

Surface Plasmon Resonance Biotechnology for Antimicrobial Susceptibility Test

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1. Introduction

Infectious diseases are a leading cause of morbidity and mortality in hospitalized patients. This fact has placed 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 microorganisms. Laboratories are expected to perform these tasks in a cost-effective and efficient manner. Two common methodologies for antimicrobial susceptibility testing in a clinical laboratory are Kirby-Bauer disk diffusion and variations of broth microdilution. The principle is based on the detection of bacterium reproduction ability under the influence of antibiotics. Therefore the testing time is determined by the doubling time of tested bacteria. These methods then usually take from one day to weeks to complete the examination. The long incubation period is inevitable for these conventional methods. Such a waiting period is not short for clinical doctors who urgently need the information to adjust the therapeutic strategy. Therefore it is important to explore new template and technology to perform an antimicrobial susceptibility test.

Surface plasmon resonance biosensing technique is well known for its characteristics of label-free, ultra-sensitive, and real-time detection capability. Thus this technique is considered as the candidate of the new platform. Surface plasmon polaritons (SPPs) was first theoretically predicted by Ritchie in 1957 (Ritchie,1957) based on the analysis of surface electromagnetic modes. The SPPs in general can be generated by electrons (Powell & Swan, 1959) or by light (Otto, 1968) under a proper excitation condition. For SPPs excited by light, in general, the dispersion characteristic of SPPs does not allow the energy of a propagation wave coupled into this surface mode: The spatial phase of a propagation wave is always smaller than that of the surface mode with the same optical frequency on a dielectric-metal interface. Thus an evanescent wave generated by a p-polarized light beam through a prism is suggested to obtain an extra spatial phase and then excite SPPs on the other surface of the metal layer. An alternative method to provide the additional spatial phase is through the aid

of a grating, of which the sub-wavelength periodic structure can provide additional spatial phase. For the past two decades, SPPs excited by light has been widely applied to the study of biomaterial processes, which include biosensors, immunodiagnosics, and kinetic analysis of antibody-antigen interaction (Davies, 1996; Rich & Myszka, 2005). The main application of SPR biosensors on biomedical science is to analyze the binding dynamics between specific antibody and antigen (Davies, 1996; Rich & Myszka, 2005; Safsten et al., 2006; Misono & Kumar, 2005). Since the mode characteristics of SPPs depend on the refractive index of the material within the dielectric-metal interface of about one hundred nanometers, the refractive index of the material determines the resonance incident angle of light, the coupling efficiency, the coupling wavelength, and the optical phase of the reflected light. All the physical quantities can be measured by 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 & Chen, 2004). This also guarantees a very small amount of sample needed for the detection of the refractive index change through a SPR method.

Most of the biomedical applications of SPR focus on detection and identification of biomolecules. Extended applications have been applied to the detection and sorting of cells or bacteria based on the same principle (Takemoto et al., 1996). The capture of the desired biomolecules with or without cells or bacteria attached is 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 Rebecca L. Rich and David G. Myszka's Survey (Rich & Myszka, 2005). For the methods using antibody-antigen binding, specific antibody is required and finding the specific antibody is usually not straight forward. This is the reason that characterization of antibody is still the main reports from utilization of SPPs. This is also an important reason that a method utilizing antibody-antigen interaction is difficult to use for antimicrobial susceptibility test. Different from the studies mentioned above, the method introduced in this chapter does not require pre-coating of specific antibodies. This method is then more versatile and can be used to detect reactions of drugs appearing on cell membranes or cell walls. While current antimicrobial susceptible testing methods take one day or more for a clinical laboratory to report the testing results (Poupard et al., 1994; Levinson & Jawetz, 1989), utilizing surface plasmon resonance significantly reduces the time duration to less than or about one hour of antibiotics treatment based on our experimental study. Antibiotics which modify or damage the cell walls of bacteria, thus, alternate the refractive index of bacterium surfaces.

Differentiation of susceptible strains of bacteria from resistant ones by using surface plasmon resonance (SPR) technique is discussed in this chapter. This technique detects the refractive index change of tested bacteria subject to antibiotics treatment in real time. Instead of detection the antimicrobial susceptibility through the cell doubling time, the SPR biosensor technology is used to detect the biochemical change of tested bacteria. A much shorter time to obtain the test result is achieved. Because of the feasibility of this antimicrobial test method using surface plasmon resonance biosensors, development of new biosensors is also very important.

Escherichia coli JM109 resistant/susceptible to ampicillin and *Staphylococcus epidermidis* resistant/susceptible to tetracycline were chosen for the antimicrobial susceptibility test in this study. Since the surface plasmon resonance is highly sensitive to the change of the

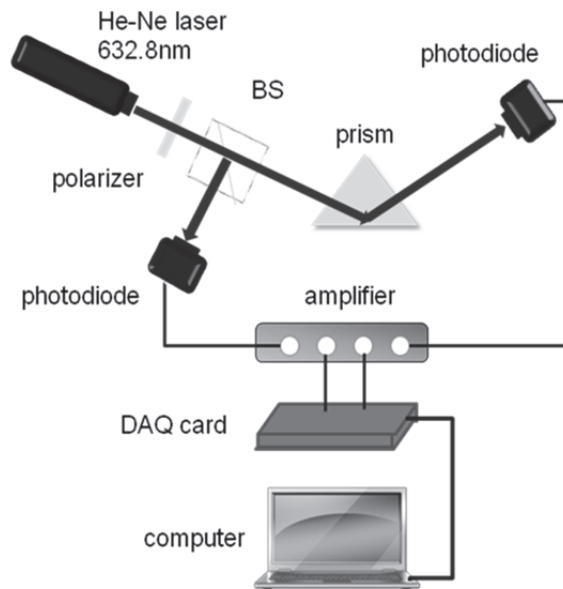
refractive index of cells near the cell-metal interface, ampicillin as the antibiotic inhibiting the synthesis of cell walls was used for the examination of *Escherichia coli* JM109. This is designed for the measurement of direct effect of antibiotics on cells. Different from ampicillin, tetracycline works as an inhibitor of protein synthesis. The influence of tetracycline on cell walls and cell membranes is then indirect. Therefore, *Staphylococcus epidermidis* used as another type of bacteria susceptible/resistant to tetracycline was used for the measurement of indirect effect of antibiotics on cells.

2. Devices and methods

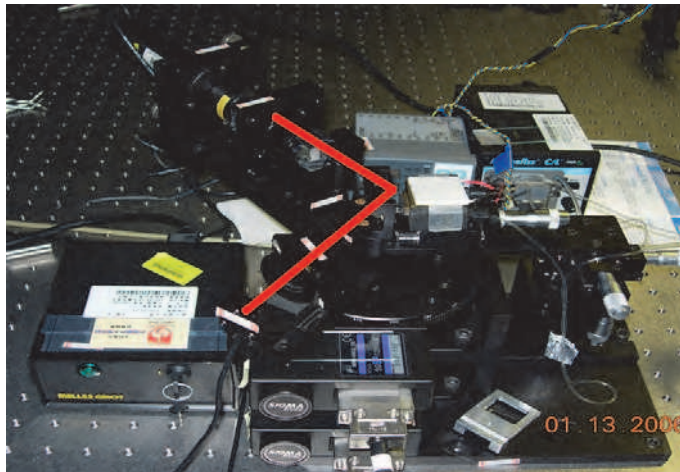
The detection principle can be realized on the detection of biochemical change of bacteria subject to antibiotics through the detection of their refractive index. This change on the refractive index of bacteria is achieved by an SPR biosensor. A chemical treatment of Poly-L-Lysine on the surface of the Au thin film in the SPR biosensor is used to trap bacteria. The Poly-L-Lysine layer does not provide specific binding to select specific bacterium strain so that a pre-purification to select tested bacteria is required for the test. After the tested bacterium strain is trapped on the Poly-L-Lysine layer, antibiotic is applied to examine the antimicrobial susceptibility.

2.1 Surface plasmon resonance biosensor

The experimental setup for the examination of drug resistance of the bacteria is shown in Fig. 1(a). The setup is the combination of the two parts: one is for the excitation of the surface plasmon and the other is the flow cell chamber. For the excitation of the surface plasmon, a Helium-Neon laser is used as the light source to provide the laser beam with wavelength 632.8 nm. Since surface plasmon can only be excited by p-polarized light, a polarized beam splitter is used to separate the p-polarized and s-polarized light. The s-polarized light is used as the normalization factor to eliminate the deterioration of measurement accuracy caused by the laser instability. After the polarized beam splitter, the p-polarized light is injected onto the Au thin film through a prism to generate surface plasmon. The required phase matching condition to excite the surface plasmon is provided by the proper incident angle and the prism, which provides an extra spatial phase along the gold film surface through its refractive index of the prism. Matching oil is applied between the prism and the glass substrate coated with the Au thin film to avoid occurrence of multiple reflection between the prism and the glass slide. The excitation efficiency of the surface plasmon by the p-polarized laser beam is measured through the silicon photodetector which receives the reflected p-polarized beam from the Au thin layer. When the surface plasmon resonance angle is reached, the energy of injected laser beam was transformed into the surface plasmon polaritons. Thus, the laser beam reflected from the Au layer reaches minimum. The photocurrent generated from the photodetector is amplified and transformed into a voltage signal via 16-bit A/D converter (Adventech PCI-1716). The intensity, normalized to the intensity of the s-polarized beam, of the reflected p-polarized beam as a function of the incident angle is obtained by the computer. Incident angle was controlled by a motorized rotation stage through a controller. The other arm that is for receiving reflection was controlled accordingly by another rotation stage to measure the power of the reflected beam. The resolution of the system on the change of refractive index of the dielectrics is 1.4×10^{-4} refractive index unit (RIU), which corresponds to the value of the SPR angle shift as 0.00867 degree.



(a)



(b)

Fig. 1. SPR biosensor used for the experiment. (a) The configuration of SPR biosensor used in the study. The SPPs was excited by 632.8nm He-Ne laser. A polarizer is used to enhance the extinction of the laser beam polarization. A polarized beam splitter (BS) direct the s-polzaried light into a detector for normalization of laser intensity fluctuation. The p-polzaried light is used to excite SPPs. The reflectance of the light is direct to the second detector for measurement of resonance angle, and thus measure the refractive index change of bacteria subject to antibiotics; (b) Picture of the home-made SPR biosensor. The solid red line indicates the laser beam.

2.2 Cell chamber

A flow cell chamber was constructed on the SPR system described above to provide the bacteria for testing, DI water for washing, and the antibiotics for the examination of drug resistance. An O-ring is attached to the chamber to prevent the liquid leakage. A thermister of 10K Ω is used to monitor the temperature of the chamber and a TE cooler is used to control the temperature by receiving the temperature information from the thermister. The temperature of the cell chamber was controlled with the fluctuation less than 0.1 $^{\circ}$ C, which is achieved by a temperature controller usually used for controlling the temperature of laser diodes. As is depicted in Fig 2, the target bacteria are first injected into the chamber through the flow channel and attach on the gold film by the adhesion of the Poly-L-lysine. Antibiotics are then added to test if the cell walls or membranes are affected.

2.3 Bacterium adhesive coating

Poly-L-Lysine has been demonstrated as an effective tissue adhesive for use in various biochemistry procedures. Poly-L-Lysine solution is diluted with deionized water prior to the coating procedure. The flat glass deposited with Au thin film was immersed in poly-L-lysine solution (concentration = 200 ug/ml) for from a couple of hours to 24 hours to interact with Au thin film as the preparation of the biochips. Different time intervals provide different adhesion of Poly-L-Lysine to the bacteria and antibiotics. After incubation, cells can be immobilized on the Au-coated glass.

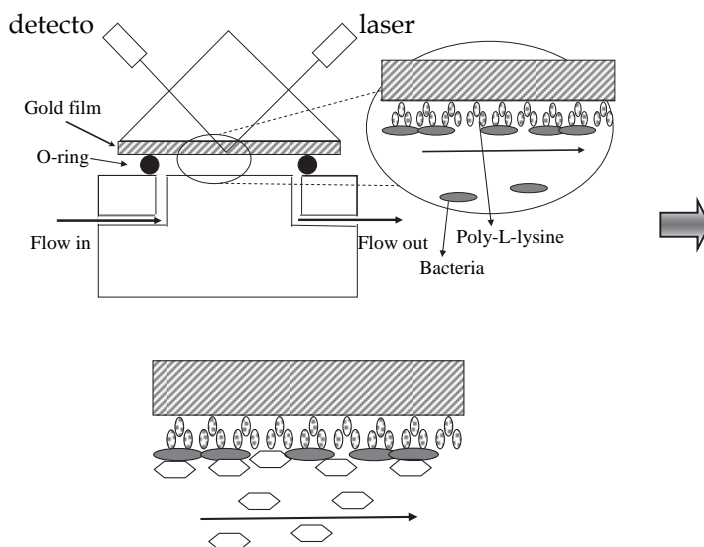


Fig. 2. Schematic illustration of the SPR device and the mechanisms of the experiment

2.4 Bacterium preparation

Preparation of Escherichia coli resistant to ampicillin Penicillin is called β -lactam drugs. An intact ring structure of β -lactam ring is essential for antibacterial activity; cleavage of the ring by penicillinases (β -lactamase) inactivates the drug (Levinson & Jawetz, 1989;

Macheboeuf et al., 2006). The antibiotics bacteria strain, *E. Coli* JM109, we use was generated by transform of ampicillin resistant plasmids to translate β -lactamase to cleave the ring of ampicillin. The *E. Coli* strain was picked out by loop and planted in 5ml LB broth over night. *Preparation of S. epidermidis resistant to tetracycline* The *S. epidermidis* were picked out by loop and were planted in 5ml LB broth over night (20 hours) and then transferred into 100ml LB broth (5 hours) for further experiment.

2.5 Scanning Electron Microscope (SEM) imaging

The glass slide with Au thin film and bacteria was placed in critical point drying (CPD) machine (Samdri-PVT) and filled with Ethanol of 100%. After that liquid CO₂ was used to replace the ethanol. The Au thin film with bacteria can then be detached from the glass slide for SEM imaging. Before taking the images, the sample was coated with Au for better conductivity. A scanning electron microscope JEOL JSM-5300 is used for the SEM images.

3. Antimicrobial susceptibility test

To test the drug resistance of bacteria using the SPR system, as depicted in Fig. 3, sterilized DI water was first injected into the flow cell chamber for 30 minutes 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 cell chamber for the bacteria to cover the Au metal film. Another washing procedure is applied to remove the bacteria that are not bound to the poly-L-lysine coating. After that an antibiotic solution was injected. The angle of surface plasmon resonance through the entire procedure was recorded as a function of time.

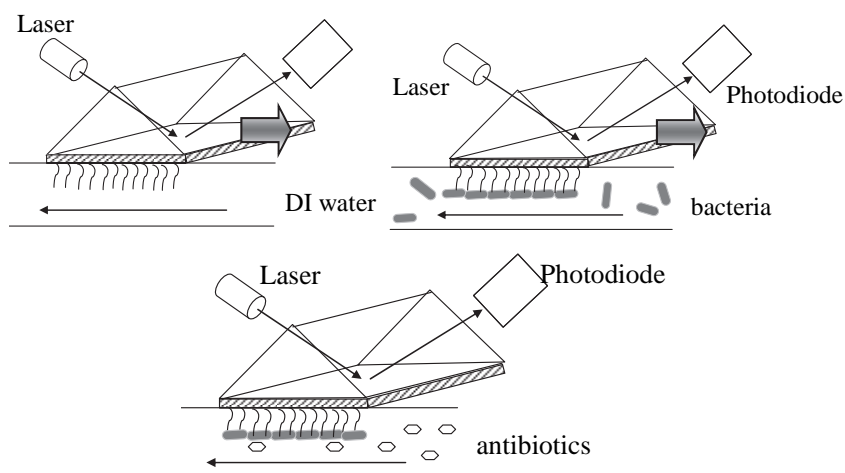


Fig. 3. Illustration of the experimental procedure. The first step is to stabilize the system and make sure that the system is operated under a constant temperature; the second step is to inject the bacteria into the cell chamber for bacterium attachment. After the bacteria are attached on the Au thin film, a LA broth is injected into the chamber for washing out the unbound bacteria. The third step is to inject the antibiotics.

Antibiotics are classified into several categories depending on its mechanisms on the interruption of cell activities, namely cell wall synthesis, cell membrane synthesis, protein synthesis, folic acid biosynthesis, DNA gyrase, and RNA polymerase.

3.1 Gram negative bacterium – *E-Coli*

3.1.1 Injection with LB

Since surface plasmon resonance is very sensitive to the refractive index change of the cells attached on the thin gold film, ampicillin as the antibiotics interrupting cell wall synthesis is chosen in this experiment. The mechanism of ampicillin is depicted in Fig. 4. As is shown in Fig. 4(a), the cell wall and membrane of *E. Coli* consist of outer lipid bilayer and inner plasma membranes. Between the two bilayers, the peptide (peptidoglycan) and cross-link (peptide-bond) form a rigid layer to constitute cell walls. As is shown in Fig. 4(b), the generation of cross-link is achieved by the assistance of transpeptidase. The mechanism of ampicillin is to interrupt the activity of transpeptidase and then to interfere cell growth and proliferation [6], shown in Fig. 4(c). When the susceptible strain of *E. Coli* JM109 is subject to the action of ampicillin, the cell walls are modified by the antibiotics. This modification changes the resonance condition of surface plasmon. The change of the resonance condition is revealed on the detector through angular interrogation.

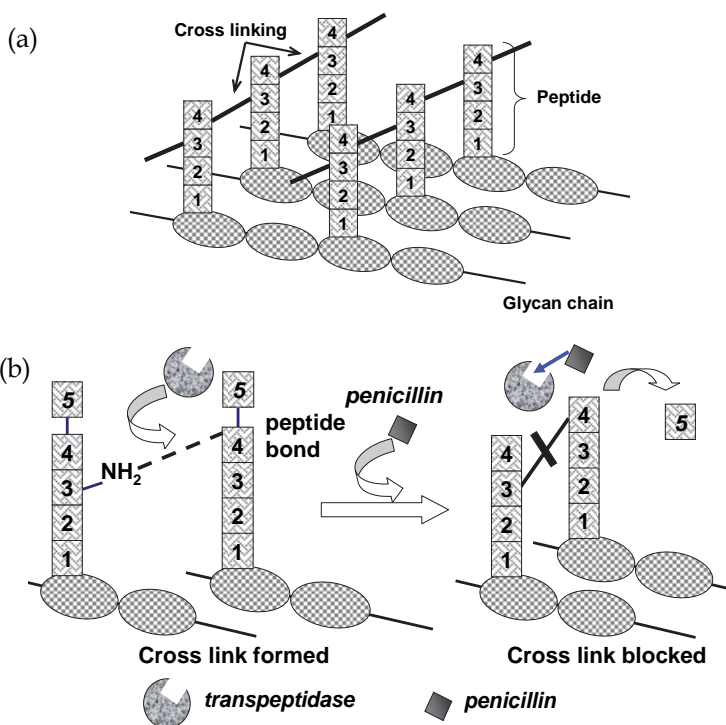


Fig. 4. (a) Cell wall structure; (b) Ampicillin mechanism

The SPR angle of antibiotic resistant strain of *E. Coli* JM109 over the operation procedures described above is shown in Fig. 5(a) and that of antibiotic susceptible strain is shown in Fig. 5(b). The shift of the SPR angle has been referred to the value of the SPR angle before the *E. Coli* was injected into the cell chamber. As shown in Fig. 5(a), the SPR angle increases when the bacteria are injected into the cell chamber. After the amount of the bacteria attached to the Au thin film coated with poly-L-lysine is saturated, DI water is injected to remove the unbounded bacteria. The SPR angle drops dramatically during this procedure. After that the 3 $\mu\text{g}/\text{ml}$ ampicillin is injected to the cell chamber. The value of SPR angle, changed by the refractive index of the bacteria, is recorded over time. The same procedure is applied on the susceptible strain and the result is shown in Fig. 5(b). The result shows that, after 30 minutes treatment of ampicillin, the decrease of the SPR angle for the resistant and the susceptible strains is -0.00154 and -0.01608 in respective. The angle shift is about ten times difference between the resistant strains and the susceptible strains. It indicates that the ampicillin causes the structure of bacteria cell walls loose or even breakdown and thus decreases the refractive index of the cell wall of the susceptible *E. Coli*. Since the antibiotic resistant strain is more resistant to ampicillin, the refractive index of its cell wall does not decrease as much as the susceptible one's does.

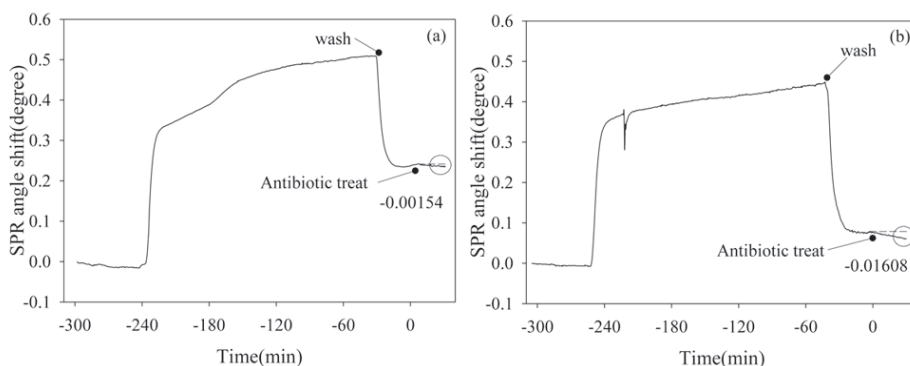


Fig. 5. Kinetic plot of SPR angle shift. The bacteria was treated by ampicillin for 30 minutes: (a) Ampicillin resistant case; (b) Ampicillin susceptible case (Chiang et al., 2009).

This difference of the resonance angle shift can be more pronounced when the concentration of the ampicillin increases to $100\mu\text{g}/\text{ml}$. As was shown in Fig. 6, the angle shift of the ampicillin-resistant strain of *E. Coli* was almost a constant during the treatment of antibiotics. However, the angle shift of the susceptible strain increased significantly over time. This demonstrates that the angle shift in the case of susceptible strain is indeed caused by the treatment of antibiotics.

The damage degree of the ampicillin, with concentration of $3\mu\text{g}/\text{ml}$, on the cell walls of the antibiotic susceptible strain is examined by SEM. The *E. Coli* before the treatment of the ampicillin is shown in Fig. 7(a). The antibiotic resistant and susceptible *E. Coli* after the antibiotic treatment are shown in Fig. 7(b) and 7(c) in respective. The comparison of the SEM pictures reveals that no significant change on the appearances of the resistant strains and the susceptible strains is observed. It can be concluded that the SPR detection method is more sensitive than SEM scanning; the change detected by the SPR sensor is not shown in the SEM pictures. After 5 hours treatment of ampicillin, the susceptible strains shrank, which was verified by SEM.

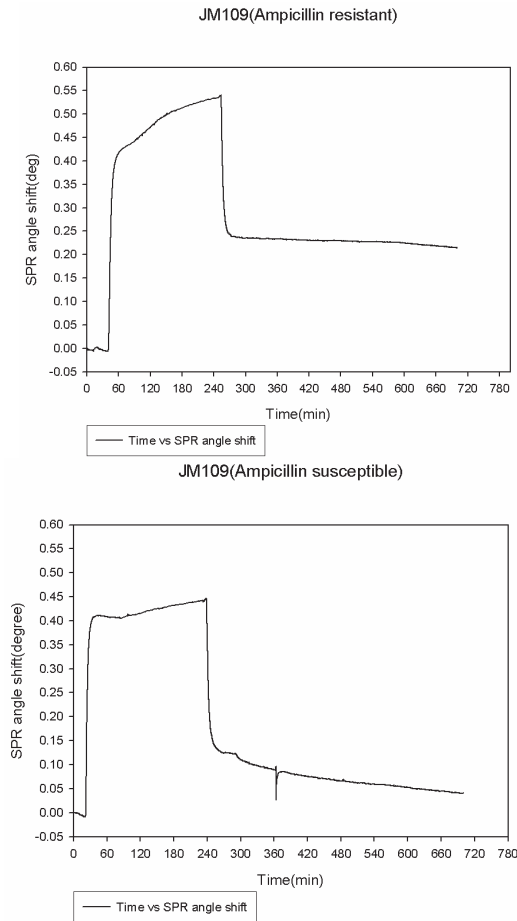


Fig. 6. Kinetic plot of SPR angle shift. The bacteria were treated with ampicillin of 100ug/ml for 300 minutes: (a) Ampicillin resistant case; (b) Ampicillin susceptible case

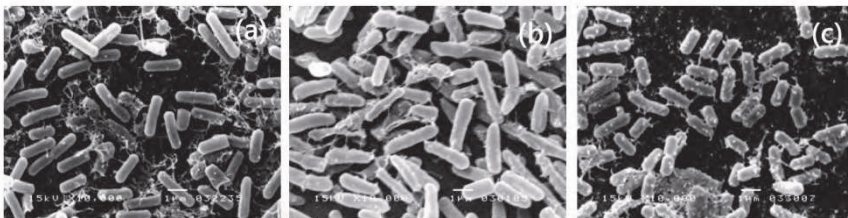


Fig. 7. SEM scanning pictures: (a) E-coli without antibiotic treating, (b) ampicillin resistant strains after 30 minutes treatment of antibiotics, (c) ampicillin susceptible strains after 30 minutes treatment of antibiotics. (Chiang et al., 2009)

In order to examine the reproducibility of the result, totally ten sets of resistant and susceptible strains of *E. Coli* JM109 were examined and the result was listed in Fig. 8. It shows that the detection of the susceptible strains is 100% correct within the limited examination number and that of the resistant strains is 90%. The incorrect set could be caused by the fall off of the gold film since gold has bad adhesion on glasses. Further verification is conducted on this issue. The angle shift difference between the resistant strains and the susceptible strains is ranged from two times to more than ten times. The variation of the result could 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. Nevertheless an acute criterion can be set to separate these strains through the SPR scanning method proposed here.

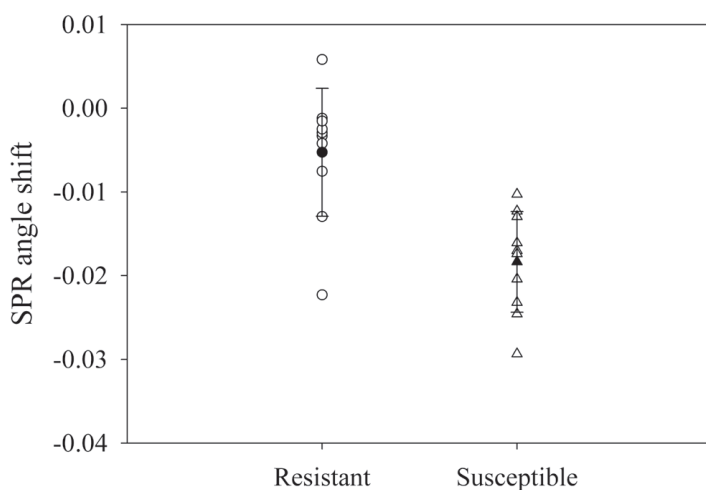


Fig. 8. Result of ten sets of resistant and susceptible strains of *E. Coli* subject to 3 ug/ml ampicillin. Solid circle indicates the average value of the angle shift in the case of resistant strain; Solid triangle indicates the average value of the angle shift in the case of susceptible strain. (Chiang et al., 2009)

3.1.2 Injection with DI water

In order to increase the accuracy of the antimicrobial susceptibility test. The coating time of Poly-L-Lysine was optimized from 24 hours to a few hours. Meanwhile, the LB injected with bacteria and for removing the unbound bacteria was replaced by DI water for reducing the interference of LB. After the adjustment, the amount of unbound or unstably bound bacteria was reduced significantly. As was shown in Fig. 9, the rinse procedure of DI water did not decrease the SPR angle from the saturation phase of bacterium adhesion as much as the situation in the injection with LB protocol. The ampicillin of 50ug/ml was applied from the time points indicated by the arrows. As shown in Fig. 9 (a), the resistant strain of *E. Coli* showed a positive angle shift right after the starting point of the ampicillin treatment and

stay almost unchanged. The sudden increase on the angle shift is the result of adding ampicillin.

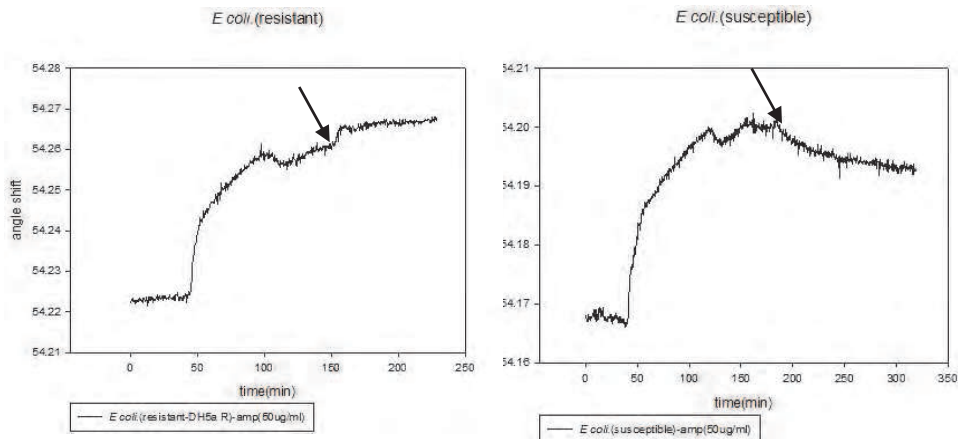


Fig. 9. Kinetic plot of SPR angle shift. The bacteria *E. Coli* were treated with ampicillin of 50ug/ml for 100 minutes: (a) Ampicillin resistant case; (b) Ampicillin susceptible case. The arrows indicate the time to start the treatment of ampicillin.

The result has demonstrated that the improved method has better accuracy in comparison with the method mentioned in the section 3.1.1. The same method using ampicillin of different concentrations, listed as 25 ug/ml, 50 ug/ml, and 100ug/ml, was also performed and the result was shown in Fig. 10. The resistant strain and susceptible strain of *E. Coli* were tested and ampicillin-only with bacteria was used as the control group. The tested groups subject to ampicillin of 25 ug/ml was marked by green solid circles; The tested groups subject to ampicillin of 50 ug/ml was indicated by red solid circles; The tested group subject to ampicillin of 100 ug/ml was indicated by blue solid circles. It revealed that the resonance angle in the resistant strain group increased because of the higher refractive index of ampicillin in comparison with that of DI water. Although the ampicillin can slightly increase the resonance angle, the resonance angle in the susceptible strain group still decreased due to the loss of cell walls, which has larger effect than that from higher refractive index of the ampicillin. This result showed that this method is suitable for the ampicillin with concentration ranged from 25 ug/ml to 100 ug/ml. We did not test ampicillin with the concentration above 100 ug/ml. For ampicillin with concentration lower than 25 ug/ml, the result became not trustable at this moment. Further study is required to push the lower limit.

3.2 Gram positive bacterium – *Enterococcus*

The protocol of DI water injection can also be used for gram positive bacteria. This tested object is *Enterococcus*. Similar result was obtained in the test, which was shown in Fig. 11. Following the same protocol, the resonance angle of SPP excitation first increased due to the higher refractive index of ampicillin in comparison with that of DI water. After the injection of the ampicillin, the angle shift of the resistant strain remained positive. However, angle shift of the susceptible strain gradually decreased to negative. The result of angle shift clearly distinguished the resistant strain from the susceptible strain.

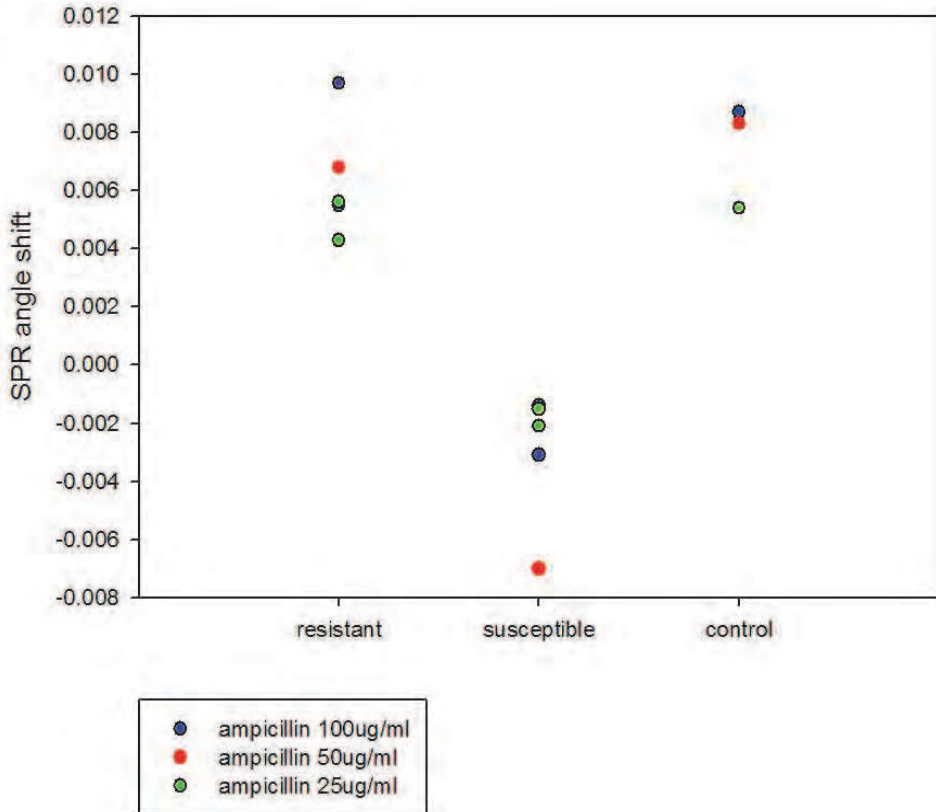


Fig. 10. Result of resistant and susceptible strains of *E. Coli* subject to ampicillin of different concentrations. Solid blue circle indicates the value of the angle shift in the case of 100 ug/ml for resistant strain, susceptible strain, and control group; Solid red circle indicates the value of the angle shift in the case of 50 ug/ml for resistant strain, susceptible strain, and control group; Solid green circle indicates the value of the angle shift in the case of 25 ug/ml for resistant strain, susceptible strain, and control group.

3.3 Different antibiotics - tetracycline

An interesting question has arisen if the same method can be used to detection antimicrobial susceptibility by antibiotic with different mechanism. For this purpose and also served as a blind test, another bacterium, *Staphylococcus Epidermidis*, is used. Tetracycline is 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 (Malacinski & Freifelder, 1998). It is important to emphasize that the surface plasma wave penetrates the contacting bacteria surface of about 100 nanometers, it is only sensitive to the change of the refraction index within this depth. For the antibiotics that interrupt the synthesis of protein, SPR biosensing technique may not be able to detect any change of bacteria subject to the treatment of tetracycline since the influence of tetracycline passed to the surface of the cells is then indirect. The change of the SPR angle of the two unknown strains is shown in Fig. 12(a) and 12(b). As shown in Fig. 12, the change of the SPR angle for one of the strains is irregular after the treatment of the 10 ug/ml tetracycline and that of the other strain showed slightly monotonic decrease over time. Based on the curves shown in Fig. 12, it is judged that the strain tested in Fig. 12(b) is the susceptible strain and the other is the resistant strain. The result is consistent with the antimicrobials property of the strains. This showed that this method can also be used to detect antimicrobial susceptibility of microorganisms subject to antibiotics with mechanisms other than working on cell walls.

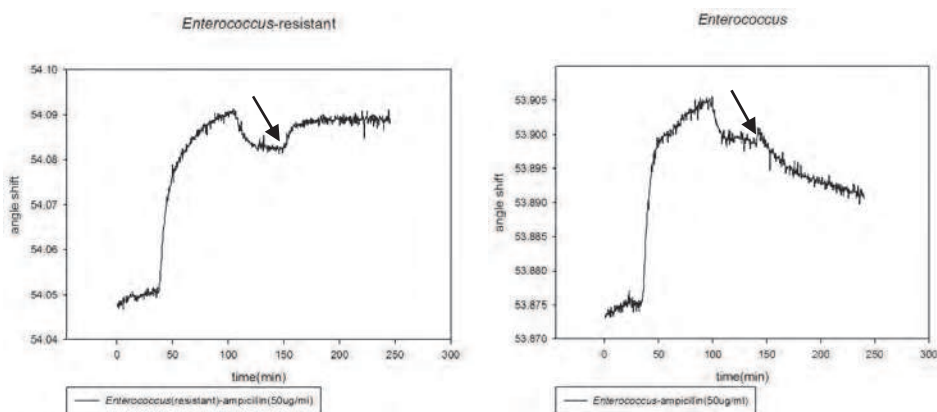


Fig. 11. Kinetic plot of SPR angle shift. The bacteria *Enterococcus* were treated with ampicillin of 50 ug/ml for 100 minutes: (a) Ampicillin resistant case; (b) Ampicillin susceptible case. The arrows indicate the time to start the treatment of ampicillin.

It is important to mention that the serum is not supplied into the system, the growth rate of *E. Coli* should not be a limited factor to generalize the potential of this method to other bacteria with longer growth time. An observation of bacteria on microscope has confirmed this point.

3. Conclusion

We have reported two innovative antimicrobial susceptible testing methods utilizing surface plasmon resonance. One is injection with LB liquid. The other is injection with DI water. In the study, the drug resistance of the gram negative bacteria, *Escherichia coli* JM109, and that of gram positive bacteria, *Enterococcus*, can be detected through the methods. This method is not limited to the antibiotics with mechanism working on the cell walls. It can be used to perform the test when antibiotics works on protein synthesis. The drug resistant of the *S. epidermidis* were successfully detected. Although the principle of the SPR testing method is based on the refractive index change of the cell-metal interface of about 100 nanometers, the resistance of the *S. epidermidis* to the tetracycline, which disturbs the protein synthesis, is still detectable by this method. This method can differentiate susceptible strain from resistant strain in a few hours and has a potential to further reduce the testing time to less than one hour if the cell adhesion time to the Au thin layer can be reduced. This method largely decreases the cost of time waste on examination and increase the chance for patient to survive.

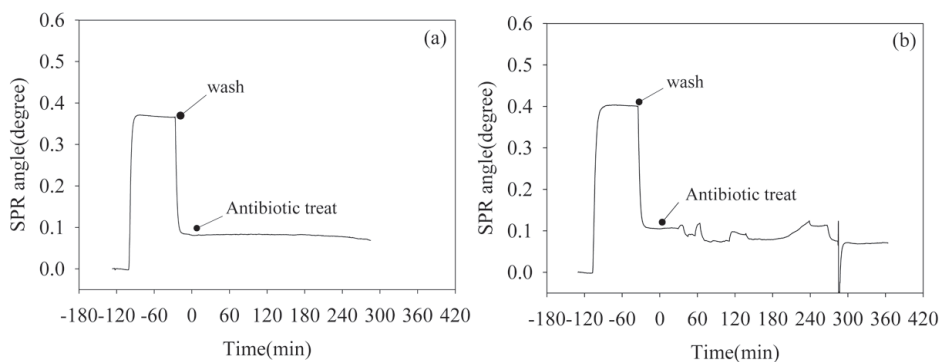


Fig. 12. Kinetic plot of SPR angle shift. The bacteria *E. Coli* were treated with ampicillin of 50 ug/ml for 100 minutes: (a) Ampicillin resistant case; (b) Ampicillin susceptible case. The arrows indicate the time to start the treatment of ampicillin. (Chiang et al., 2009)

4. Acknowledgment

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