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Rapid diagnostic susceptibility testing of bacteria and fungi from clinical samples using silicon gratings

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ABSTRACT

Bacterial and fungal infections persistently plague society and have amounted to one of the most prevalent issues in healthcare today. Thus, significant research effort is directed towards developing rapid diagnostic techniques for determination of the correct antibiotic (or antifungal) for a patient-tailored therapy. We have developed a rapid phenotypic antimicrobial susceptibility testing (AST) in which photonic 2D silicon microarrays are employed as both the optical transducer element and as a preferable solid–liquid interface for bacterial/fungal colonization. We harness the intrinsic ability of the micro-architectures to relay optical phase-shift reflectometric interference spectroscopic measurements (termed PRISM) and incorporate it into a platform for culture-free, label-free tracking of bacterial/fungal colonization, proliferation, and death. For example, bacteria proliferation within the microtopologies results in an increase in refractive index of the medium, yielding an increase in optical path difference, while cell death or bacteriostatic activity results in decreasing or unchanged values. The optical responses of bacteria, including clinical isolates and samples derived from patients at neighboring hospitals, to various concentrations of relevant antibiotics are tracked in real time, allowing for accurate determination of the minimum inhibitory concentration (MIC) values within 2-3 hours in comparison to assay times of >8 hours (using standard broth microdilution techniques or state-of-the-art clinical automated systems). This has opened the door to the observation of unique bacterial behaviors, as we can evaluate bacterial adhesion, growth, and antibiotic resistance on different micro-architectures, different surface chemistries, and even different strains. Motility, charge, and biofilm abilities have been explored for their effect of bacterial adhesion to the microstructures as we further develop our method of rapid, label-free AST for full clinical application.

Keywords: antimicrobial resistance, antibiotic, susceptibility testing, bacteria, fungi, antifungal resistance.

1. INTRODUCTION

1.1 Antimicrobial resistance

Antibiotics and antifungals have revolutionized human and veterinary medicine, as well as the agriculture industry. However, the abuse and misuse of these agents has led to a global crisis of antimicrobial resistance, which is predicted to be the number one cause of death by 2050, causing 10 million deaths, unless a global response is mounted [1]. Two main approaches have been taken to tackle this impending crisis, including the development of new antimicrobial agents and the implementation of better antimicrobial stewardship, in which physicians and the general public have been advised to decrease their antibiotic intake. The latter approach has stimulated innovative research for developing a test to predict antimicrobial resistance in isolated organisms, and in particular, the development of rapid antimicrobial susceptibility testing (AST).

1.2 Antimicrobial/antifungal resistance and their susceptibility testing

To determine the appropriate choice of antibiotic or antifungal agent to administer to a patient, clinical microbiology laboratories use various methods of AST [2] and antifungal susceptibility testing (AFST) [3]. The minimum inhibitory concentration (MIC) of an antimicrobial or antifungal agent against a pathogen is determined using these tests.

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For bacteria and fungi, the MIC value is determined as the point in which the antibiotic has effectively inhibited the growth of the bacteria. Clinical breakpoints, determined by AST, are used by clinical labs to advise a patient on a proper therapy. Within these breakpoints, a concentration of antibiotic can be considered “susceptible” with a high likelihood of a positive therapeutic outcome, “intermediate” with an uncertain therapeutic effect, or “resistant” with a high likelihood of therapeutic failure.

Several methods are considered as gold standards for comparison purposes when developing a new AST method, including agar dilution testing, broth microdilution (BMD) testing, disk diffusion testing, and the bioMérieux Etest. While these tests are relatively inexpensive to perform, they must be performed manually and take approximately 18 ± 2 hours to perform (corresponding to overnight) [2]. These methods are not considered as rapid AST methods, but do provide reliable values for MIC determination. Currently, in the United States, the Food and Drug Administration (FDA) has cleared five automated systems for clinical use, including the VITEK[®] 2 (bioMérieux), Phoenix[™] (Becton Dickinson Diagnostic Systems), Microscan WalkAway[®] (Siemens Medical Solutions Diagnostics), Sensititre ARIS 2X (Trek Diagnostic Systems) [4, 5], and most recently, in 2017, the PhenoTest BC Kit (Accelerate Diagnostics, Inc) [6]. These instruments can analyze dozens of bacteria samples in one run and some can even identify the type of bacteria in the sample. However, these automated systems generally take greater than 8 hours to determine the MIC values of each antibiotic, which often leads to clinicians running the samples overnight [4, 7]. The growth of bacteria is generally tracked by changes in turbidity, colorimetric changes, and fluorescence in these instruments. An abundance of research has been invested in improving AST methods in order to develop quicker, cheaper, more reliable platforms than the standard commercial methods and automated instruments. These methods can be tedious, while others are expensive and incompatible with multiplexed systems to analyze multiple samples in one experiment. Again, some of these methods do not directly measure the volume or size of bacterial growth, but measure secondary markers induced by metabolic processes, such as fluorescence [8-10] or pH [11]. Although currently established automated, high-throughput systems provide significant advantages, their relatively long time-to-readout is often insufficient for proper management of critically ill hospitalized patients. When the readout time is more than a day, a patient will often be treated with a broad-spectrum antibiotic, which may be ineffective, leading to poor outcomes, including greater morbidity and mortality.

1.3 The concept of phase-shift reflectometric interference spectroscopic measurements (PRISM)

With the explosion of interest in nano- and micro- fabrication research, many have harnessed the electro-optical properties of nanomaterials as platforms for AST. These sensing platforms also allow for control over the material, which may play a role in the growth and behavior of bacterial populations over time [2]. While cell-based biosensing techniques employing diffraction gratings [12, 13] and reflectometric interference spectroscopy [14-16] on silicon transducers are widely known, they typically operate in sub-wavelength regimes and/or rely on the effective medium model in which the spacing between topological features, d , is much smaller than the wavelength of the incident light, λ . Thus, bacteria (and more so fungi) monitoring with these methods become difficult as the microorganisms, cannot fit within the interstitial space of the gratings. Our group employs two-dimensional phase gratings, based on microstructured silicon, with topological features characterized by $d \gg \lambda$ to ensure that the studied microorganism can comfortably fit and preferentially colonize within the transducing element [17-19]. Using periodic silicon features larger than $1 \mu\text{m}$ allows for bacteria to be effectively trapped in the matrix and proliferate, while maintaining the ability to perform real-time phase-shift reflectometric interference spectroscopy measurements (PRISM). Because bacteria become trapped within the micro-topologies, it is possible to monitor bacterial growth rates by PRISM, making it a suitable candidate platform for AST. In the case of microstructures, such as wells or pillars with the incident light normal to the surface, the silicon topologies acts as a photonic 2D phase (lamellar) grating. By applying a fast Fourier transform analysis to the reflectance intensity as a function of wavelength, one can derive the optical path distance for microstructures. This method allows for sensing the change in refractive index of the medium over time, which can correspond to the microorganism presence, in the micron-sized pores or pillars.

2. MATERIALS AND METHODS

2.1 Fabrication of PRISM chips

PRISM chips containing either lamellar arrays of micro- pillars or pores were fabricated used standard lithography and reactive ion etching techniques at the Micro- Nano- Fabrication and Printing Unit, Technion – Israel Institute of Technology. Developed whole wafers were coated with photoresist to protect the microstructures and diced into $4 \text{ mm} \times 4 \text{ mm}$ chips by an automated dicing saw (DAD3350; Disco, Japan). The silicon chips were washed with acetone to remove

the photoresist and oxidized for 1 h in ambient air at 800°C in a furnace (Lindberg/Blue M 1200 °C Split-Hinge, Thermo Scientific, USA). Amine-modified chips were generated by incubation in 2% v/v (3-Aminopropyl)triethoxysilane (APTES) in 50% methanol for 1 h. Lectin-modified chips (termed “WGA” for wheat germ agglutinin) were prepared as described previously [17,19]. Briefly, oxidized chips were silanized in 2% v/v N’-(3-trimethoxysilylpropyl)diethylenetriamine (50% ethanol, 0.6% of acetic acid) for 1 h, followed by subsequently incubation in a solution of succinic anhydride (10 mg mL⁻¹ in acetonitrile, 4% v/v diisopropylethylamine) for 3 h. Amine activation was promoted by incubation in NHS (10 mg mL⁻¹) and EDC (20 mg mL⁻¹) dissolved in MES buffer (supplemented with 10% DMSO) for 1 h. In the final step, WGA was conjugated to the surfaces by storing each chip with 30 µL of WGA solution (1 mg mL⁻¹ WGA in 10% DMSO) for up to 1 week at 4°C until used. Prior to use, substrates were rinsed with PBS.

2.2 Bacterial and fungal cultures

Bacteria: Escherichia coli (E. coli) K-12 was generously donated by Prof. Sima Yaron (Technion – Israel Institute of Technology). All cultures were maintained on Mueller-Hinton agar. Each bacterial strain was cultured individually in Mueller-Hinton broth (MHB) overnight at 37°C, followed by sub-culturing in a 1:100 dilution until an optical density value of 0.5 was attained.

Fungi: Aspergillus niger (A. niger) was isolated from contaminated onion into 1% potato dextrose agar (PDA) and incubated in dark for 10 days at 25°C. Conidia from the mature culture were re-cultured by streaking onto Sabouraud dextrose agar (SDA) plates supplemented with chloramphenicol at 30°C and for 2 – 5 days (until sufficient sporulation was reached). Subsequently, the conidia were gently removed from the agar plate and resuspended in sterile double distilled water. Conidial density was quantified by a hemocytometer (Neubauer improved cell counting chamber), and the respective conidial densities for PRISM and BMD were obtained by dilutions in 2% Roswell Park Memorial Institute medium (RPMI 1640).

2.3 Antibiotic and antifungal susceptibility testing

AST was performed in a heated manifold housing injection channels and controlled by a motorized stage. All AST assays are performed in two stages after obtaining a baseline signal: First, a bacterial suspension is injected into each channel and given 15 minutes to settle within the microstructures. Second, antibiotic solution made in nutrient broth is injected into each channel. Reflectance signal is continuously monitored throughout the experiments. In all assays, a bifurcated fiber optic fitted with a collimating lens is positioned normal to the photonic chips, both illuminating the chip via a broadband light source and transmitting reflected light to a CCD spectrometer for further frequency analysis. Using frequency analysis of the resulting reflectance spectra, the optical path difference between the two parts of the incident light beams was calculated, as $2nL$. The results are presented in terms of $\Delta 2nL$ (%), or the percent change of $2nL$ over time: was calculated during bacterial adhesion stages as:

$$\Delta 2nL(\%) = \frac{2nL - 2nL_0}{2nL_0} \times 100\% \quad (1)$$

in which $2nL_0$ is the value of $2nL$ at the time when bacteria suspended in MHB was first introduced into the flow channel ($t = 0$ of the accumulation time).

AFST is carried out in a similar manner to the AST assays and using the same optical setup. First, a conidia suspension is injected into each channel and given 15 minutes to settle within the microstructures. Second, antifungal solution (e.g., voriconazole and amphotericin B) is injected into each channel. Reflectance signal is continuously monitored throughout the experiments.

2.4 Scanning electron microscopy

Prior to imaging, all PRISM chips were fixed and stored at 4°C in 2.5% glutaraldehyde. For scanning electron microscopy (SEM), samples were washed in subsequent 50-70-100% ethanolic solutions, each for 5 minutes and then dried under a stream of nitrogen. Samples were analyzed at 1 keV using a high-resolution SEM (Ultra Zeiss Plus).

3. RESULTS AND DISCUSSION

3.1 Antibiotic susceptibility testing using PRISM

We developed a platform for label-free AST using PRISM chips [17]. Figure 1 depicts representative optical responses collected from a micro-pillar type photonic chip. The chip is inoculated with *E. coli* K-12 and is then exposed to a high concentration ($1 \mu\text{g mL}^{-1}$) of the cephalosporin-type antibiotic, ceftriaxone (CRO). As a parallel control, a similar chip is exposed to only nutrient medium without antibiotics. In the latter case, colonization and proliferation of bacteria within the grating leads to a change in the average refractive index of the filling medium correlating to measurable changes in the $2nL$ value, represented as an increase in the $\Delta 2nL$ (%). The corresponding SEM image of the chip confirms colonization and proliferation of the rod-shaped bacteria within the interstitial space of the Si pillars. In contrast, when bacteria are exposed to antibiotics, cell death or bacteriostatic activity within the gratings result in decreasing or unchanged $2nL$ values, all of which is captured in real time. Indeed, the corresponding micrograph of the chip depicts distorted cells and their debris. With this platform, MIC values for the model pathogen *E. coli* K-12 were determined within 2-3 hours when starting from a bacterial concentration of 10^7 cells mL^{-1} . The MIC values determined by PRISM were comparable to those obtained by BMD testing and the automated VITEK 2 system [17].

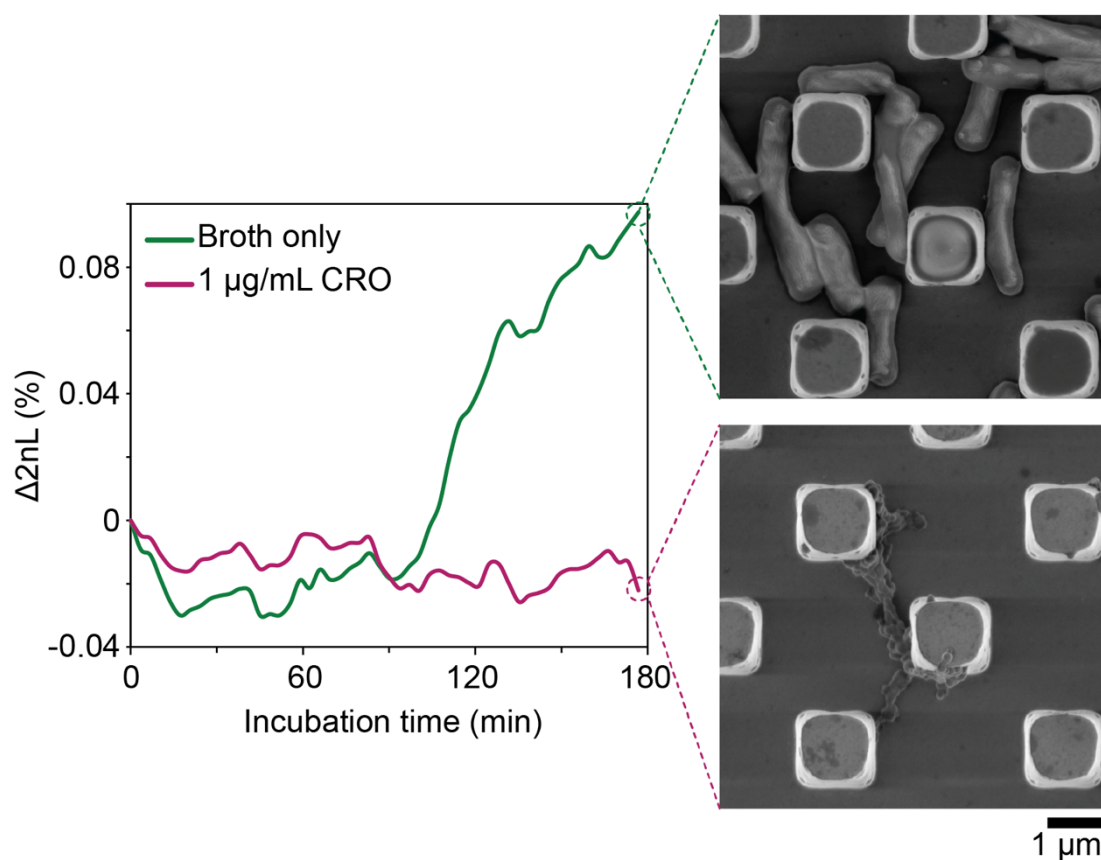


Figure 1. Real-time PRISM curves of pillar gratings, where $\Delta 2nL$ values were recorded every 3 min over a 3 h time period without the use of fluorescent labels. The PRISM curve shows the bactericidal effect of the antibiotic ceftriaxone at a concentration of $1 \mu\text{g mL}^{-1}$ on *E. coli* bacteria. Corresponding scanning electron micrographs of bacteria within the micro-topologies with and without the antibiotic CRO were sampled 180 minutes of *E. coli* cells colonizing within PRISM chip.

The system was further advanced and adapted to handle fungal suspensions and to perform AFST assays. In this case, microporous silicon gratings provide a solid-liquid interface for the capture the micron-sized *Aspergillus* conidia within

their porous structure, as depicted in Figure 2. We demonstrate that we can optically monitor fungal growth of *A. niger* in the presence of various concentrations of the clinically relevant antifungal agents and determine of the MIC values. We further confirm that the PRISM MIC values are comparable to the outcome of standard broth microdilution.

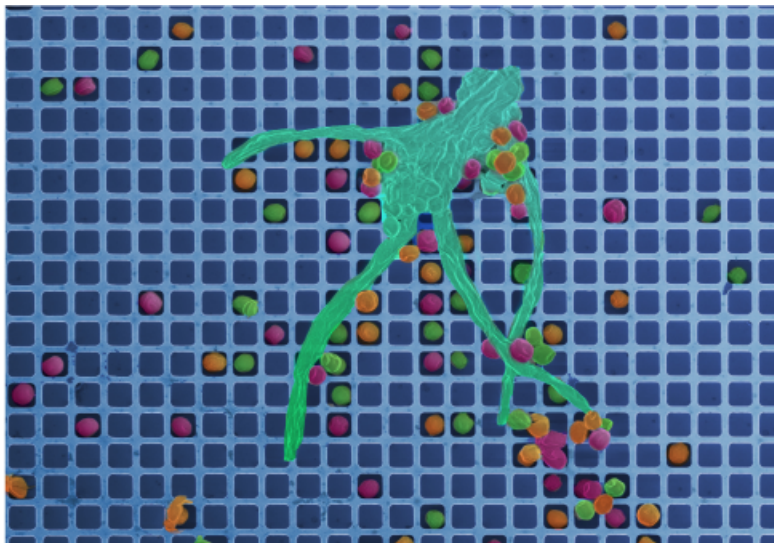


Figure 2. A false-colored scanning electron micrograph of *A. niger* colonizing within PRISM chips with a microporous structure.

4. OUTLOOK

The optical responses to various concentrations of clinically relevant antibiotics have been optically tracked by PRISM, allowing for the MIC values to be determined and compared to both standard BMD testing and clinic-based automated AST system readouts. This method has a total assay time of 2-3 h versus >8 h with automated AST systems. Furthermore, we have extended this work to perform AFST for a model fungal species, *A. niger*. The PRISM AFST assay allows for rapid detection of fungal growth and provides a rapid and label-free alternative to standard antifungal susceptibility testing methods such as broth microdilution and agar diffusion methods.

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