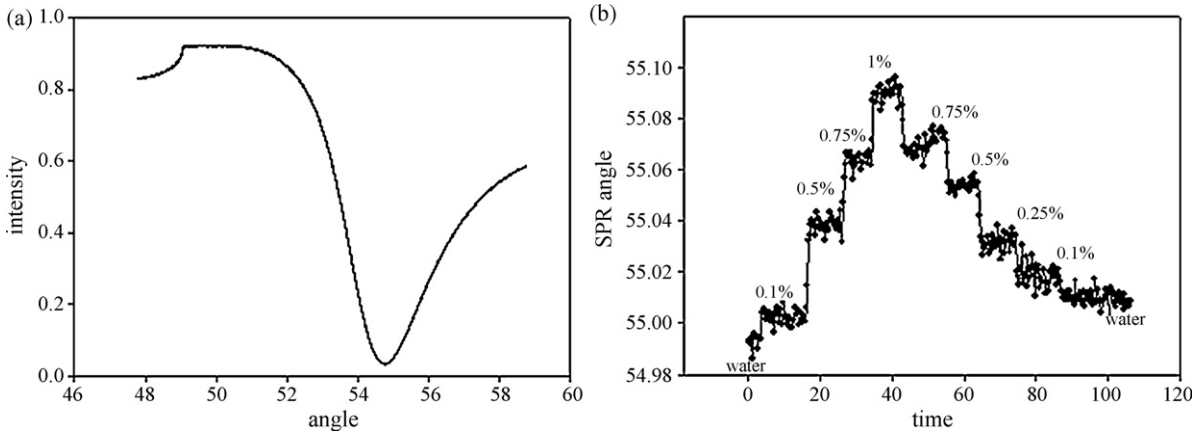

Fig. 2. (a) Intensity of the reflected laser beam as a function of the incident angle. This reflection indicates the coupling efficiency of the surface plasmon polaritons. Smaller reflection implies larger coupling efficiency; (b) sensitivity test of the system. Sucrose was used as the reference of standard refractive index. Sucrose with 0.1% concentration has the refractive index different from water by 1.4×10^{-4}.

The incident angle of the light to reach the maximum excitation efficiency is named as the resonance angle or the surface plasmon resonance angle. This resonance angle shifts when the refractive index of the bacterium portions fell within about 300 nm of the bacteria–Au interface changes.

Incident angle of the laser beam was controlled by a motorized rotation stage through a controller, and a silicon photodetector was controlled accordingly by another motorized rotation stage to measure the power of the reflected laser beam. These two rotation stages (Sigma Koki, SGSP-120YAW) have the angular resolution 2.5×10^{-3} degrees when controlled by the two-axis controller (Sigma Koki, SHOT-202). The intensity of the reflected beam as a function of the incident angle was obtained by a computer via 16-bit A/D converter (Adventech PCI-1716).

To avoid direct Au coating on the prism, an Au thin layer with thickness 47.5 nm was coated on a glass substrate made of SF11 as the platform of the surface plasmon polaritons. This coating was achieved through sputtering machine and the thickness of Au thin layer was measured by an atomic force microscopy (AFM). Matching oil was applied between the prism and the glass substrate to avoid multi-reflection occurring between them.

A cell chamber equipped with a fluidic channel was constructed on the SPR platform to provide the bacteria for testing, to flow DI water for stabilizing and for washing unbound bacteria, and to inject the antibiotic for performing the antimicrobial susceptibility test. An O-ring was attached to the chamber to prevent the leakage of liquid. A thermistor was buried in the metal chamber through a small hole barely intruding the cell chamber to monitor the temperature of the cell chamber and a TE cooler was placed on the side surface of the chamber to stabilize the temperature at 37 °C by receiving the temperature information from the thermistor. The temperature fluctuation is less than 0.1 °C. As is depicted in Fig. 1(b), the targeted bacteria were first injected into the chamber through the fluidic channel and were bound on the gold film with the aid of Poly-L-lysine which provided adhesion. The Poly-L-lysine layer was pre-coated on the Au thin film. Antibiotics were then sent to the cell chamber through the fluidic channel. Influence of antibiotics on the cell walls or membranes of bacteria were picked up by surface plasmon polaritons.

A scanning electron microscope (SEM) JEOL JSM-5300 was used for the SEM imaging of the bacteria before and after antibiotic treatment.

2.2. Poly-L-lysine

Poly-L-lysine has been demonstrated as an effective tissue adhesive material in various biochemistry procedures. Poly-L-lysine solution, a hydrobromide from SIGMA, was diluted with deionized water prior to the coating procedure. Flat glass slides deposited with Au thin film were immersed in Poly-L-lysine solution

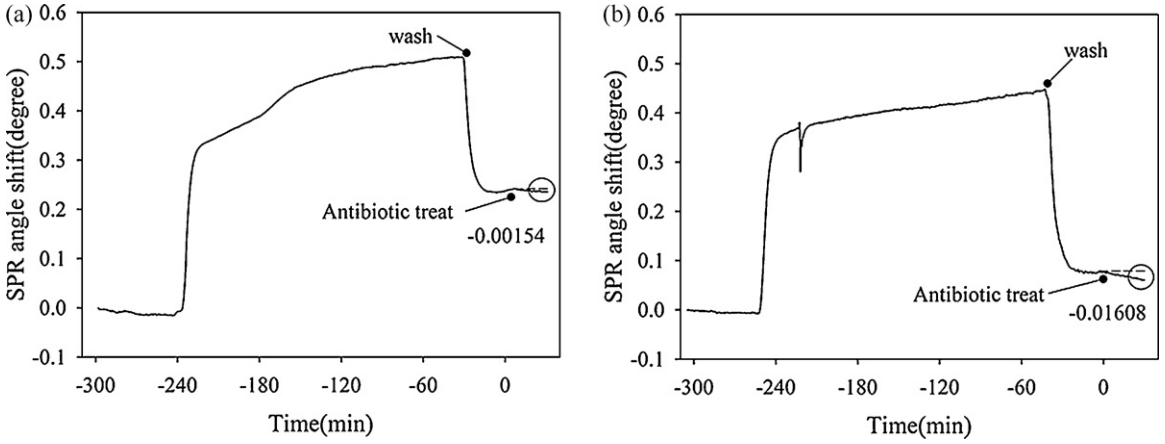

Fig. 3. Kinetic plot of SPR angle shift for the antimicrobial susceptibility test of *E. coli* to ampicillin for 30 min: (a) angle shift of the ampicillin-resistant strain is around −0.00154° after the treatment for 30 min; (b) angle shift of the ampicillin-susceptible strain is around −0.01608° after the treatment for 30 min.

(concentration = 200 μg/ml) for at least 24 h to ensure good quality of coating on the Au thin film. This procedure can be considered as the preparation of the biochips and will not prolong the antimicrobial susceptibility test. After incubation, cells can be immobilized on the Au-coated glass slides through the adhesion ability of the Poly-L-lysine layer.

2.3. Preparation of E. coli resistant to ampicillin

The bacteria strain, *E. coli* JM109, resistant to ampicillin was generated by transforming ampicillin-resistant plasmids into the cells. The plasmids then translate to β-lactamase, which can cleave the ring of ampicillin (Levinson and Jawetz, 1989; Macheboeuf et al., 2006). The *E. coli* strain was picked out by loop and was planted in 5 ml LB broth culture for 12–16 h with 182 rpm at 37 °C. The pH of the bacteria media (LB broth) was maintained at pH 7.0–7.5 with Hepes buffer.

2.4. Preparation of S. epidermidis resistant to tetracycline

Similar to the preparation of ampicillin-resistant *E. coli*, the strain of *S. epidermidis* resistant to tetracycline was generated by transforming tetracycline-resistant plasmids into the cells. After plasmids translation, the bacterium strain was picked out by loop and was planted in 5 ml LB broth for 20 h. The bacterium strain was then transferred into 100 ml LB broth for 5 h for further experiment.

3. Results and discussion

SPR biosensing in general can be achieved through intensity interrogation, angular interrogation, phase interrogation, and wavelength interrogation. In this system, the detection method of the SPR biosensor is based on the angular interrogation. The coupling efficiency as a function of the incident angle was measured and is illustrated in Fig. 2(a), which was experimentally measured by this device. This intensity shown in Fig. 2(a) was obtained by normalizing the intensity of the reflection beam to the laser output, which was monitored by another silicon photodetector through a beam splitter placed in front of the He–Ne laser. This normalization procedure is used to eliminate the intensity fluctuation of the laser output. The sensitivity of this system was measured by the concentration of sucrose, which served as the material providing standard reference of the refractive index. The measurement result is shown in Fig. 2(b). Since some sucrose molecules remained on the surface of the Au thin film as a residue when the concentration of sucrose decreased, the value of the SPR angle on the decrease portion is usually higher than that on the increment portion. The residue effect becomes more pronounced when the concentration of sucrose approaches to zero. This is a typical phenomenon when a sensitivity test is performed on a SPR biosensor. It is known that the refractive index of 0.1% sucrose solution exceeds that of pure water by approximately 1.4×10^{-4} at 632.8 nm. As is shown in Fig. 2(b), this SPR biosensing system can resolve slightly better than $\Delta n = 1.4 \times 10^{-4}$. That is to say, the sensitivity of the SPR biosensing system on the change of refractive index of the dielectrics is slightly smaller than 1.4×10^{-4}. This sensitivity corresponds to the value of the SPR angle shift as 0.00867°, which is more than three times of the resolving power of the rotation stages in the system.

Since surface plasmon polaritons penetrate into cells approximately in the order of 100 nm, the biosensor is very sensitive to the change of the refractive index of cell walls and membranes attached on the Au thin film within that range – no matter what causes the change. The mechanism of ampicillin is to interrupt the activity of transpeptidase, which assists the formation of peptide-bond combined with peptidoglycan to constitute cell walls, and then to interfere cell growth and proliferation (Macheboeuf et al., 2006). Therefore, for the measurement of direct influence of antibiotics, ampicillin was used as the antibiotic for the antimicrobial susceptibility test. *E. coli* JM109 strains were used as the tested objects.

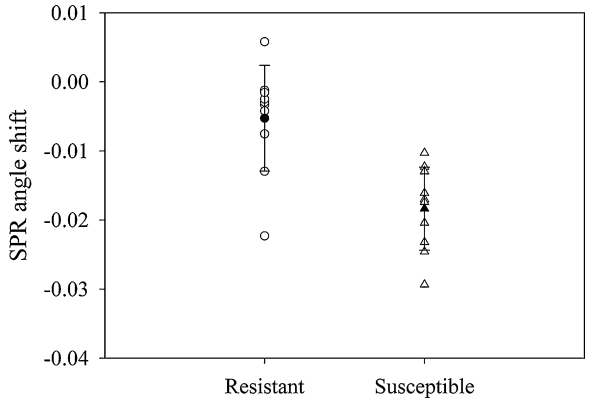

Fig. 4. SPR angle shift of 10 sets of resistant/susceptible strain: angle shift of the resistant strain *E. coli* is indicated by the open-circle and its average value is indicated by a solid-circle with standard deviation bar; angle shift of the susceptible strain *E. coli* is indicated by the open-triangle and its average value is indicated by a solid-triangle with standard deviation bar. The averaged angle shift for the resistant strains is around −0.00527° and the standard deviation is around 0.00763°. The averaged angle shift for the susceptible strain is around −0.01835° and the standard deviation is around 0.00602°.

3.1. Examination of the E. coli strains susceptible/resistant to ampicillin

To test the drug resistance of *E. coli* JM109 using the SPR system, sterilized DI water was injected into the cell chamber for 30 min to stabilize the system after the biochip coated with Poly-L-lysine was assembled. Following the stabilization procedure, the incubated LA broth was injected into the fluidic cell chamber for about 60 min to cover the Au thin layer. Another wash procedure was followed to remove the bacteria that were not bound to the Poly-L-lysine layer. When the bacteria binding reached a steady state, the ampicillin solution with concentration 3 μg/ml was injected. The resonance angle of surface plasmon through the entire procedure was recorded as a function of time.

The SPR angle of antibiotic-resistant strain of *E. coli* JM109 over the operation procedure described above is shown in Fig. 3(a) and that of antibiotic-susceptible strain is shown in Fig. 3(b). The shift of the SPR angle was referred to the value of the SPR angle before the *E. coli* strain was injected into the cell chamber. As is shown in Fig. 3(a), the SPR angle increased when the bacteria were injected into the cell chamber. After the amount of the bacteria attached to the Au thin film coating was saturated, DI water was injected to remove the unbound bacteria. The SPR angle dropped dramatically during this procedure. After bacteria binding reached steady state, the 3 μg/ml ampicillin was injected into the cell chamber. The value of SPR angle, changed by the refractive index of the bacterium surface, was recorded over time. The same procedure was applied on the susceptible strain and the result is shown in Fig. 3(b). The result shows that, after 30 min treatment of ampicillin, the decrease of the SPR angle for the resistant and the susceptible strains were −0.00154° and −0.01608°, respectively. The angle shift is about 10 times difference between the resistant strain and the susceptible strain. The decrease of the refractive index, which is depicted by the negative angle shift, is possible from the structure loose of bacteria cell walls or even breakdown due to the effect of ampicillin. Since the antibiotic-resistant strain is more resistant to ampicillin,

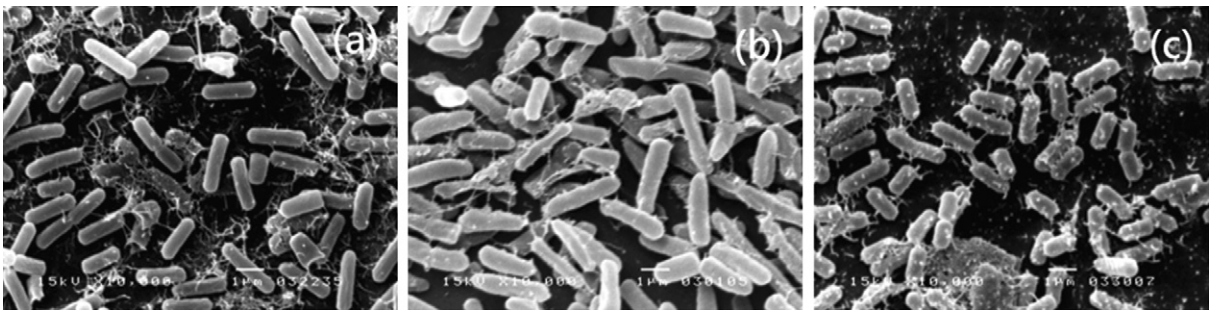

Fig. 5. SEM scanning pictures: (a) *E. coli* without the treatment of ampicillin; (b) ampicillin-resistant strains after 30-min treatment of ampicillin; (c) ampicillin-susceptible strains after 30-min treatment of ampicillin.

the refractive index of its cell walls did not decrease as much as that of the susceptible strain.

In order to examine the reproducibility of the result, totally 10 sets of resistant and susceptible strains of *E. coli* JM109 were examined. The measurement result is shown in Fig. 4. The average angle shift, indicated by open-circle, for 10 sets of the resistant strains is -0.00527° with standard deviation 0.00763° and the average angle shift, indicated by open-triangle, for that of the susceptible strains is -0.01835° with standard deviation 0.00602°. As is shown in Fig. 4, two sets of the resistant strains subject to ampicillin have the angle shift larger than expected. It therefore shown that the diagnosis of the susceptible strains is 100% correct within the limited examination number and that of the resistant strains is 80% correct. The incorrect sets could be caused by the fall off of bacteria under the water flow containing ampicillin. Further verification will be conducted on this issue. The difference of the angle shifts between the resistant strains and the susceptible strains ranged from 2 times to more than 10 times. The variation of the result could be due to the different degree of the drug resistance of the bacteria, the different distance between the prism and the Au-coated glass, and the coverage efficiency of bacteria on the surface of the thin gold film from time to time. The p-value using two-tailed condition is 4.78×10^{-4} (<0.05), which demonstrates statistical significance of the experimental results. Nevertheless an acute criterion can be set to separate these strains through the proposed SPR scanning method except the two sets of the resistant strains. The failure on these two sets of resistant strains could be due to the stability of cell adhesion under the flow of ampicillin-contained water solution.

The damage degree of the ampicillin on the cell walls of the strain susceptible to ampicillin was examined by scanning electron microscope. The *E. coli* before the treatment of the ampicillin is shown in Fig. 5(a). The antibiotic resistant and susceptible *E. coli* strains after the antibiotic treatment for 30 min are shown in Fig. 5(b) and (c), respectively. A comparison between the SEM pictures revealed no significant change in the appearance of the resistant strain and that of the susceptible strain subject to the treatment of ampicillin for 30 min. However, after 5 h of treatment of ampicillin, the susceptible strain shrank, which was verified by SEM. It can be concluded that the SPR detection method is more sensitive than SEM scanning. The change of cells within 30 min of antibiotics treatment is not observable in the SEM pictures, but can be detected by the SPR sensor.

3.2. Examination of the S. epidermidis strains susceptible/resistant to tetracycline

Another bacterium, *S. epidermidis*, was used as the test object for confirming the performance of the SPR system on the measurement of indirect influence of antibiotics on bacterium strains. Tetracycline was used as the antibiotics in this test. Different from the mechanism of ampicillin, the tetracycline is a 30S inhibitor, which blocks the binding of aminoacyl-tRNA to A-site of ribosomes and then inhibits the protein synthesis (George and David, 1998). Therefore, this test is for examining the applicability of the proposed method to another bacterium resistant to antibiotic with different mechanisms. Meanwhile, we also used this one as a blind test for the performance of our SPR biosensor on the antimicrobial susceptibility test. Therefore, the group who provided the bacteria did not inform which strain was resistant to tetracycline and which was not. Since the surface plasma wave penetrates the contacting bacteria surface of about 300 nm, it is only sensitive to the change of the refraction index within this depth. The influence of tetracycline, which interrupts protein synthesis, passed to the surface of the cells is then indirect. The change of the SPR angle of the

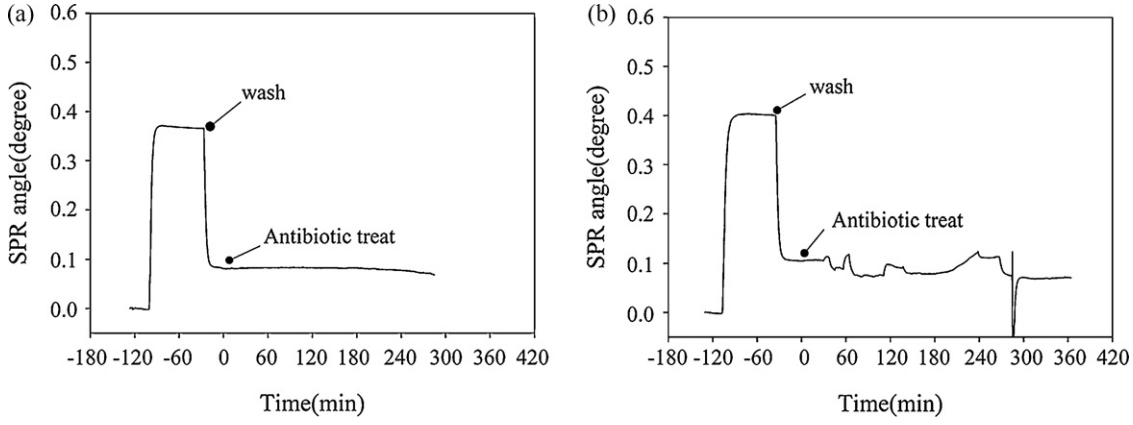

Fig. 6. Kinetic plot of SPR angle shift of *S. epidermidis* treated by tetracycline for 300 min: (a) tetracycline-resistant strain and (b) tetracycline-susceptible strain.

two unknown strains is shown in Fig. 6(a) and (b). As is shown in Fig. 6, the change of the SPR angle for one of the strains was irregular after the treatment of the 10 μg/ml tetracycline and that of the other strain showed slightly monotonic decrease over time. Based on the curves shown in Fig. 6, it is judged that the strain tested in Fig. 6(b) was the susceptible strain and the other in Fig. 6(a) was the resistant strain. The result of the blind test is consistent with the antimicrobial property of the strains. This test was repeated several times and the irregular angle shift was always observed when the tetracycline-susceptible strain was detected.

The observation on the test of *S. epidermidis* showed that the antimicrobial susceptibility of the bacteria was still detectable though the antibiotics did not directly act on the cell walls or cell membranes. Since the influence of tetracycline on the cell walls or membranes is indirect, the SPR angle change of the bacteria subject to the treatment of tetracycline over time was more dramatic than that of the *E. coli* treated by ampicillin.

In general, the potential error sources of this new SPR application can be from the mechanical stability of the system, variations in bacteria adhesions to the substrate, and the adhesion stability. However, the first two error sources can be corrected in our measurements because we are detecting the trends of dynamic changes, instead of absolute readouts at specific time points, so internal controls are included in each experiment. The stability of bacteria adhesion could affect the diagnosis accuracy on the resistant strains. It depends on the degree of instability. However, the stability issue does not appear on the test of *S. epidermidis*.

We have applied different concentrations of antibiotics to the bacteria in this study (data not shown) and noticed that the resulting SPR angle shift appeared to be a qualitative, rather than quantitative measure for bacteria antibiotics susceptibility tests, i.e., there was no apparent dose–response correlation. Although the nature underlying the SPR angle shift is currently unknown, we do know that this optical change is not due to loss of bacteria from the gold film, and that the pattern of SPR angle shift resulted from the ampicillin treatment was different from that of tetracycline. Further investigations are certainly needed to understand what causes the observed SPR angle shift when exposing the bacteria to antibiotics.

4. Conclusion

We have reported an innovative antimicrobial susceptible testing method utilizing surface plasmon resonance. Susceptible and resistant strains of *E. coli* (Gram-negative) JM109 to ampicillin and those of *S. epidermidis* (Gram-positive) to tetracycline, served as a blind test, were examined. For the drug resistant strains of both bacteria, the SPR angle was almost a constant over time. Since ampicillin directly acts on the formation of cell walls, the susceptible strain of *E. coli* revealed monotonic decrement over time on the SPR angle. Totally 20 sets of resistant/susceptible strains of *E. coli* were examined and the *p*-value using two-tailed condition is 4.78×10^{-4} (<0.05), which demonstrates statistical significance of the test. Since tetracycline is an intracellular antibiotic, interestingly, the result of the susceptible strain of *S. epidermidis* showed very irregular shift of the SPR angle. The experimental result has shown the feasibility of utilizing SPR biosensors for fast antimicrobial susceptibility test. However, the report here focuses on distinguishing the susceptible and the resistant strains. Quantitative analysis of drug resistance has to be studied and established. Since the detection was not based on cell duplication but biochemical change, the growth rates of bacteria should not be a limiting factor to generalize the potential of this method to other bacteria with longer growth time. A study on this aspect will be performed. Furthermore, a multi-channel cell chamber will be developed to perform multi-antibiotic tests in the near future.

Acknowledgments

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