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Antimicrobial Susceptibility Assays Based on the Quantification of Bacterial Lipopolysaccharides via a Label Free Lectin Biosensor

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ABSTRACT

A label free lectin biosensor developed in our laboratory that can quantitatively measure the binding between the lectin immobilized at the carbohydrate sensor surface and the lipopolysaccharide (LPS) on gram-negative bacteria was demonstrated for an antibiotic susceptibility assay. The biosensor utilizes a polythiophene interface containing fused quinone moieties glycosylated to form carbohydrate platform for the immobilization of Concanavalin A (Con A) and is capable of LPS binding measurements via orthogonal quartz crystal microbalance and electrochemical readouts (EQCM). Such orthogonal transduction provides cross-validation, better sensor sensitivity and large dynamic range of the measurements. We have applied this label free lectin biosensor for a new antibiotic susceptibility assay by characterizing the antimicrobial activities of various antibiotics (i.e. ciprofloxacin, ceftriaxone, and tetracycline) against *E coli* W1485 as a model system. The label free biosensor allows both end point and real time measurements of antibiotic effects on the bacterial cell surface LPS which is shown to correlate to their antibiotic effects. At the end point, after 18 hours incubation of bacterial cells with these three antibiotics respectively, the bacterial LPS binding signal was reduced to 23%, 27%, and 38% respectively for the three antibiotics, indicating that ciprofloxacin is the most effective against this *E coli* strain. Real time measurements at one hour time point showed a similar trend with a reduction of binding to 91%, 93%, and 95% respectively. From the binding kinetics of these measurements, the relaxation time (τ) was obtained, where higher τ value means slow binding interactions between the lectin and the bacterial LPS. The obtained order of τ, (i.e. $\tau_{ciprofloxacin} > \tau_{ceftriaxone} > \tau_{tetracycline}$) again indicated that ciprofloxacin has more bactericidal activity than the other two antibiotics with the same concentrations. Thus, we are able to

establish that the reduction in the binding of LPS with the lectin Con A sensor upon exposure to various antibiotics has a direct relation with the antibiotic dosages making this label free biosensor assay promising for therapeutic management of these drugs as well as for applications in antibiotic research and development.

INTRODUCTION

The continued evolution of antibiotic resistance in pathogenic bacteria has reached a level of public concern that is highlighted by frequent references in the media to the so-called "super-bugs".[1] Extensive research has been directed towards synergistic combinations of antimicrobial agents.[2] Such combinations are vital for treating infections that fail to respond to single therapeutic agent. However, injudicious, empiric use of antibiotics has contributed to the emergence of more resistant pathogens and has also caused septic shocks in patients. These concerns, coupled with homeland security threats, escalate the need for reliable and effective assays for antimicrobial susceptibility testing.[3] Established techniques for such assays, e.g. broth dilution and disc diffusion, involve multiple time-consuming steps including: (1) pre-culturing of isolated bacteria to enrich cell density to detectable levels (24–48 h.), (2) incubation of cells with antibiotics in 96-well plates or petri dishes (24–48 h.), and (3) determination of bacterial growth using absorption spectroscopy or by visual assessment.[4] Moreover, these assays typically require significant quantities of patient samples such as blood, sputum, or urine for analysis.[5] Molecular genetics techniques (e.g., polymerase chain reaction (PCR) and microarrays) are common, but are based on the detection of β-lactamases directly from a clinical sample.[6] Under present state of the art, the number of already described lactamases is too many to propose universal primers for their detection. The direct method based on the spectrophotometric detection of lactam hydrolysis using cell extracts[7] is labor-intensive and cannot be used routinely.[6-7] Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MS) has recently been introduced for the characterization of the antibiotic resistance mechanisms[8], but the relatively small mass of antibiotics (~ 1,000 Da) complicates their analysis because of their

interactions with the matrix and the resulting high noise levels.[9] Instrument and its maintenance costs are additional issues in this regard. Hence, in the absence of precise information about the antibiogram of particular pathogen, physicians often resort to empirical therapies that utilize broad-spectrum antibiotics. Such indiscreet use of antibiotics intensify the problem of antibiotic resistance.[10]

In contrast, biosensor assays can be a smart option for susceptibility testing, if a suitable strategy can be devised by understanding the antibiotic action mechanisms. Bacterial cells express both carbohydrate and lectin adhesin structures on their outer cell walls.[11] Certain antibiotics can act on cell wall biosynthesis which can dramatically affect the cell surface carbohydrate and lectin expression in the cell envelope, i.e. cytoplasmic membrane and cell wall.[12] These affects such as the alternation of lipopolysaccharide (LPS) chain length[13] or the alteration in lectin expressions can significantly affect the bacterial binding with the substrates. Thus, a real time information regarding these alteration can not only provide a fast bacterial susceptibility testing, but also can explain the fundamental mechanisms of the differences in mode of action of antibiotics, their influence on cell surface morphology, and antibiotic efficacies. Moreover, many physiological complications such as septic shock, which is associated to antibiotic released endotoxins (LPS), can by identified and treated.

Since antibiotics from different classes have different mechanisms of action and the antibiotic effects on bacterial morphology and viability are concentration and time dependent, we hypothesized that the effects of antibiotics on the LPS properties of a gram-negative bacteria may be class and concentration dependent. A biosensor that specifically measures the binding of gram-negative bacteria LPS will be able to quantify this effect. Therefore, the measurement of the magnitude of binding is an indirect measure of the antibiotic susceptibility under various

physiological conditions. However, in order to have this measurement done and in a precise manner, there is a need of a sensor which has high sensitivity (as there might be only very small changes in the LPS expressions), broad dynamic range (to accommodate different antibiotic actions and different physiological conditions), and an innovative mechanism to have least possible interferences from the reagents used in the test. We have recently developed such a sensor which has all these unprecedented performance characteristics and is able to detect bacteria both by their fimbriae protein in the pili and LPS expressions.[14] From these two different binding expressions, the mechanism that uses LPS structures and mediated by Concanavalin A (Con A) was shown to have more rigid character, thus leading to very high sensitivity and low detection limit (i.e. up to 50 Cells/ml). Therefore, in this work, we have utilized the function of Con A mediated LPS detection of that sensor. Moreover, the utilization of integrated quartz crystal microbalance and electrochemical readouts (EQCM) transduction mechanism provided internal validation that significantly enhanced the reliability of detection. As a representative bacteria, we have used *E. coli* which is one of the highly relevant and commonly targeted indicator for routine analysis of the contaminated water and food sources.[15] As per our original hypothesis, if the antibiotics have effects on the *E. coli*, the binding between the LPS of *E. coli* and the Con A confined to the sensor surface will be affected which will induce different electrochemical and QCM signal changes as depicted in Figure 1. According to these change of signals, the properties of the antibiotics action can be quantified.

MATERIALS AND METHODS

Chemicals and Materials

(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) was purchased from VWR International. Triethanolamine, ceftriaxone disodium salt hemi (heptahydrate) (third-generation

cephalosporin antibiotic), ciprofloxacin (≥98.0% (HPLC)) and tetracycline (≥98.0% (NT)) were purchased from Sigma-Aldrich. Fresh dilutions of the antibiotics were prepared daily in either sterile culture medium or distilled water. Concanavalin A (Con A) was purchased from Sigma. *E. coli* W1485 (ATCC 12435) was obtained from ATCC. All other reagents and materials were analytical grade and solvents were purified by standard procedures.

Biosensor Interface Fabrication and Characterization

Electrochemical polymerization of 3-((2, 5-dimethoxyphenyl)ethynyl)thiophene (abbreviated TQ) monomers can be carried out by using potentiometry, galvanometry, or cyclic voltammetry techniques. Cyclic voltammetry was employed in this work. Cyclic voltammetry uses multiple potential cycles to form the polymer film allowing the electrochemical characteristics of the growing polymer to be monitored during the polymerization process and the film can be grown more uniformly. Thus, polyTQ film was deposited on the gold electrode (surface area 0.22 cm^2) in CH_3CN with 0.1 M $LiClO_4$ as supporting electrolyte (~ 5 mL) containing about 2 mM TQ by cyclic voltammetry. Before experiment, the supporting electrolyte was deoxygenized using nitrogen for 20 min. The potential sweep range was between 0.5 and 1.2 V (vs. Ag/AgCl wire) at a scan rate of 20 mV/s for 20 cycles. The yellow-brown film modified gold electrode was washed with CH_3CN and water. The cyclic voltammograms of polymerization were recorded with *GAMRY* electrochemical workstation. The biointerface was fabricated after electrochemical polymerization of polythiophene containing fused quinone moiety on the gold electrode, and then the thiol modified mannose was linked to the quinone moiety on the basis of 1,4-reductive Michael-type addition, which formed quinone functionalized mannose modified gold electrode. The modified gold electrode was exposed to 300 nM Con A for 1 hour and formed the Con A

sensor for the following study (The detailed fabrication process is described in our earlier work[14] and additional information including the characterization of the biointerface by Electrochemical impedance Spectroscopy (Figure S1, supporting information (SI)), Atomic Force Microscopy (AFM) (Figure 2) and FTIR (Figure S2, SI) are included.

E. coli W1485 Culture and Sensitivity Test

W1485 strain of *E. coli* was used.[16] The culture of *E. coli* W1485 was grown in Levinthal broth (LB) at 37 °C for 18 h in a shaking incubator. During the growth of a bacterial culture, a succession of phases, characterized by variations of the growth rates, are conveniently distinguished and be modeled with four different phases: lag phase, log phase (i.e. exponential phase), stationary phase, and death phase.[17] In our work, the cultured bacteria sample either in log phase or stationary phase was directly diluted with HEPES buffer to the desired concentrations and frozen for further use without any washing steps[11] so that the *E. coli* is basically in non-growing phase.[18]

The antimicrobial susceptibilities of various antibiotic drugs are determined by the described Con A biosensor. For this purpose, three different antibiotics, Cipofloxacin, Ceftriaxone, and Tetracycline with different mechanisms of actions were used to study their different abilities to kill or inhibit the bacterial growth. Both end point and real time measurements are performed. In the end point measurement, four samples containing 2×10^5 cells/mL of *E. coli* obtained from log phase in fresh LB culture were prepared. Ceftriaxone (final concentration is 0.1mg/L), ciprofloxacin (final concentration is 0.1mg/L) or tetracycline (final concentration is 10mg/L) was added into each sample, respectively. The four samples were incubated for 18 h, 37 °C, 280 rpm on an orbital environmental shaker.[18] Finally, 1 μL of the *E. coli* culture from the each prepared sample was introduced to the Con A biosensor in l mL of 10

mM HEPES buffer (pH 7.4) for QCM and electrochemical biosensor measurements, respectively. The sample without addition of any antibiotic was used as control. For the real time measurements, Con A sensor was mounted in a biosensor cell containing l mL of 10 mM HEPES buffer (pH 7.4). Real time QCM measurements were obtained for each experimental step. First, 1μL of 5×10^8 cells/ml *E. coli* obtained from the stationary phase was added into the biosensor cell. When frequency reached a constant value, it was washed with l mL of 10 mM HEPES buffer to remove the unbound *E. coli*. Second, ciprofloxacin, ceftriaxone or tetracycline with the final concentration of 30 mg/L was added to the Con A sensor cell, respectively. The frequency changes vs. time and the damping resistance vs. time were recorded by QCM. By obtaining the damping resistance through fitting the Butterworth-van-Dyke equivalent circuit, we can determine whether the surface layer shows viscoelastic characteristics. After the ciprofloxacin, ceftriaxone or tetracycline was incubated with *E. coli* for three hours, the electrochemical signal changes were recorded by the same biosensor but measuring the electrochemical signal instead.

We have performed experiments to study the concentration of the antibiotics and the length of the incubation time of antibiotics for their ability to kill or inhibit the growth of the bacteria. Different concentrations of each antibiotic were added to 5×10^8 cells/ml *E. coli* respectively, and then incubated at 37°C and 280 rpm on the shaker. After three hour incubation, 1 μL of antibiotic treated *E. coli* culture was added to the Con A sensor cell containing l mL 10 mM HEPES buffer (pH 7.4) for QCM measurements, respectively. To study the effects of length of the incubation time, ceftriaxone, ciprofloxacin or tetracycline at concentration of 10mg/L was added into 5×10^8 cells/ml *E. coli*. 1 μL of each antibiotic treated *E. coli* was taken at different incubation times (i.e. 0, 1, 2, 3h) and was added into the Con A senor cell containing l mL of 10 mM HEPES buffer (pH 7.4) for QCM measurements.

RESULTS AND DISCUSSION

Biosensors for Antimicrobial Susceptibility Tests

Figure 1 schematically describes the modes of actions of three different antibiotics (i.e., Ciprofloxacin, Ceftriaxone, and Tetracycline) used in this work. The interaction of antibiotics with the bacteria cause the alterations of the outer membrane of bacterial cell wall, and subsequently the properties of LPS, located in the outer layer of the outer membrane, with the magnitude of that effect depending upon the antibiotic used. For instance, **Ciprofloxacin**, a quinolone derivative with a broad antibacterial spectrum, destabilize the LPS structures in the bacterial outer membrane.[19] **Ceftriaxone**, one kind of lactamase-stable cephalosporins, inhibit the bacterial cell wall synthesis by acting as suicide substrates.[20] This results into a rapid fragmentation of the bacteria and ultimate release of LPS. **Tetracyclines** on the other hand, are broad-spectrum protein synthesis inhibitors.[21] Tetracyclines also caused the LPS release, however, it is unclear what mechanism is responsible for their effect on LPS of bacteria.[19a] Regardless of the cause of its effect on LPS, it consequently affects the bacterial binding with the sensor due to lesser binding sites available.

We recently developed a carbohydrate biosensor which can efficiently measure the bacterial binding via both their LPS and fimbriae protein using orthogonal electrochemical and QCM transduction mechanisms. The details of the detection principles of these responses have been given in the earlier paper.[14] Briefly, a biosensor using quinone fused mannosylated polythiophene biointerface is fabricated for the detection of gram negative bacteria *E. coli* targeting its LPS structures as well as pili binding using combined EQCM methods. We used thiophene because of its good thermal and chemical stability and relative ease of

functionalization with quinone (Q)/hydroquinone (HQ) functionality. Polythiophene with π-conjugated quinone moieties displays low formal potential around zero volts vs. Ag/AgCl reference electrode. By taking advantage of the addition and substitution reactions of quinones with nucleophiles, particularly thiol and amino groups, we were then able to incorporate carbohydrate functionality to the polythiophene units. We further characterized the morphology of the biointerface by AFM (Figure 2) and performed chemical analysis of the biointerface by FTIR (Figure S2 in the SI). The system thus generated has multiple orthogonalities: 1) the innovative biointerface with built-in solid state redox probe allows label free and reagentless transduction of both electrochemical and QCM mechanisms, and 2) the signals generated by these mechanisms are by themselves orthogonal; the electrochemical measurements is a signal OFF approach (i.e. an increase of analyte concentration results in a decrease of the signal) while QCM is signal ON approach (i.e. an increase of analyte concentration results in an increase of the signal) for this biointerface. All these functions of the biointerface positively interact to provide enhanced sensitivity, broader dynamic range of detection, and greater reliability via cross-validation. In the present work, the binding of Con A with LPS was used that leads to an increase in the mass loading at the interface which brings about a shift in the frequency of the QCM sensor. With increasing bacterial binding, the shift of the frequency should be increasing and vice versa, so the QCM in this sensor is a signal ON approach. Contrarily, with a similar increase in binding, the electron transport of the quinone-fused polythiophene at the interface should be hindered, thereby lowering the electrochemical signal and thus, it is a signal OFF approach. Here, we like to demonstrate the feasibility of using this lectin sensor to quantify the difference in the modes of actions of different types of antibiotics on gram-negative bacteria by relating their effect on the bacterial cell surface morphology and LPS properties upon the

antibiotic exposures. We hypothesize that subtle changes of LPS integrity and function will lead to diminished bacterial binding with the lectin sensor that can be measured real time. Consequently, a fast antimicrobial susceptibility test can be established to determine antibiotic efficacy in vitro in an efficient manner.

In order to validate that the described biosensor can be used for an antimicrobial susceptibility test for the chosen antibiotics, we prepared 2×10^5 cells/mL of *E. coli* samples in fresh LB culture and then incubated with antibiotics ceftriaxone (final concentration = 0.1mg/L), ciprofloxacin (finial concentration = 0.1mg/L) or tetracycline (final concentration = 10mg/L) for 18 h, at 37 °C and 280 rpm in a shaking incubator. The concentrations used are based on the literature and current clinically used dosages. The sample without any antibiotic was used as a control. *E. coli* W1485, a wild type of *E. coli* K-12, was used as the model gram negative bacterial analyte in the work. *E. coli* W1485 is a "semirough" bacterial strain in which the intact LPS core is capped by a single O-antigen subunit consisting of glucose, N-acetylglucosamine, galactose and rhamnose in the ratio 1.8: 1.0: 0.7: 0.6, which has been confirmed by SDS–PAGE method.[11] Con A binds specifically at α-D-mannosyl and α-D-glucosyl residues (two hexoses differing only by the alcohol on carbon 2) in terminal position of ramified structures from B-Glycans (reach in α-mannose, or hybrid and bi-antennary glycanes complexes). Therefore, the multivalent binding of Con A to the *E. coli* W1485 surface O-antigen glucose receptor facilitates the strong adhesion of *E. coli* W1485 to the mannose immobilized on the QCM surface. Afterwards, these samples were analyzed for their QCM and electrochemical sensor responses which are shown in Figure 3. As shown in Figure 3A, the blank sample with no antibiotics provided the highest QCM signal and highest damping resistance (R) changes as the bacterial cell surface LPS in this sample are at their fully functional state, which resulted in highest

binding to the sensor interface (curve d). Here the QCM frequency shift is because of the mass loading on the QCM surface due to bacterial adhesion and the change of damping resistance is a result of increasing softness of the interface which causes the damping of the oscillation wave. The detailed explanation of these phenomena can be found elsewhere,[22] however, we have already shown that in case of ConA-LPS binding[14]; the frequency-resistance ratio is high enough to consider this binding as rigid, and the mass loading as the dominant effect whereas the softness of the interface can be considered negligible. Once the samples having various antibiotics (curve a, b and c) were introduced, we observed much lesser shift in frequency as well as smaller damping resistance change which quantitatively depended upon the antibiotic used. By normalizing these frequency shifts to that obtained from the control (inset in Figure 3A), it was found that the bacterial binding was reduced to 38%, 27%, and 23% for the given concentrations of tetracycline, ceftriaxone, and ciprofloxacin respectively. These results suggest that the antibiotics inhibited the growth of *E. coli* in log phase and affected the LPS integrity and function at bacterial cell surface so that fewer number of *E. coli* bound to the Con A sensor, compared to the control sample without antibiotics. In order to further confirm the results by an internal validation method, the quinone-fused polythiophene solid probe allows simultaneously analyzing the same binding events via square wave voltammetry (SWV) and the results are shown in Figure 3 (B). Here, the Con A sensor displayed increasing SWV signals (curve a, b and c) when antibiotics were added, compared to control experiment (curve d). The pattern of the responses was also related to what was obtained from QCM. Normalization of the SWV signal showed a 7.2, 4.8, and 2 folds increase of the signal as compared to control experiment with addition of 0.1mg/L of ciprofloxacin, 0.1mg/L of ceftriaxone or 10mg/L of tetracycline, respectively (Inset, Figure 3B). From these results, it was clearly indicated that all three

antibiotics are able to alter the LPS properties and could inhibit the *E. coli* growth. However, they differ in their strengths for this inhibition, thus having different consequences in terms of binding. Ciprofloxacin is slightly more active than ceftriaxone against *E. coli* at the same concentration, and both ceftriaxone and ciprofloxacin are superior to tetracycline against *E. coli*. Electrochemical approach displayed more distinctive difference of antibiotics susceptibilities than that shown in QCM, however, electrochemical measurement is an end point calibration. QCM, on the other hand, can be used to monitor the process real time and thus can determine the binding kinetics between Con A on the sensor surface and *E. coli* pretreated by various antibiotics. Therefore, this combined quartz crystal microbalance and electrochemical readouts (EQCM) approach has multiple advantages in addition to cross-validating the measurement results.

Real Time Analysis of Bactericidal Activities of Antibiotics

Once we have established that different types of antibiotics have differing abilities to alter the LPS properties and subsequent reduction in its binding and this can be used as an indicator for the antibiotic effects for killing the bacteria or inhibiting its growth, we further tested the sensor ability to quantitatively measure the same parameters real time. For that purpose, Con A sensor was exposed to 1μL of 5×10^8 cells/ml *E. coli* to capture the bacteria via Con A-LPS binding. The unbound *E. coli* was removed by washing with 1 mL of 10 mM HEPES buffer. Antibiotic samples were then added into the sensor cell with the final concentration of 30 mg/L and the frequency changes were measured real time (Figure 4A). After an incubation of three hours, the electrochemical signals were also recorded (Figure 4B). In the three separate measurements shown in Figure 4A, it can be clearly seen that the initial parts of the curves which are related to *E. coli* immobilization are almost coinciding to each other. This real time QCM

measurement also allows us to observe the reproducibility of the sensor performance. But in the last parts of the curves, when different antibiotics are added, their variable effects in killing the bacteria and reducing their binding caused a variable shift of the frequency. This shift was opposite in direction to what was obtained by bacterial addition which is indicative of reduced binding. These responses (shown in the inset) provided us the quantitative details of this effect. During the two hours of period after the antibiotic addition, we had 350Hz to 320Hz (0.91 fold), 350Hz to 325Hz (0.93 fold) and 370Hz to 350Hz (0.95 fold) decrease in frequency, for ciprofloxacin, ceftriaxone, and tetracycline respectively. Due to real time nature of this experiment, the frequency and damping resistance change were much smaller than that of Figure 3 confirming the antibiotic drug effect on LPS is a slow process but our high sensitivity sensor allows such subtle changes to be measured. Moreover, the results of the QCM and Electrochemical measurements are validating each other again. The mass effect is diminishing as compared to the control measurement due to the lesser binding as a result of real time antibiotic effect, whereas the electrochemical signal is increasing for the same reason. Table S1 describes the magnitudes of both the frequency shifts as well as the resistance changes for all the experiments. It can be clearly noted that changes in frequency and the damping resistance are always in the same direction, thus these changes are correlated to each other. For instance, the frequency shifts for the data in Figure 4 are negative, and so are the resistance changes in these experiments. However, among three antibiotics, ceftriaxone shows the highest changes in resistance in both the end point susceptibility testing as well as the real time study in this experiment. This can be related to its mechanism of action shown in Figure 1. Ceftriaxone causes the bacterial cell to finally lyse, thus generating more fluid contents than the others, which contribute more towards the resistance changes. Thus the damping resistance data in these

experiments can also be hinting towards final fate of the bacterial cells. Similarly as susceptibility experiment, SWV sensor signal for *E. coli*/Con A-QCM modified gold electrode without addition of any antibiotic displayed the lowest value of 0.37μA. After incubation with ciprofloxacin, ceftriaxone or tetracycline with the same concentration of 30mg/L, the modified gold electrode displayed the signal of 0.89μA (2.41 fold), 0.92μA (2.50 fold) and 0.65μA (1.76 fold), respectively. These results indicate that LPS may become destabilized in the presence of antibiotics and ultimately released from the *E. coli* so that the *E. coli* becomes unbound from the Con A sensor. The result also demonstrates that tetracycline has the least bactericidal activity against *E. coli* than ciprofloxacin and ceftriaxone with the same concentration while ciprofloxacin and ceftriaxone display almost similar bactericidal effects on *E. coli*. Once we can determine these effects real time, we can quantitatively figure out the most effective drug, its time of response, and even its effective concentration to be for the best therapeutic management.

The Effects of Concentration and the length of Incubation Time on Bactericidal Activities of Antibiotics

Since antibiotics from different classes have different mechanisms of action and antibiotic effects on bacterial morphology and viability are concentration and time dependent, we hypothesized that the ability of antibiotics to kill the bacteria or inhibit its growth may be class and concentration dependent, which will affect the interaction between the LPS and Con A sensor. In order to further study the effects of concentration and length of incubation time on the bactericidal activities of antibiotics, different concentration of antibiotics were added into the broth containing *E. coli* and incubated for different length of times. Figure S3 shows frequency as a function of time of the Con A sensor interacted with *E. coli* pretreated by ceftriaxone (A), ciprofloxacin (B), and tetracycline (C) with different concentrations, respectively. This data also

shows the similar trends as those originally obtained from the susceptibility tests, i.e. the ΔF decreased with increasing the concentration of the antibiotics. Figure S3 (D)-(F) show the histogram of ΔF or ΔR changes vs. antibiotics with different concentrations which are again correlated to each other with ceftriaxone showing consistent increase in resistance with increasing its concentration. This is probably due to more and more cell lysis thereby intensifying the fluidic environment. Moreover, this data can be used to find out the dosage information for different antibiotics. For instance, ciprofloxacin at concentration of 1 mg/L shows a frequency of 112 Hz which quickly reaches to 73 Hz, and 70 Hz at concentrations of 10 mg/L and 30 mg/L respectively. Thus there is no big change in the step from 10-30 mg/L. This means that the concentration of 10 mg/L is its most effective concentration to act against this bacterial strain. On the other hand, both ceftriaxone and tetracycline show consistent changes in frequency while changing the concentration which implies that even the dosages at 30 mg/L level can be administered as well. The results of Figure S3 also indicate that the concentration has distinctive effect on the bactericidal activity of the three kinds of antibiotics against *E. coli* and ciprofloxacin is particularly effective against *E. coli* than the other two antibiotics with the same concentrations.

In order to further compare the effect of concentrations on the bactericidal activities of the antibiotics, the binding kinetics between Con A sensor and *E. coli* were studied. The binding between Con A sensor and *E. coli* can be described by eq. 1. The amount of the complex *E. coli*/Con A formed at time t after the injection is given by eq 2 and 3, where ΔM_{max} is the maximum binding amount of *E. coli*/Con A, ΔM is the measured binding amount, t is the time after injection. τ is the relaxation time associated with *E. coli* binding which is calculated from curve fittings of the ΔF during the binding process. The higher τ value means the longer binding

time required between the Con A and *E. coli*. Table 1 summarized the effects of concentration of antibiotics on the relaxation time τ. From the Table 1; we can observe that τ increased with increasing the concentration of antibiotics while at the same concentration, $\tau_{ciprofloxacin} > \tau_{ceftriaxone} > \tau_{tetracycline}$, which indicated that the rate of binding between Con A and *E. coli* pretreated by ciprofloxacin is smaller than that between Con A and *E. coli* pretreated by ceftriaxone and tetracycline. The result further confirmed that ciprofloxacin has more bactericidal activity than the other two antibiotics with the same concentrations.

$$[\text{Con A}_{\text{attached to gold}}] + [\text{E } coli] \longrightarrow [\text{E } coli / \text{Con A}_{\text{attached to gold}}] \quad (1)$$

$$[\text{E } coli / \text{Con A}_{\text{attached to gold}}]_t = [\text{E } coli / \text{Con A}_{\text{attached to gold}}]_\infty (1 - e^{-(1/\tau)t}) \quad (2)$$

$$\Delta M_t = \Delta M_\infty (1 - e^{-(1/\tau)t}) \quad (3)$$

The effect of incubation time on the bactericidal activities was studied further and the results are presented in Figure S4. At the same incubation time, the ΔF shift induced by ciprofloxacin is smaller than that induced by ceftriaxone or tetracycline but the damping resistance changes are very minimal for all three antibiotics. The result indicates that the sensor is very sensitive to measure the time effects on the bactericidal activity of the three kinds of antibiotics against *E. coli* and ciprofloxacin has a greater killing effect on *E. coli* than does ceftriaxone and tetracycline under the same conditions even though there are little structure changes of the bacterial cells at varying incubation time (i.e. the minimum change of the damping resistance signal).

The results of Figure S3, Table 1 and Figure S4 demonstrate that ciprofloxacin damaged LPS more rapidly and in greater level than that of ceftriaxone and tetracycline which possibly resulted from their different mechanisms of action. Ciprofloxacin may chelate divalent cations which normally bridge adjacent LPS and phospholipid molecules and stabilize the molecules in

the bacterial outer membrane.[19b] LPS may become destabilized and ultimately released from the *E. coli* when divalent cation bridges are removed from the outer membrane.[23] So *E. coli* couldn't bind to the Con A sensor using LPS as linker. Ceftriaxone doesn't interact with divalent cations on the bacterial outer membrane. Instead, ceftriaxone passes through water-filled porin channels in the outer membrane into the periplasmic space and interacts with penicillin binding proteins and finally cause cell lysis. The release of LPS by the ceftriaxone may be the result of fragmentation of the bacteria. Because of the multiple mechanism, ceftriaxone released LPS slower and in lesser amounts than ciprofloxacin in our work. Martin E. Evans and Matthew Pollack reported that it is unclear what mechanism is responsible for the release of LPS from bacteria by tetracycline.[19a] They thought that the mechanism of LPS release by tetracycline may be similar to that of ciprofloxacin. According to result of Figure S3 (D) in the work, the similar ΔF changes induced by tetracycline and ceftriaxone indicate that the mechanism of LPS release by tetracycline may be similar to that of ceftriaxone. In most studies dealing with antibiotic-induced LPS release, bioreactive endotoxin has been measured by means of the Limulus amoebocyte lysate (LAL) assay which involves an enzymatic reaction triggered by the core region of LPS.[24] Endotoxin levels determined by means of the LAL assay therefore reflect not only the concentration of LPS but also the accessibility of the inner core which may be unfolded during exposure to cell-wall active drugs. However, some antibiotics (i.e. aminoglycoside) are known to suppress the LAL reaction, which limit the application of LAL. Reproducible ELISA also can be used to detect LPS. However, antibodies have some limitations such as their production in vivo; limited target analytes; limited shelf lives and temperature-sensitive to get denatured. A QCM is known to provide a very sensitive mass measuring device and dispense with the time- and cost- demanding labeling step and also

eliminates any possible interference of the "true" binding process due to the presence of the labels.[11] In our work, the obtained results confirmed feasibility of QCM for promising detection of LPS release. Furthermore, the use of integrated electrochemical measurement in the form of EQCM benefited the reliability of the whole analytical method by providing internal validation of the results.

The minimum inhibitory concentration (MIC), the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation is generally regarded as the most basic laboratory measurement of the activity of an antimicrobial agent against an organism. The order of antimicrobial activities, (i.e. ciprofloxacin>ceftriaxone>tetracycline) obtained from our lectin biosensor above is consistent with the trend of reported MICs (shown in Table 2). As shown in Table 2, MICs of tetracycline are distinctly higher than other two antibiotics against different strains. MICs of ciprofloxacin are slightly smaller than ceftriaxone. Theoretically, the antimicrobial activity of ceftriaxone is higher than ciprofloxacin because of their different antimicrobial mechanisms. The trends of antibiotics bactericidal activity are similar to the reported MICs trends, which also demonstrates that EQCM method is an efficient method for the determination of MIC.

CONCLUSIONS

A novel integrated label free lectin EQCM biosensor is utilized to evaluate the antimicrobial susceptibility of various antibiotics on the basis of quantitatively evaluating the binding between lectin Con A and LPS on the wall of *E. coli.* A measurement of subsequent unbinding upon the antibiotic addition causing the release of LPS, made this sensor capable of real time analysis. Three typical kinds of antibiotics were used as models to study the bactericidal activities of antibiotics against *E. coli*. Measurements were performed both after sample incubation as well as

real time to provide the proof of concept. In both the cases, the biosensor demonstrated that all three kinds of antibiotics effectively inhibit the growth of *E. coli*. However, ciprofloxacin was slightly more active than ceftriaxone against *E. coli* W1485 with the same concentration, and both ceftriaxone and ciprofloxacin were superior to tetracycline. Variations in antibiotic concentrations and incubation times further elaborated the bactericidal phenomena and enabled to study the binding kinetics and the relaxation time. In our opinion, this is a rare example of using biosensor in this fashion to find out the antibiotic actions and such studies can form the basis of real time analysis of antibiotic selection, their therapeutic management, and the control over their empiric use which usually give rise to antibiotic resistance. Moreover, this sensor can be used to analyze the impact of antibiotic treatment, both for those bacteria that are sensitive to and those that are resistant to the antibiotics. One of the largest drawbacks to use culture in order to detect bacteremias in septic patients is that frequently these patients have already been given empiric antibiotics by the time the blood is drawn. The presence of antibiotics in the blood is frequently inhibitory to the growth of pathogens, at times even if the pathogen is resistant to the antibiotic. The reported biosensor can detect the presence of pathogens and identify them in conditions including the presence of antibiotics which is of even greater value. Going even further, bacteria which have undergone lysis due to antibiotics may still be detectable via this biosensor as it finds fragments of the bacteria containing the appropriate pili or LPS. Such new dimensions of biosensor based detection can lead to new researches towards drug discovery of infectious diseases.

ASSOCIATED CONTENT

Supporting Information

Additional information including fabrication the Con A sensor and characterization of Con A sensor and the effects of concentration and incubation time on bactericidal activities of antibiotics as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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FIGURES

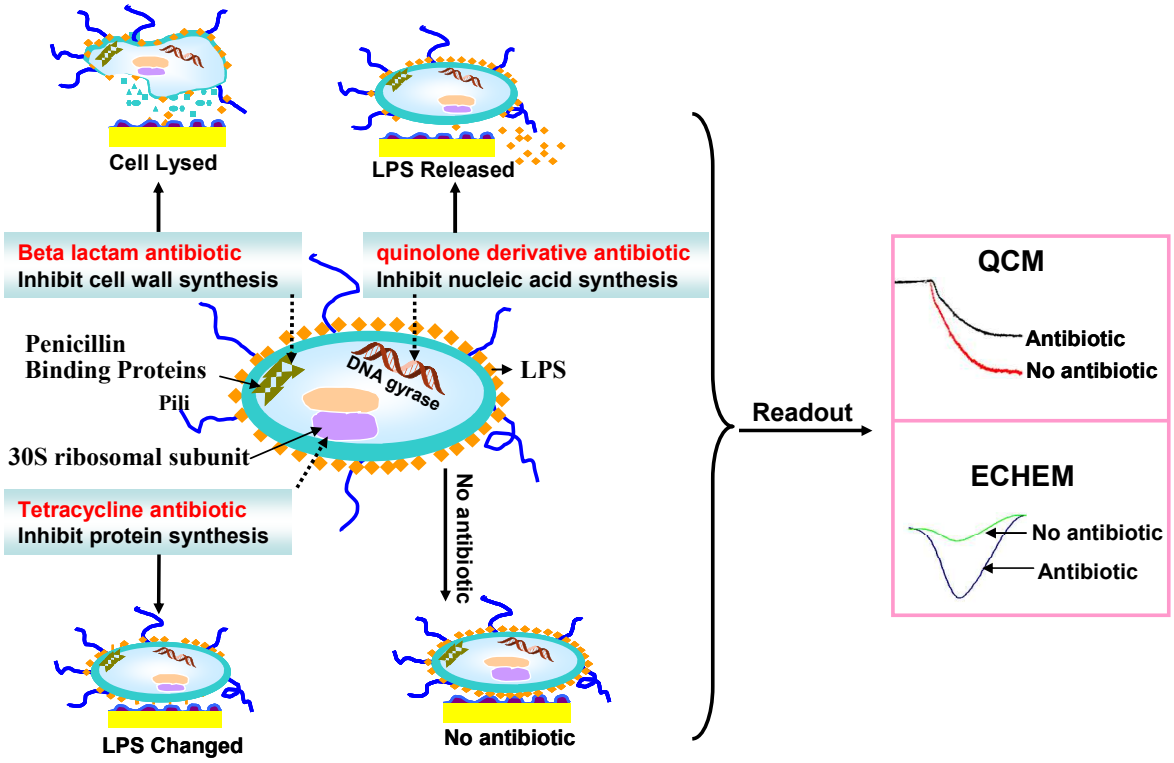


Figure 1: Schematics of the mechanisms of action of the three antibiotics studied on the bacterial cell wall. The magnitudes of the alterations vary with the type of antibiotics which affect the bacterial binding to the lectin biosensor. Consequently, a representative shift in both electrochemical and QCM signals should be observed.

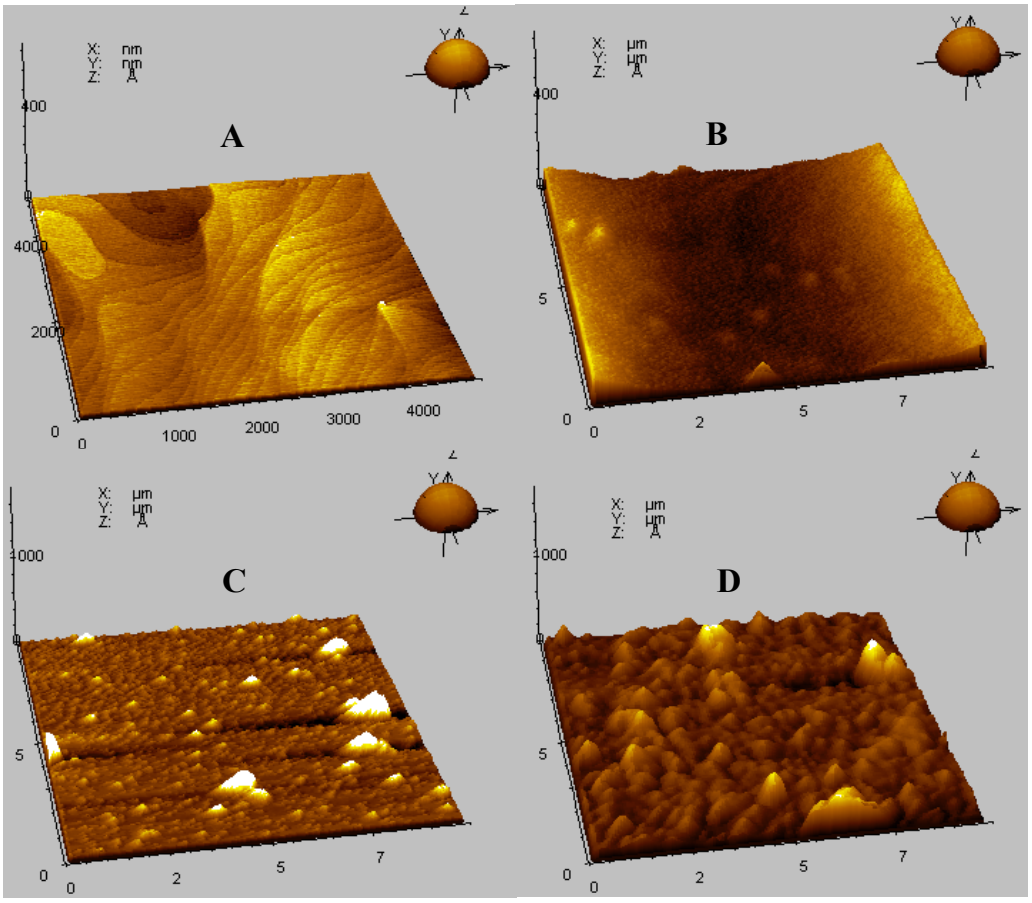


Figure 2. AFM topographic images of (A) bare Au (111) surface, (B) TQ modified Au (111) surface, (C) SM/TQ modified Au (111) surface, (D) ConA/SM/TQ modified Au (111) surface.

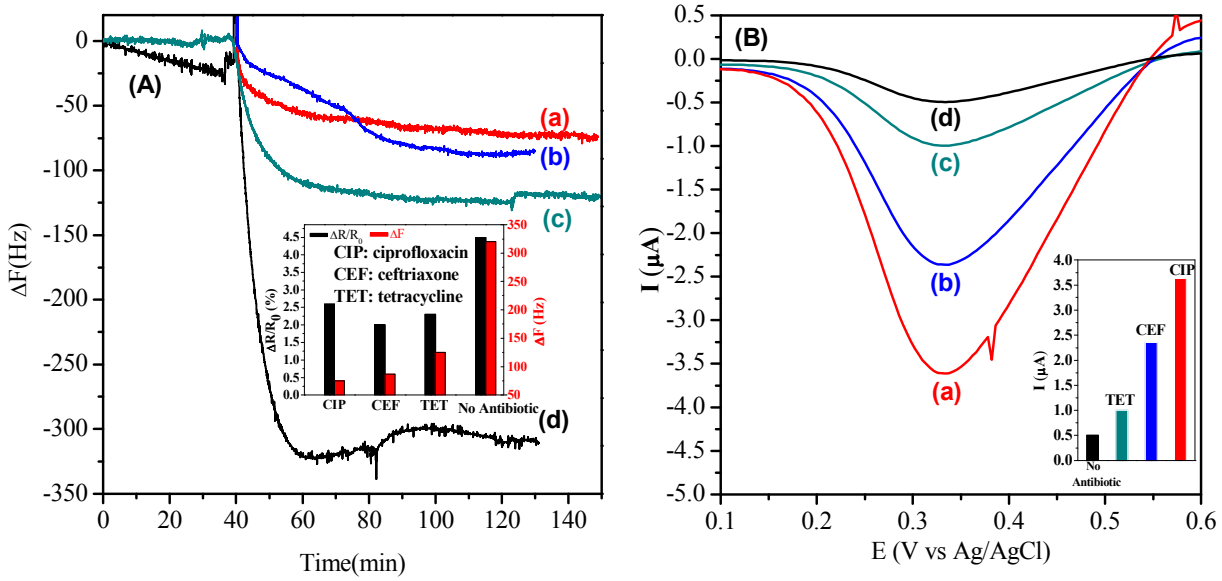


Figure 3. (A) Frequency change vs time curve and (B) Square wave voltammetry (SWV) responses when Con A sensor was exposed to *E.* coli pretreated by 0.1 mg/L ciprofloxacin (a), 0.1 mg/L ceftriaxone (b) and 10 mg/L tetracycline (c) and blank samples without addition of any antibiotics (d) in log phase, and then incubated for 18 h, 37 °C, 280 rpm on an orbital environmental shaker, respectively, in HEPES buffer (pH 7.4) with 1 mM Mn^{2+} and 1 mM Ca^{2+}. The insets indicate the sensor responses in comparison to control measurements both for QCM and electrochemical approach.

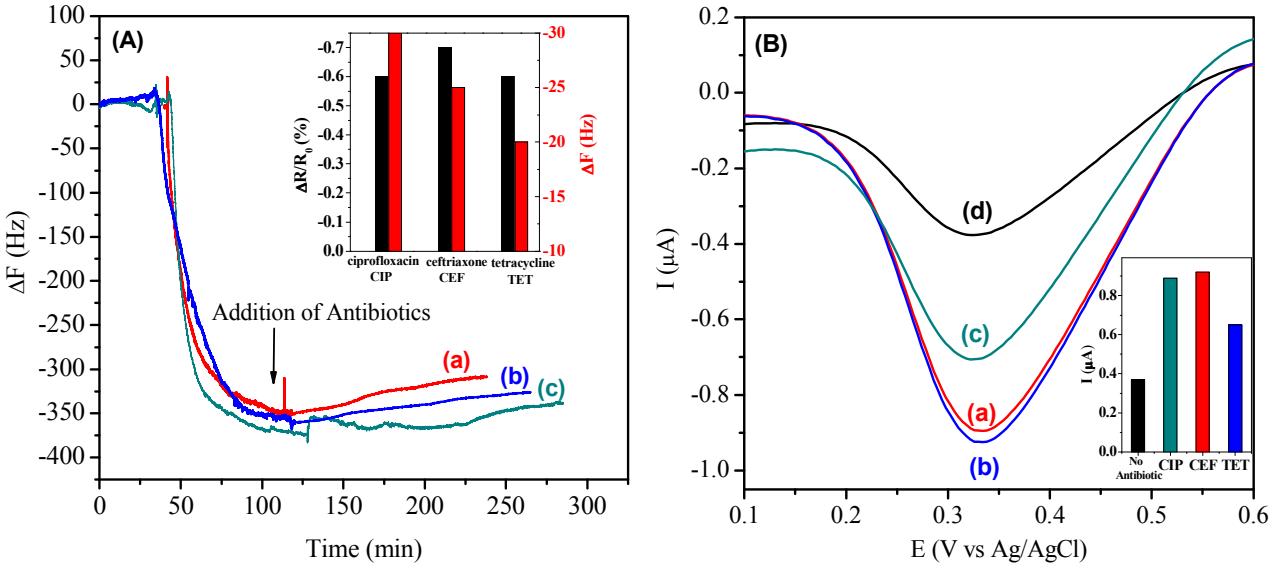


Figure 4. (A) Frequency change vs time curve when Con A mediated sensor was exposed to *E. coli* W1485 obtained from stationary phase, and then 30 mg/L ciprofloxacin (a), ceftriaxone (b) or tetracycline (c) was added into the QCM cell after *E. coli* were attached on the Con A sensor. And (B) the corresponding square wave voltammetry (SWV) responses with 30 mg/L ciprofloxacin (a), ceftriaxone (b) and tetracycline (c) and without addition of any antibiotics (d), respectively in HEPES buffer (pH 7.4) with 1 mM Mn^{2+} and 1 mM Ca^{2+}. The insets show the sensor responses for both these approaches.

Table 1 The effects of concentration of antibiotics on the τ (relaxation time)

τ (min) Antibiotic / Concentration	ceftriaxone	ciprofloxacin	tetracycline
1 (mg/L)	15.5	21	11.2
10 (mg/L)	18.4	23.5	13.9
30 (mg/L)	22.5	23.6	19.8

Table 2 Comparison between MIC in references and the results in our work

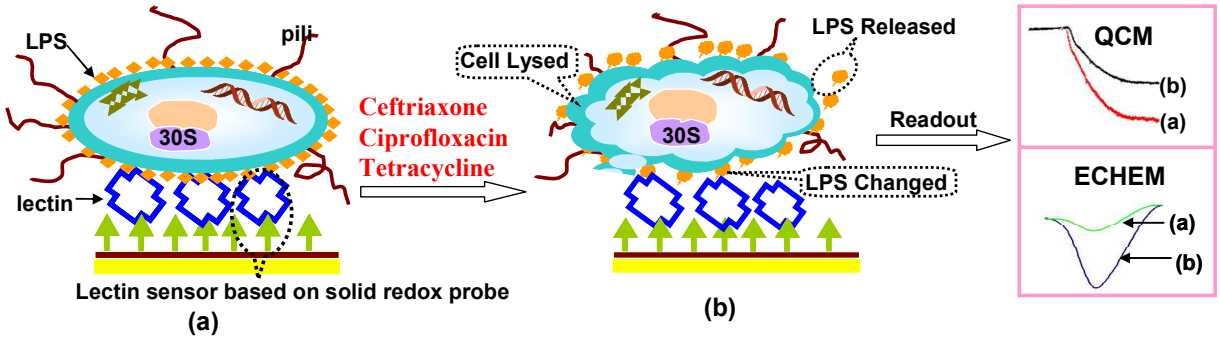
Antibiotics		Ceftriaxone[a]	Ciprofloxacin[b]	Tetracycline[c]
Minimum Inhibitory Concentration (MIC, mg/L) in references		0.024-0.195[25]	0.0075-0.015[18, 25b, 26]	0.5-4.5[21, 25b, 27]
LPS Activity in our work	Susceptibilities Tests	27%	23%	38%
	Real Time Analysis of Bactericidal Activities	93%	91%	95%
	relaxation time	$\tau_{ciprofloxacin} > \tau_{ceftriaxone} > \tau_{tetracycline}$		

a: Ceftriaxone: 0.0125-25 mg/L (E coli (47))[28], 0.06-8 mg/L (E coli (305))[29];

b: ciprofloxacin: 0.015 to 0.002 mg/L (E. *coli* Neumann) and 0.03 to 0.002 mg/L (E. *coli* KL16) with increased the pH from 6.0 to 8.0[30];

c: Tetracycline: 0.16 mg/L (ATCC 25923)[31].

For TOC Only



Lectin sensor based on solid redox probe

(a) (b)