Rapid Detection of Bacterial Resistance to Antibiotics

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ABSTRACT

Antibiotic-resistant pathogens are a major health concern in everyday clinical practice. Since their detection by conventional microbial techniques requires minimally 24 hours, a tool that could reveal their presence in less than one hour would be indispensable in the management of infections. In this study, we present a novel nano-mechanical sensor that is capable of distinguishing movement at the nanoscale and that permits a monitoring of the dynamics of a sample during its exposure to environmental modifications. The high sensitivity of this technique allows the detection of low concentrations of bacteria and a rapid identification –few minutes instead of hours or days - of the antibiotics to which the germ is resistant and of the dynamic effects of antibiotics on susceptible and resistant bacteria. This methodology opens a way to rapid determination of bacterial antibiograms. Moreover, it can also be applied to investigate other biological specimens such as proteins or cells.

Keywords: Bacterial resitances, nanomechanical sensors, cantilevers, Nano-motion.

1 INTRODUCTION

To suppress the emergence and propagation of antibiotic-resistant bacteria is one of the major health issues of the present century. Bacterial pathogens are renowned for their capacity to develop resistance to antibiotics [1]. Moreover, due to the widespread misuse of antibiotic drugs, this trend has recently exploded, with the consequence that multidrug-resistant isolates are nowadays frequently encountered. When infections become resistant to first-line medical therapies, more costly alternatives must be instigated to avoid mortality. The greater expense of these treatment modalities and the longer course of the illness, involving perhaps hospitalization, pose a great financial burden on health-care systems, society and individuals.

An early detection of the presence of microorganisms and a determination of their susceptibility to available antibiotics, are essential for an efficient reduction in mortality and morbidity. Using currently-available microbial techniques, useful information concerning the susceptibility of common, fast-growing pathogens to antibiotics cannot be acquired within fewer than 1-3 days, and for slow-growing ones, up to one month's time may be required [2]. In recent years, several alternative techniques have been developed to measure the growth or viability of microorganisms. These often take advantage of surrogate markers that gauge the metabolic activity of the bacteria (e.g., the mycobacterial growth indicator tube [3-5], the resazurin-reduction microplate assay [6], or the BacTiter-Glo™ microbial cell-viability assay [7,8]).

At the same time, different PCR-based genotypic or molecular methods for predicting drug resistance have also been developed, automated and commercialized (e.g., the MTB/RIF™ assay [9, 10]).

To circumvent the different limitations of the available techniques, we have recently exploited one of the working principles of the atomic force microscope (AFM) [11] to develop a nano-mechanical oscillator sensor that is capable of detecting the presence of living bacteria and of indicating their susceptibility to antibiotics [12]. In recent years, micro- and nano-mechanical oscillators, such as AFM cantilevers, have become a new class of sensors. The development of very delicate oscillators and their coupling with sensitive displacement detectors have resulted in the emergence of many extraordinarily powerful experimental tools [13]. The high sensitivity of such devices has been exploited for the detection of very small masses [14-16] or for nano-stress sensing in molecular biology [17-19]. Such nano-mechanical oscillators are employed also as artificial noses to detect the presence of specific molecules in gaseous and fluid environments [20-23]. The main limitations of this technique lie in the complexity of the device's assemblage. Another drawback of the system is that its sensitivity is greatly reduced when measurements are made in fluid environment [24].

2 RESULTS

2.1 Description of the procedure

In this work, we present a novel nano-mechanical sensing technique that is capable of detecting the movements of specimens in the Angstrom-to-micrometer range within a physiological medium. The methodology permits a monitoring of the evolution of the dynamics of the specimen (such as bacteria) during its exposure to chemical or physical modifications in the environment. The high sensitivity of this nano-mechanical sensor allows the detection of very low concentrations of bacteria and a rapid identification – within a few minutes instead of several hours or days - of the antibiotics to which the germ is sensitive or resistant.

The core of the system is a nano-mechanical oscillator which is inserted into an analysis chamber, which is fitted with an inlet and an outlet in order to allow flushing different liquids. The dynamic deflections of the oscillator are detected and recorded using a transduction unit. Specifically, we have used a standard AFM cantilever as a sensor, and have exploited the illumination-detection system of a commercial AFM (PicoForce, Bruker) to detect its oscillations.

Prior to its insertion into the analysis chamber, the surface of the sensor was chemically treated with (3-aminopropyl)triethoxysilane (APTES) to promote a non-specific attachment of the bacteria [25]. Living bacteria were injected into the analysis chamber and left to incubate for 10 minutes, to ensure their firm attachment to the cantilever. Attachment was confirmed using an optical microscope that was focussed on the sensor, as well as using an AFM.

Once the sensor had been introduced into the analysis chamber, the latter was flushed with PBS. Before the on-set of data-capture, and after each exchange of fluid, the injection system was turned off (typically for 5 minutes) to stabilize the chamber and to reduce the drift in the measurements.

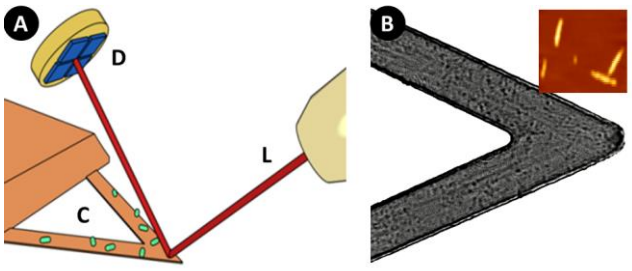

Figure 1: Schematics of the set-up. Left panel: Schematic representation of the set-up. C: cantilever bearing attached bacteria; L: beam of laser light focused on the cantilever; D: 4-segment detector used to register the fluctuations of the sensor. Right panel: Optical image of the cantilever bearing attached bacteria; inset: AFM image (8 x 8 μm2) of the attached bacteria

Deflections of the sensor that were caused by thermal fluctuations were then measured for 2 minutes (headed "*PBS*"), and were used as the baseline for the entire experiment.

The solution containing the living bacteria was then introduced into the analysis chamber. An average incubation time of 10 minutes at ambient temperature was needed to ensure the firm attachment of the bacteria to both sides of the cantilever and to stabilize the system. Thereafter, the analysis chamber was thoroughly rinsed with PBS to remove loosely-adhering and any floating bacteria. The nano-scale vibrations of the sensor were collected for at least 6 minutes, divided into 2-minute data-chunks, under the heading "*Bacteria in PBS*".

Following these measurements, the PBS in the analysis chamber was exchanged with a standard bacterial growth medium (LB). This step was needed to promote the growth of the bacteria, since ampicillin acts particularly efficiently on microorganisms in this state. The time to lysis is indeed proportional to the growth rate [26]. The data were collected in 2-minute chunks, analysed and headed "*Bacteria in LB*".

Next, ampicillin-supplemented LB (10 mg/ml) was injected into the analysis chamber. The deflections of the sensor were recorded for more than 10 minutes, divided in 2-minute chunks, analysed and headed "*Ampicillin*".

Finally, the antibiotic solution was flushed out of the analysis chamber with LB. This step was included to confirm the persistence of the antibiotic's effect. Usually, no more than 10 minutes were needed to detect the cessation or the amplification of the sensor's fluctuations, depending on the dynamics of the resistance of the chosen bacteria. This set of data appeared under the heading "*LB wash*".

To confirm the outcome of the experiment, each used sensor was incubated overnight at 37°C in growth medium (LB). At the close of this period, the turbidity of the solution served to indicate the presence of living bacteria on the cantilever.

2.2 Experimental results

Several different experimental set-ups were investigated to probe the capabilities of the technique (Figure 1, left panel). Each experiment was repeated at least 10 times to verify the consistency of the recorded data

The first set-up was involved the investigation of an ampicillin-sensitive strain of *Escherichia coli* (DH5α) , a Gram negative, rod-shaped class of bacteria which cause, for example bacteraemia, abscesses and urinary-tract infections. Initially, experiments using the broth macrodilution method [27] were conducted to characterize the chosen bacterial strain; including the determination of the minimum inhibitory concentration (MIC) of ampicillin against DH5α, which was 2 mg/ml. This antibiotic of the β-lactam class interferes with the synthesis of peptidoglycan, which is an essential component of the bacterial membrane.

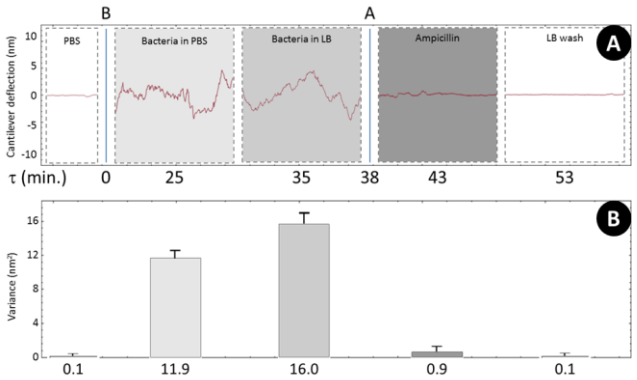

Figure 2: Sensitivity of *E. coli* to ampicillin. Upper panel: deflection of the sensor. The boxes (1 minute in the presence of PBS and 2 minutes in the presence of the other media) depict the fluctuations of the sensor after exposure to the different media: "*PBS*", "*Bacteria in PBS*", "*Bacteria in LB*", "*Ampicillin*" and "*LB wash*". The lines marked "*B*" and "*A*" indicate the junctures at which the bacteria ("*B*") and ampicillin ("*A*") were injected. Lower panel: the corresponding variances.

Ampicillin thus causes lysis of the cell membrane, particularly in growing bacteria, resulting in their death [28].

For the cantilever-sensing experiments, a small aliquot of living bacteria (~105 cells) was introduced into the analysis chamber and permitted time for attachment. Optical and atomic-force analyses of the surface of the sensor (Figure 1, right panel and inset) revealed the firm attachment of several hundred bacteria after an incubation period of 10 minutes. Thereafter, the analysis chamber was sequentially flushed, over a total timespan of 30 minutes, firstly with phosphate-buffered saline (PBS, pH 7.4, Sigma Aldrich), containing no nutrients, secondly with lysogeny broth (LB, Sigma Aldrich), a bacterial growth medium, thirdly with LB containing ampicillin (at a concentration of 10 mg/ml), and fourthly with pure LB once again, to flush out the antibiotic. The deflections of the sensor were recorded and analysed during each exchange of fluid.

The data are depicted in Figure 2. Before the bacteria were introduced into the analysis chamber ("*PBS*"), the amplitude of the deflection (Figure 2, upper panel) was very slight. After their introduction, this parameter increased ("*Bacteria in PBS*" and "*Bacteria in LB*"), and then returned to very low values following the injection of the antibiotic ("*Ampicillin*"). The values remained very low after the antibiotic had been flushed out of the analysis chamber ("*LB wash*").

To evaluate the effective variation in the amplitude of the sensor's fluctuations, the deflection curves obtained during each fluid exchange were statistically analysed by calculating their variance (Figure 2, lower panel). This analysis confirmed an increase in variance after the introduction of the bacteria and maintenance of the high level whilst they were still living.

After the introduction of ampicillin, the variance dropped to very low values. To stabilize the fluid in the analysis chamber, the measurement headed "*Ampicillin*" in Figure 2 was performed 5 minutes after the introduction of the antibiotic. By this time-point, the variance of the deflections had decreased almost 20-fold. Removal of the antibiotic by flushing the chamber with LB elicited no increase in the vibrations. Indeed, the values were comparable to those obtained when no microorganisms were present, thereby indicating that the bacteria had probably been killed.

The continued presence of the bacteria, and their concentration, were monitored by optical microscopy during the entire course of the experiment. Upon its termination, the used cantilevers were placed overnight in growth medium at 37°C to confirm the death of the bacteria.

To ascertain whether the technique was capable of detecting resistance to antibiotics, a second set of experiments were performed. A plasmid harbouring an ampicillin-resistant gene namely the pGL3-basic vector (Promega, Madison, WI), was inserted into the same strain of *E. coli* (DH5α). The MIC of ampicillin towards this modified strain was found to be more than 30-fold higher.

The bacteria were introduced into the analysis chamber, permitted to attach to the sensor, and sequentially exposed to PBS, LB and ampicillin-supplemented LB (10 mg/ml). At 10 mg/ml, the concentration is higher than the MIC for the susceptible *E. coli* strains, but lower than that for the resistant ones. Future studies will focus particularly on this parameter, with a view to confirming that the technique is indeed capable of detecting the effects of different concentrations of antibiotics.

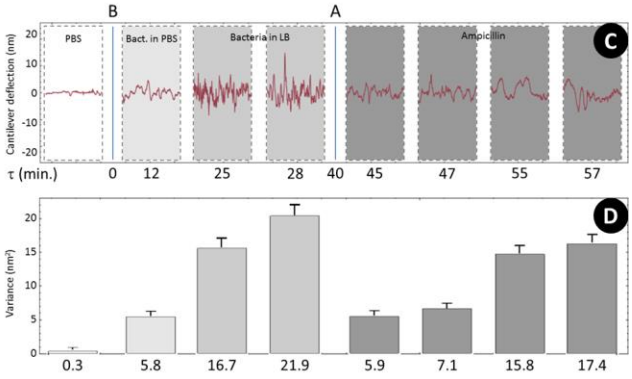

Figure 3: Resistance of *E. coli* to ampicillin. Upper panel: the deflection of the sensor. The boxes (2 minutes in the presence of each medium) depict the fluctuations of the sensor after exposure to the different media: "*PBS*", "*Bacteria in PBS*", "*Bacteria in LB*" and "*Ampicillin*". The lines marked "*B*" and "*A*" indicate the junctures at which the bacteria ("*B*") and ampicillin ("*A*") were injected. The measurements in presence of ampicillin were repeated to confirm that the fluctuations increased in the presence of the antibiotic. Lower panel: the corresponding variances.

In the presence of the living bacteria the variance of the sensor's deflection increased, as shown in figure 3. When the bacteria were exposed to a nourishing medium (LB), the amplitude of the vibrations were further augmented, probably reflecting an increase in the metabolic activity of the attached cells. Upon exposure to the antibiotic, the amplitude of the vibrations dropped almost immediately. As before, the first measurement was performed 5 minutes after the injection of ampicillin. At first, the variance in the amplitude of the deflections decreased 4-fold. Subsequent measurements showed that, even in the presence of the antibiotic, the variance in deflection amplitude was slowly increasing and had been partially recovered 20 minutes after the ampicillin injection.

3 CONCLUSIONS

Nano-mechanical sensors are nowadays employed in multiple fields and in particular in molecular biology [14-23]. Their use as high-resolution nano-sensors has been frustrated by some technical limitations of the technique. For instance, while several studies have been focused on measuring the average deflection of a cantilever sensor caused by absorption stress on one of its sides [29], only very few were performed in liquid environments [24].

To-date, there is no report in which these sensors have been used to detect the dynamic fluctuations of specimen, and in particular of living biological samples. We have demonstrated that a simple set-up, consisting of a micro-fabricated cantilever and a deflection-detecting system, is capable of monitoring the nanometric-scale motions/vibrations of living bacteria. This novel technique can yield relevant information much more rapidly than is currently possible using conventional methodological tools (within minutes rather than hours or days); it has the potential to revolutionize the determination of bacterial antibiograms. We have tested the efficiency of the technique by characterizing the dynamic effects (in minutes) of ampicillin on susceptible and resistant bacteria.

We have not yet pin-pointed the exact origin of the measured deflections. However, a comparison of the findings relating to motile (*E. coli*) and non-motile (*S. aureus*) bacteria indicate that internal movements are the most probable source of the oscillations.

With this tool, we have now at our disposal a means of rapidly screening of bacterial responses to antibiotics and of establishing antibiograms within a matter of just a few minutes instead of days or weeks, depending upon the replication speed of the microorganism in question2.

Parallelization and miniaturization of the device will further enhance its detection speed and ensure its widespread adoption in research departments, hospitals and medical offices. Finally, the potential of the technique is not limited to the field of microbiology; novel experiments are presently underway to study chemical reactions in proteins, to detect the properties of mammalian cells and their susceptibility to drugs.

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