

Direct-from-Sputum Rapid Phenotypic Drug Susceptibility Test for
Mycobacteria

A Thesis
presented to
the Faculty of the Graduate School
at the University of Missouri-Columbia

In Partial Fulfillment
of the Requirements for the Degree
Master of Science

by
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DECEMBER 2018

The undersigned, appointed by the dean of the Graduate School, have examined the Thesis entitled

**Direct-from-Sputum Rapid Phenotypic Drug Susceptibility Test for
Mycobacteria**

Presented by Timothy Butler

A candidate for the degree of Masters of Science in Biological Engineering and hereby certify that, in their opinion, it is worthy of acceptance.

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Acknowledgements

Obtaining my masters degree in biological engineering proved to be without difficulties, and would not be complete without mentioning the following people. I would first like to thank my parents, Jim and Cindy Butler, for their continuous encouragement to better myself academically. I wouldn't be the person I am today without my dad encouraging me to think critically while remaining humble and without my mom always pushing me to do more and that no situation is too tough to overcome.

Next, I would like to give my gratitude to Dr. Shramik Sengupta. It has truly been a pleasure volunteering, and working in your research lab. Comparing from when I started in 2014, to now having a master's degree, the difference is stark, but not without recognition to the person who was always patient to teach me, to allow me to make mistakes and grow from them. Since then, I have come to understand what it takes to run a research lab and believe that the time spent with you will prove to be helpful in my professional career.

A huge shout-out goes to my colleagues. I would not be this far into my thesis without my lab-mates grinding through the same work. It has been a pleasure working toward a common goal in Dr. Sengupta's lab and I will miss the guidance and assistance that was offered throughout my undergraduate and graduate degree in both research and life.

Finally, my girlfriend, Taylor Thompson. Thank you for your unwavering support throughout this time. I know at times this degree has tried us both, but I wouldn't

have wanted anyone else by side on those troubling days. I appreciate your consistent upbeat attitude and the amount of love you give.

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1. Abstract

Background

The spread of multi-drug resistant tuberculosis (MDR-TB) is a leading global public-health challenge. Because not all biological-mechanisms of resistance are known, culture-based (phenotypic) drug-susceptibility testing (DST) provides important information that influences clinical decision-making. Current phenotypic tests typically require pre-culture to ensure bacterial loads are at a testable level (taking 2-4 weeks) followed by 10-14 days to confirm growth, or lack thereof.

Methods and Findings:

We present a 2-step method to obtain DST-results within 3 days of sample collection. The first involves selectively concentrating live mycobacterial cells present in relatively large volumes of sputum (~2-10ml) using commercially-available magnetic-nanoparticles (MNPs) into smaller volumes, thereby bypassing the need for pre-culture. The second involves using microchannel Electrical Impedance Spectroscopy (m-EIS) to monitor multiple aliquots of small volumes (~10 μ L) of suspension containing mycobacteria-cells, MNPs and candidate-drugs to determine whether cells grow, die, or remain static under the conditions tested. m-EIS yields an estimate for the solution “bulk capacitance” (C_b), a parameter that is proportional to the number of live bacteria in suspension. We are thus able to detect cell death (bactericidal action of the drug) in addition to cell-growth.

We demonstrate proof-of-principle using *M. bovis* BCG and *M. smegmatis* suspended in artificial sputum. Loads of ~ 2000 – 10,000 CFU of mycobacteria

were extracted from ~5ml of artificial sputum during the decontamination process with efficiencies of 84% -100%. Subsequently, suspensions containing $\sim 10^5$ CFU/ml of mycobacteria with 10 mg/ml of MNPs were monitored in the presence of bacteriostatic and bactericidal drugs at concentrations below, at, and above known MIC (Minimum Inhibitory Concentration) values. m-EIS data ($\square C_b$) showed data consistent with growth, death or stasis as expected and/or recorded using plate counts. Electrical signals of death were visible as early as 3hours, and growth was seen in < 3days for all samples, thus allowing us to perform DST in < 3days.

Conclusion:

We demonstrated “proof of principle” that (a) live mycobacteria can be isolated from sputum using MNPs with high efficiency (almost all the bacteria that survive decontamination) and (b) that the efficacy of candidate drugs on the mycobacteria thus isolated (in suspensions containing MNPs) could be tested in real-time using m-EIS.

2. Introduction

2.1. Motivation

Tuberculosis (TB) is one of the world's significant public health challenges. In 2017, 1.6 million people died due to TB, and 10 million new infections were recorded (1). Since TB is both fatal and contagious, timely diagnosis and treatment is the key to containing the spread of TB.

One of the major challenges to easy and effective treatment is the emergence of drug resistant strains of *Mycobacterium tuberculosis* (Mtb), the organism causing TB. There is a set of 4 drugs that are considered "first line" in the treatment of TB: Rifampicin (a.k.a. rifampin) (RIF), Pyrazinamide (PZA), Ethambutol (EMB), and Isoniazid (2, 3) [Sometimes, Streptomycin (STR) is also included in this list (3)]. Of these, RIF and INH are the first and second most common drugs typically prescribed, and strains of Mtb showing resistance to them individually are designated RIF-resistant and INH-resistant, respectively. A strain of Mtb is considered to be multi-drug resistant (MDR) if it is resistant to both (3). Also there exist other "second-line" drugs that are able to treat TB, but are not preferred for a variety of reasons ranging from cost, to side effects. These include fluoroquinolones as well as other drugs, some of which need to be injected. A strain of Mtb that is resistant not only to INH and RIF, but also to the fluoroquinolones, and at least one of the 3 most common injectable second-line drugs (i.e., amikacin, kanamycin, or capreomycin) is designated as an Extremely Drug Resistant (XDR) strain.

In 2015, the year's annual report on TB issued by the World Health Organization (WHO) included a supplement devoted to the emergence and spread of drug resistance (4), and in 2017, the WHO issued updates to the previously issued supplement on drug resistance (5). According to these reports, 4.1% of new and 19% of previously treated TB cases were estimated to have RR (Rifampicin Resistant) or MDR TB. Additionally, 6.2% of MDR-TB cases had additional drug resistance and were considered Extensively Drug Resistant TB (XDR-TB). Only 54% of MDR-TB patients who had started treatment in 2014 were successfully treated while 16% of patients died and 8% of patients' treatments failed (21% were lost to follow-up or not evaluated). This translates to about 600,000 new cases of MDR/RR-TB cases emerging in 2016 with 240,000 cases of death due to MDR/RR-TB.

The primary diagnostic method for detecting TB in low resource settings is the Sputum Smear Microscopy. This method involves exposing the collected sputum to stains that adhere to *M. tuberculosis* (Mtb) cells and looking under a microscope for the stained cells. Although this method is inexpensive, it has a relatively high limit of detection, around 10^5 CFU/mL of Mtb (6), and thus has a high rate of false negatives. This is especially true for patients who are asymptomatic or mildly symptomatic whose Mtb loads may be as low as 1000 CFU in 2-5mL of sputum (2). Another limitation of this method is that it cannot be used for Drug Susceptibility Testing (DST). In order to both detect Mtb cells and assay for drug resistance, two broad classes of methods are available: genotypic (molecular) and phenotypic (culture based).

2.2. Genotypic (Molecular) Systems

Genotypic systems detect the species/groups of microorganisms in a biological sample by detecting the presence of DNA with a characteristic sequence. Drug resistance is detected on the basis of finding DNA encoding genes/mutations known previously which are responsible for resistance to one or more drugs. Examples of this system include the Xpert MTB/RIF™ from Cepheid and Line Probe Assays (LPAs) from companies like Hain Biosciences. The Xpert™ yields information regarding the presence of Mtb DNA and that of a gene encoding resistance to RIF. LPAs are available from Hain Bioscience in Germany (7, 8) and detect mutations in the *rpoB* gene, which is associated with RIF resistance, as well as mutations in the *katG* gene and the *inhA* promoter region, which are associated with INH resistance. Another assay, called MTBDRsl™, which is also from Hain, identifies certain genetic mutations that make Mtb resistant to fluoroquinolones.

These systems have a number of advantages that propelled them to being recently adopted in a number of settings, including low-resource environments. Their most important trait is that they are rapid. For instance, the Xpert™ is able to provide an answer in 2-6 hours (9), and the LPAs from Hain have a turnaround time of 24-48 hours (8). Another important trait of these systems are that they are automated and hence have low user error and, if needed, can handle large numbers of samples.

These systems have two major drawbacks. The first is the relatively high cost, which requires significant subsidies to reach users in low-resource

environments. While in the US and Western Europe, the Xpert MTB/RIF instrument retails for around \$60,000 and individual test cartridges retail for around \$65 (10). The Foundation for Innovative Diagnostics (FIND) coordinates volume discounts from the manufacturer and subsidies from the UN (WHO), governments and various private philanthropies, such as the Gates Foundation, make the instruments available for around \$17,000 and cartridges for around \$10 each to select users in low-resource environments (11). Similarly, LPA strips are made available at a price of approximately \$10. However, as the WHO notes (7, 8) performing the LPA test requires other laboratory consumables and supplies which may push the cost to between \$20 and \$30 a test, even after subsidy.

The other main drawback is that the genetic tests provide only a limited picture of the organism's drug susceptibility profile, especially from the point of effective treatment. For instance, the Xpert detects resistance to rifampicin only, which the genetic basis is well-understood, and the LPAs detect some, but not all, cases of isoniazid resistance since there are multiple resistance pathways for resistance to INH (12). Complicating the picture further, a mutation to the *katG* gene (S315T) that is detected by the LPAs, which is known to result in elevated values of Minimum Inhibitory Concentrations (MIC) to INH, but the degree to which the MIC is elevated varies significantly among isolates (7). This implies that certain isolates harboring the mutation can still be treated with INH, but others cannot. Moreover, the absence of known mutations does not imply that the drug of choice will be effective. The CDC states in its guidelines to clinicians (13).

“Further, not all biological mechanisms of resistance are known. As a result, if no mutations are detected by the molecular assay, resistance cannot be ruled out. **Therefore, it is essential that conventional growth-based drug-susceptibility tests are done and used in conjunction with molecular results.**”

2.3. Phenotypic systems

Phenotypic systems are the systems in which Mtb is detected and/or characterized with respect to its drug susceptibility based on direct or indirect observations of cellular growth and/or bacterial metabolism in a chosen media. The most common matrix tested for the presence of Mtb is sputum. Since there are large numbers of microorganisms of various types present in the sputum of all patients, a decontamination protocol is typically performed prior to any phenotypic test. The protocol involves the use of a decongestant (such as NALC – N-acetyl-L-cysteine) to make the sputum less viscous and a killing agent (such as sodium hydroxide) that kills all microorganisms except the extremely hardy mycobacteria like Mtb. Once decontaminated, the sample (decontaminated sputum) is added to a growth media, and inserted into an Automated Culture Based Diagnostic System like the MGIT (Mycobacteria Growth Indicator Tube) from Becton Dickinson (BD) or the Trek ESP system from Thermo-Fischer.

Due to low operational costs and rugged performance, Automated Culture-based Diagnostic Systems remain the workhorses for multiple applications, including the detection of TB (MGIT and Trek ESP), and

subsequent phenotypic DST. These automated culture systems rely on detecting living bacteria by looking for changes brought about by bacterial metabolism to the properties of suspension, such as O₂/CO₂ levels, pH, electrical conductivity, temperature, etc. Their major drawback is that, because the amounts of metabolite released by the bacteria are low, they have a high “threshold” of detection. In other words, these systems require that the concentrations of the bacteria in the suspension need to rise to around 10⁸ CFU/mL before a positive culture can be identified (14). This can be time consuming since bacteria such as Mtb has a long doubling time and may be present in the sputum at in low loads, around 1000 CFU in 2-5mL (2). Thus, it can sometimes take many days (weeks) before the culture is at high enough levels for detection.

Drug Resistance Testing is typically carried out after the Mtb has been detected in the Automated Blood Culture System. Aliquots from the positive MGIT tube is transferred to other tubes, where the media has been supplemented with antibiotics, and observed over a period of 1-2 weeks to detect growth (or lack thereof) in the presence of antibiotics. This, coupled with the fact that the DST proper starts many days after the sputum sample is collected, result in the time to results for the phenotypic DST being ~ 1 month, or longer.

To overcome the need for “pre-culture” prior to DST, a method called Microscopic Observation of Drug Susceptibility (MODS) has been developed (15). The test uses 24- well plates with four wells for a single patient specimen: two wells are drug free, while the other two wells contain Rifampicin (16) and

Isoniazid (2). After inoculation, the plates are sealed in zip-lock bags and then incubated. If the pathogen in sputum is present, their growth in the liquid medium is observed under an inverted light microscope and morphologically examined for patterns specific to Mtb. This typically takes 7-14 days to observe growth in the wells without drugs and to confirm lack of growth in those with drugs (17) and the cost per test was estimated to be about \$5 in low-resource environment of Peru (18). Microscopic Observation Drug Susceptibility assay (MODS) has hence been recommended by the WHO as an affordable method for relatively rapid detection and DST.

Despite its advantages, MODS is not as widely used in resource-limited settings as was initially anticipated due to concerns for biosafety, reliance on the skill of laboratory personnel in interpreting results, and efficiency for handling large number of samples. While concerns about biosafety can be alleviated using commercially produced kits with sealed wells, such as those produced by hardy Diagnostics (18), and a certain degree of automation can be introduced in the capture of images (19), this method still requires that every well be visually inspected by a well-trained laboratory professional. This puts a requirement of both time and human resources that often make it unfeasible to handle a large number of sputum samples that may need to be tested in high-TB burdened and low-resource settings.

The phenotypic approach presented here seeks to (a) overcome the need for pre-culture by using a magnetic-nanoparticles (MNP) based method to collect virtually all the mycobacteria cells present in a relatively large volume of sputum

(2 – 5 ml) into a relatively small volume (~200 μ L) of buffer/growth medium within a short period of time (~20 minutes) (b) overcome biosafety concerns by using microfluidic technology to partition the 200 μ L of media containing mycobacteria cells into sixteen individually isolated micro-reservoir (wells) of 10 μ L volume, each containing different amounts of candidate drugs, and (c) overcome the need for subjective judgment by a trained clinical worker by using an extremely sensitive electrical method (m-EIS) that monitors the suspensions in the wells continuously, and is able to report not only the growth, but also the death of cells in real time.

Because this method pre-concentrates the mycobacteria prior to culture, and is able to monitor cell death in real time due to the action of antibiotics, it potentially enables the phenotypic susceptibility tests to be performed at time-scale comparable to the “molecular” methods, and with the automation and the automation and scalability of the latter, but at a fraction of the cost. Figure 1 shows an outline for the timelines of each method.

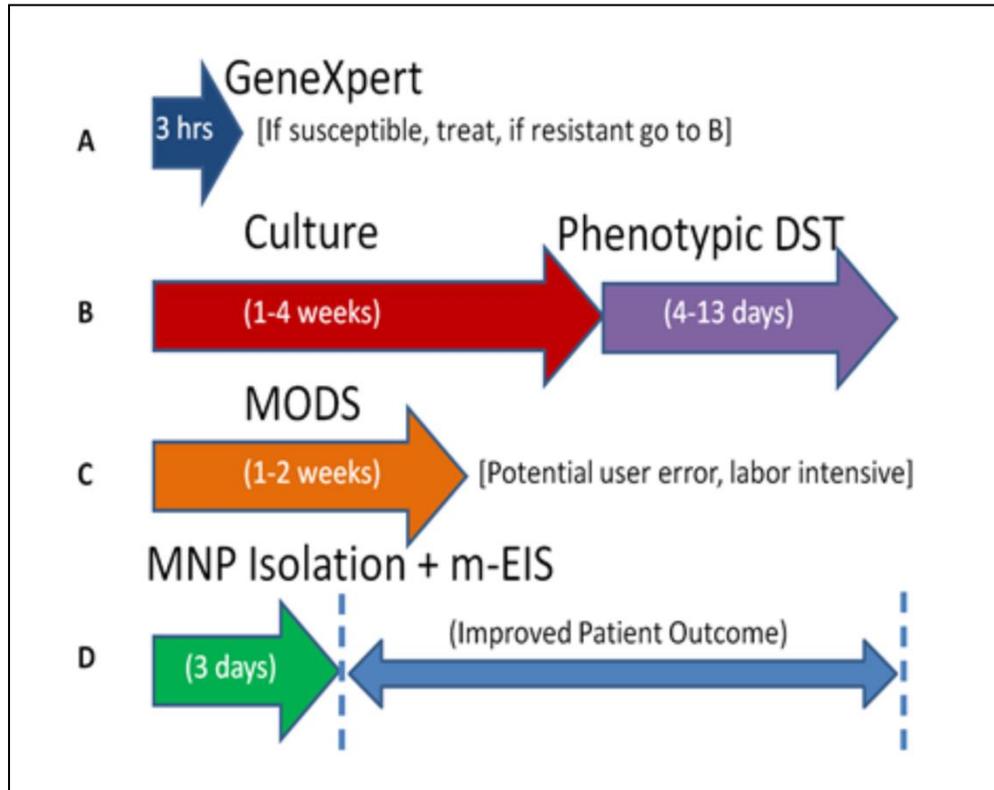


Figure 1. Timeline of available approaches to obtaining drug susceptibility information for TB.

(A) GeneXpert genotypic approach that only identifies Rifampicin susceptible pathogens. If RIF-resistant, the sample is channeled to path B. (B) Traditional culture based phenotypic approach (MGIT etc.) (C) Microscopic Observation of Drug Susceptibility (MODS). (D) Proposed approach (isolation using MNPs + growth/death/ stasis assay using EIS).

2.4. m-EIS

Our lab has previously developed and patented (20, 21) a novel method to monitor the proliferation or death of microorganisms in suspension, which we refer to as microchannel Electrical Impedance Spectroscopy (m-EIS). It relies on the fact that, in the presence of high frequency alternating current (AC) electric field, the membranes of cells become polarized and store charge; thereby acting like electrical capacitors (22). These capacitances at the individual cells contribute to the overall “bulk capacitance” of the suspension, or net charge stored in the interior. The amount of charge stored by a bacterium is about 100X that of an equal volume of aqueous solution (23). Therefore, even at low concentrations (volume fractions), bacteria in a suspension contribute significantly to the latter’s bulk capacitance. Also, it should be mentioned that only living cells with intact membranes contribute to bulk capacitance. A cells death is accompanied by loss of membrane potential and electrical polarization (24).

Measuring the bulk capacitance is not straight-forward since the electrical behavior of aqueous solutions containing polarizable species, like cells and proteins, is extremely complex. Such systems can be modeled electrically by the circuit shown in Fig. 3 (a) (25). In this equation, charge stored at the electrode-solution interfaces is accounted for by the two electrode interface capacitances (C_e) and charge stored by elements dispersed in the interior, by “bulk capacitance” (C_b). Measuring changes in C_b is challenging since C_e is roughly 1000 times larger. The key innovation (26) is a method that enables the changes

in C_b to be detected, despite the “screening effect” of charges at the electrode-effective bulk resistance (R_b) of the suspension. This increases the impedance of the bulk ($R_b C_b$) to be comparable to that of the interface at realizable frequencies allowing an appreciable voltage drop over the bulk-suspension. Therefore, the charge-storage in the bacteria contributes significantly to the measured impedance. The long narrow channel causes more of the electrical “lines of force” to interact with the suspended bacteria. The measured impedance (Z) at 500 frequencies (ω) between 1 kHz and 100 MHz is fit to the equation, and C_b is estimated (along with R_b , R_e and C_e).

The application of this technology has been demonstrated for food quality assessment (26) and blood culture (27). For blood culture, the “times to detection” (TTDs) were often days shorter than the current market leader. The threshold concentrations of detection are 10^3 - 10^4 CFU/mL, while compared to current technologies 10^8 CFU/ml (14).

Further, since single cells lose their membrane potential when they die and no charge accumulates at the membrane in absence of a potential, m-EIS is able to observe cell death in real time. This insight has been previously used to determine minimum inhibitory concentrations (MICs) for multiple bacteria-antibiotic pairs (28). Besides being rapid, taking 4 hours for *E. coli*, *S. aureus* and *Pseudomonas* detection, and accurate, correct MIC values were obtained for well-characterized stains; it is able to distinguish between bacteriostatic effects, in which the bacteria can no longer replicate, from bactericidal, which will kill the bacteria cell. Moreover, cell death can be observed using m-EIS for initial loads

at or above the “threshold” concentrations of around 10^3 CFU/mL. This was shown for *E. coli* and *Pseudomonas* (29). Not only does the m-EIS method require a lower threshold concentration, but it also works with small volumes of sample (~10 μ L). It thus requires very small number of cells (< 100 CFUs) to work. Further (as will be demonstrated in this work), it is unaffected by the presence of inert material like MNPs, even if they happen to be charged or polarizable. The presence of inert species merely contributes to the background, and since m-EIS looks for a *change in* bulk capacitance from the baseline (time $t=0$) value brought about either by cell proliferation or cell death to determine the effect of the candidate drug on the mycobacterial cells, they do not affect the interpretation of the data as long as their number remains constant. Due to a combination of needing low cell numbers to work, and an ability to work in the presence of MNPs, m-EIS is ideally suited to monitor cell growth/death after the cells present in the entire sputum sample have been concentrated down into a small volume using MNPs.

3. Materials and Methods

3.1. Rationale and overview

The goal of developing this method is to obtain rapid and accurate phenotypic DST results. The main barriers to these results are (a) the relatively low load of mycobacteria that can be present in sputum, which leads to the requirement that it be pre-cultured and (b) the relatively slow metabolic rate (doubling time) of the mycobacteria, which makes it time consuming to distinguish a suspension

containing cells that grow despite the presence of drugs, from those in which cells cease to grow, or die. We overcome the former by isolating and concentration cells into a small volume using MNPs, and the latter by using a sensitive electrical method that is able to monitor cell growth/death/stasis in small volumes in real time.

Since *M. tuberculosis* is a biosafety Level 3 (BSL-3) organism, and our lab is only certified for BSL-2 ones, we chose to use BSL-1 and BSL-2 analogs. *M. smegmatis* is a BSL-1 organism, whose membrane is very similar to that of *M. tuberculosis* (30, 31), and *M. bovis BCG* is a BSL-2 organisms that has a doubling time of ~20 hours (32), which is comparable to the ~24 hour doubling time of *M. tuberculosis* (30, 31). To simulate a patient sample from clinical labs, artificial sputum was created in lieu of actual patient sputum. Since naturally-occurring sputum also contains non-mycobacterial microorganisms (gram positive and gram negative bacteria), we also added to our sputum *S. aureus* and *P. aeruginosa* to represent the effects of the presence of other commensal/pathogenic bacteria in the sputum.

An overview of the experimental protocol followed is depicted in Figure 2. Once synthetic “infected” sputum is created with gram-positive, gram negative, and mycobacteria, it is subjected to a decongestion and decontamination protocol similar to one that a patient sample would undergo. After decongestion and decontamination, MNPs are used to collect mycobacteria. The efficiency of the decontamination process, and the collection efficiency for mycobacteria surviving decontamination are evaluated using plate counts. Mycobacterial

suspensions containing loads of microorganisms and MNPs similar to those obtained at the end of the isolation process are then exposed to candidate antibiotics at known concentration, and the efficacy of the m-EIS method to observe cell proliferation/death in real time are then evaluated. To establish the fact that the m-EIS measurements can be conducted despite the presence of MNPs, we also conduct similar tests without the presence of MNPs. More detailed information containing the individual steps are provided below (including data collection, analysis and statistical analysis).

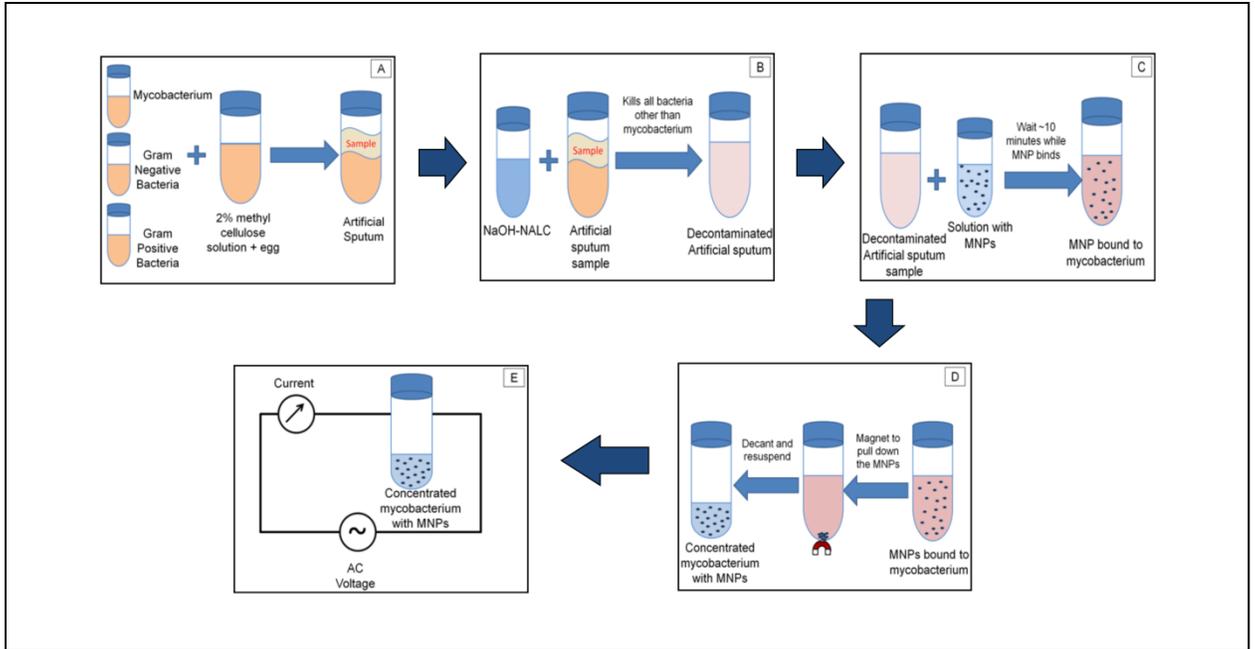


Figure 2. Experimental Protocol

(A) the preparation of artificial sputum with mycobacteria + gram positive and gram negative bacteria (B) the digestion/decontamination (C) Addition of MNP and allowing them to bind to the mycobacteria (D) Isolation of the mycobacterium into a pellet and its resuspension (E) Periodic electrical assay using m-EIS.

3.2. Bacterial Preparation

All cultures used during the experiment were purchased through ATCC and subcultured in the lab. For the in-vitro experiments, two different types of mycobacteria were chosen as surrogates in lieu of *M. tuberculosis*, viz.

Mycobacterium smegmatis (ATCC 700084) and *Mycobacterium bovis BCG*

(ATCC 3574). To simulate the bacteria found naturally in sputum from patient

samples, *Staphylococcus aureus* (ATCC 29213) and *Pseudomonas aeruginosa*

(ATCC 27853) were selected as representative gram positive and gram negative

bacteria, respectively. *M. smegmatis* was cultured in Middlebrook 7H9 (Fluka

Analytical, St. Louis Missouri) and *M. bovis BCG* was cultured in Middlebrook

Albumin Dextrose Catalase (ADC) supplements (HIMEDIA, Mumbai, India). Both

S. aureus and *P. aeruginosa* were cultured in tryptic soy broth (TSB) media

(Sigma Aldrich, St. Louis, Missouri). All cultures were incubated at 37°C. To

obtain the proper concentrations for the experiments, A UV-VIS

spectrophotometer (Azzota S M 1000) was used to find the optical density (OD)

of the samples at 600nm or 570nm. From literature, it is known that OD₆₀₀ values

of 0.1 and 0.05 correspond to concentrations of 1×10^7 CFU/ml for *M. smegmatis*

and *M. bovis BCG*, respectively (33, 34). Similarly, the OD₅₇₀ value for 1×10^7

CFU/ml of *S. aureus* is reported to be 0.15 (35), and that of $\sim 1 \times 10^8$ *P.*

aeruginosa is reported to be 0.1 (36) . All bacteria were grown to the above

mentioned target concentrations, and then serially-diluted to obtain

concentrations desirable for introduction into the artificial sputum matrix.

3.3. Artificial Sputum Preparations

In order to accurately represent the patient sample, we created artificial sputum for the extraction experiments. The artificial sputum was prepared according to protocols available in literature (37-39). This sample was a 1% (w/v) aqueous methylcellulose solution which had a starting volume of 1L. This sample was then sterilized using an autoclave and 1 emulsified egg was added to the mixture to be used as artificial sputum for our experiments. To properly simulate bacterial collection from sputum, to bacterial concentrations of *S. aureus* and *P. aeruginosa* was 10,000 CFU/ml. The *M. smegmatis* and *M. bovis BCG* concentrations were 10,000 CFU/mL. For the experiment, 5mL of sputum and bacteria was used to simulate the sample. Further representation of this step can be seen in Figure 2A.

3.4. Decontamination and Digestion

This process is conducted to ensure that (a) all non-mycobacterial species are eradicated from the collected specimen, and (b) living mycobacteria are released from the matrix (aqueous buffer) and can grow freely in suspension. The most commonly used digestion/decontamination reagent consists of a mixture of N-acetyl-L-cysteine (NALC) and Sodium Hydroxide, where NALC acts as the digestion agent and the NaOH acts as the decontaminating agent, For the experiments presented here, the standard NaOH-NALC decontamination process suggested in literature (40) was used with minor modifications, viz. we used a slightly lower concentration of NaOH of 1%, vs. the 2% commonly used to

[2], along with a shorter duration of exposure (10 minutes for *M. smegmatis* and for *M. bovis BCG* vs 15 minutes for *M. tuberculosis*). This was due to the fact that *M. smegmatis* and *M. bovis BCG* are less hardy than *M. tuberculosis*, making them more susceptible to the decontamination process.

The efficacy of the process (both in terms of eliminating non mycobacterial species, and in allowing the mycobacteria to survive) was evaluated by plating aliquots obtained at the beginning and at the end of the decontamination and extraction process. At the beginning of the process, 5ml of the artificial sputum containing *S. aureus*, *P. aeruginosa* and *M. smegmatis/M. bovis BCG* at $\sim 10^4$ CFU/ml was prepared. To verify this, 100 μ L aliquots of the “infected” artificial sputum were plated on Tryptic Soy Agar (TSA) and 7H10 plates (for samples containing *M. smegmatis* and other non-mycobacterial species), or TSA and Lowenstein-Jensen (LJ) plates (for samples containing *M. bovis BCG* and other non-mycobacterial species) after appropriate dilutions. Colony counts from the TSA plates indicate the numbers of non-mycobacterial species present, and those on 7H10/LJ plates indicate the numbers present for mycobacteria.

To the artificially prepared “infected sputum”, an equal volume (5ml) of 2% NaLC-NaOH was added, mixed well and allowed to sit for 10 minutes. At the end of this 10 minute period, the sample was neutralized by adding 450 μ L of ~ 12 M HCl. Once again 100 μ L aliquots from the suspension were plated on TSA, 7H10 and/or LJ agar plates in order to estimate the numbers of mycobacteria and non-mycobacterial species surviving.

3.5. Extraction using MNPs

The MNPs used for the experiments reported here were commercially available RapiPrep-TB™ beads (Microsens Biotechnologies, London UK) that have already been optimized to bind to *M. tuberculosis* cells present in sputum (41-43).

These beads are supplied in a suspension wherein the concentration of beads is 2.5 mg/ml. 4ml of this suspension is added to the ~10ml of neutralized decontaminated sputum, gently mixed by inverting by hand for ~10 times, and allowed to sit for 10 minutes. After this duration, it was mixed again by inversion, and the tube placed against a permanent magnet for 1 minute. This draws virtually all the MNPs to the side of the tube, forming a pellet, which also contains mycobacteria adhered to the MNPs. The supernatant is discarded, and the pellet is re-suspended in 1ml of buffer. 100µL aliquots of this resuspension are plated on TSA/ 7H10/ LJ plates to estimate numbers of live cells collected. It may be noted that if virtually all the MNPs are collected into the pellet, the re-suspension would have an MNP concentration of ~10 mg/ml, which is the concentration used during the m-EIS studies.

The real world clinical patient sputum samples (which contain mycobacteria along with other commensal microorganisms) can be processed similarly for decontamination and extraction using magnetic nanoparticles. An overview of the patient sputum sample processing protocol prior to use in m-EIS for antibiotic susceptibility testing can be seen in Figure 3.

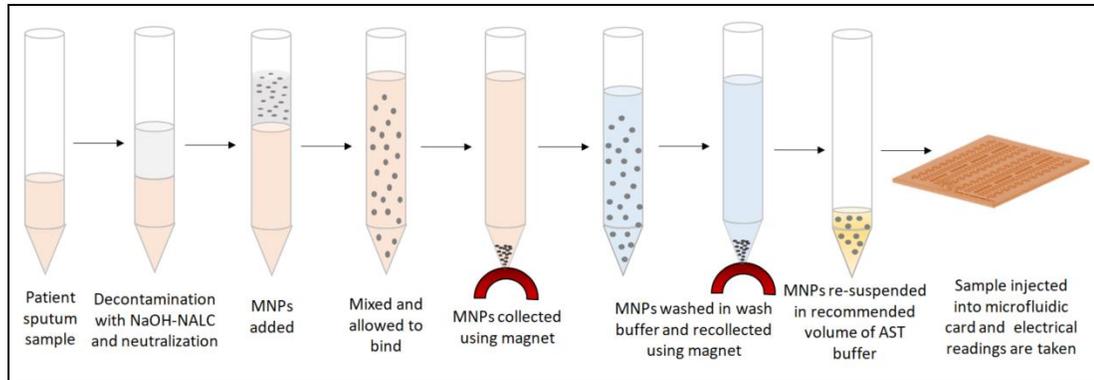


Figure 3. Real World Clinical Patient Sputum Sample Processing Protocol

Schematic of proposed approach involving isolation of mycobacteria using MNPs to bypass the preculture, allowing the assay of growth/death using m-EIS measurements to be completed quickly.

3.6. Choice of Drugs and Concentrations Tested

The drugs tested in this study were a subset of common/first-line drugs typically used against *M. tuberculosis*, and whose effects on *M. smegmatis* and/or *M. bovis BCG* at various concentrations have been studied previously by others. As shown in Table 1, drugs were chosen to represent the various effects that the drugs can have with respect to microbial survival: viz. cidal (where the drug kills the microorganism), static (where the net number of live microorganisms does not change), and ineffective (where the microorganism keeps proliferating despite the presence of drug). For each cidal/static drug, three concentrations were chosen: one significantly below the known Minimum Inhibitory Concentration (MIC) range, one within the known MIC range, and one much above the same.

For *M. smegmatis*, the drugs selected are: amikacin (bactericidal, with a known MIC of 0.5 mg/L (44)), rifampicin (known to be ineffective (45)), and ethambutol (known to be bacteriostatic, with an MIC of 1 mg/L (44)). For *M. bovis BCG*, the drugs selected are: amikacin (bactericidal, with a known MIC of 0.125 mg/L (46)), pyrazinamide (known to be ineffective (47)), and ethambutol (known to be bacteriostatic, with an MIC range of 2mg/L to 4 mg/L)(48)).

Organism	Drug (type)	Concentration Tested (µg/mL)		
		< MIC	MIC	> MIC
<i>Mycobacterium smegmatis</i>	Amikacin (cidal)	0.0625	1	16
	Ethambutol (static)	0.125	4	32
	Rifampicin (ineffective)	[upper limit of range typically tested] 16		
<i>Mycobacterium bovis</i> BCG	Amikacin (cidal)	0.125	1	16
	Ethambutol (static)	0.125	4	32
	Pyrazinamide (ineffective)	[upper limit of range typically tested] 16		

Table 1.

Specific concentration of antibiotic tested for M. smegmatis and M. bovis BCG.

In a real-world situation, we anticipate that the mycobacteria isolated from human sputum using MNPs will be dispersed into growth media, and tested against multiple candidate drugs in parallel. We hence prepared suspensions with mycobacteria and MNPs in growth media where both the initial load of mycobacteria ($\sim 10^5$ CFU/ml) and the concentration of MNPs present (10 mg/ml), were similar to what one would expect to obtain at the end of the decontamination and isolation process described above. Since it was uncertain / un-verified a-priori if (a) the MNPs would affect the metabolism of cells in a manner that alters their response to the drugs, and (b) the MNPs would adversely affect the electrical measurements, parallel experiments, where MNPs were not added, were also conducted to serve as a baseline when trying to evaluate the effect of the MNPs. For any given experiment, ~ 20 ml of suspension (containing mycobacteria, growth media, candidate drugs at selected concentrations, and/or MNPs) was prepared, and incubated at 37C. Periodically, 50 μ L aliquots were taken from these liquid cultures and subjected to electrical readings.

3.7. Electrical Readings

Aliquots ($\sim 50\mu$ L) taken from the liquid culture and introduced into a microfluidic cassette containing channels with a 1mm x 1mm cross section. These cassettes consist of a glass-slide base, and a 3D printed top with engraved channels affixed using an adhesive (Epo-Tek 301TM). The 3D printed part was fabricated at the Mizzou 3D Printing Lab and had electrodes asymmetrically placed 10mm

apart, as shown in Figure 4. It thus allows the user to assay a fluid volume of ~10 μ L (~10mm x 1mm x 1mm).

The electrodes shown were connected to an impedance analyzer (Agilent/Keysight 4294A), and impedance measurements [Resistance (R) and Reactance (X)] were recorded at 128 frequencies (ω) between 1 kHz and 100 MHz. This raw data [R(ω) and X(ω)] was analyzed offline using Z-viewTM software to yield an estimate of the suspension's bulk capacitance (C_b). To study the effect of candidate drugs on the mycobacterial cells, such scans were conducted on the same sample multiple times over the duration of the study. The time interval between the readings was $\frac{1}{2}$ to $\frac{3}{4}$ hour for *M. smegmatis* and 12 hours for *M. bovis BCG* (except for the case of studying the effect of the cidal drug amikacin on *M. bovis BCG*, where readings were taken every hour). At each time-point, 5 independent aliquots were taken from the liquid culture suspension. After loading each aliquot (~50 μ L) into the cassette, electrical measurements were taken. Then the channel was cleared twice using sterile base media (7H9 for *M. smegmatis*, MGIT for *M. bovis BCG*). After the channel was cleared, the channel was loaded again with the next ~50 μ L aliquot until 5 readings were completed.

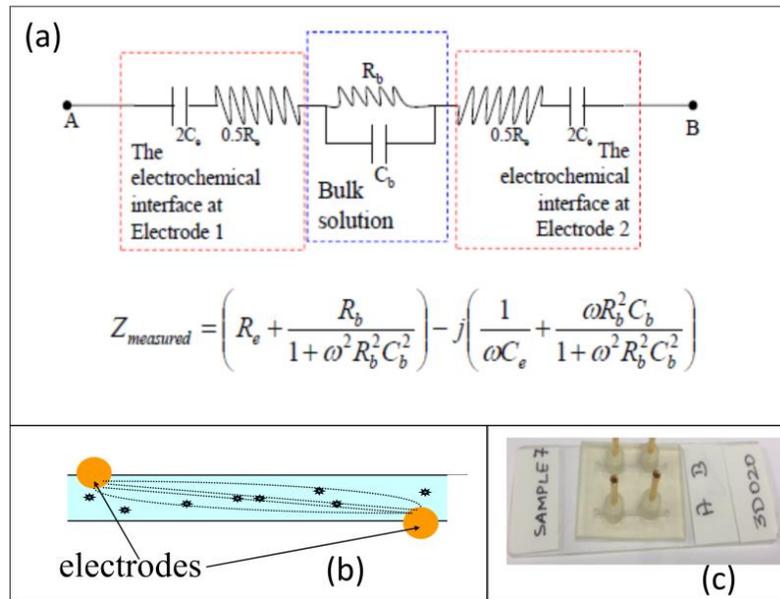


Figure 4. Schematic and Electrical Circuit Model Representation of Microchannel

(a) *Electrical Model of an aqueous suspension in contact with metal electrodes. The equation relates the real (in-phase) and imaginary (out-of-phase) components of the measured impedance (Z) and how they vary as a function of frequency and model parameters (R_e , C_e , R_b and C_b) (b) schematic, and (c) picture of microfluidic cassette.*

While different suspensions had differing initial values of C_b (presumably due to minor differences in initial load of live microorganisms and/or MNPs), we are interested in tracking changes to C_b due to cell growth/death. Hence, all readings were scaled with respect to the average value of C_b at time $t=0$.

3.7.1. Analysis of the Electrical Data

The data collected by the impedance analyzer is in the form of resistance (R) and reactance (X) values of the bacterial suspension at 128 logarithmically equi-spaced frequencies (ω) between 1 KHz and 100 MHz. As described by us previously (49) the R and X vs. ω data is fitted to the equivalent electrical circuit shown in Figure 5 using a commercially available software package (ZView™). The software accepts as an input the Z vs. ω data, and provides an estimate for all the circuit parameters, including the “bulk capacitance” (Our parameter of interest that provides a measure of charges stored in the interior of the suspension (away from the electrodes)). It may be noted that here, (a) the capacitances and resistances at both electrodes are combined into a single capacitance and resistance, respectively, and (b) both the bulk and interfacial capacitances are represented as a constant-phase element (CPE) to account for the non-ideal nature of the capacitance at cell membranes, where charges carried by ions take a finite time to accumulate, unlike an ideal capacitor where the accumulation is instantaneous. The magnitude of the bulk CPE, thus, reflects the amount of charge stored at the membranes of living microorganisms in suspension. An increase in the number of living microorganisms (brought

about by cell proliferation) should be reflected as an increase in this value (magnitude of CPE). On the other hand, a decrease in the number of living microorganisms in the suspension (due to the action of the drug present) should lead to a lower bulk capacitance (CPE_b – T)

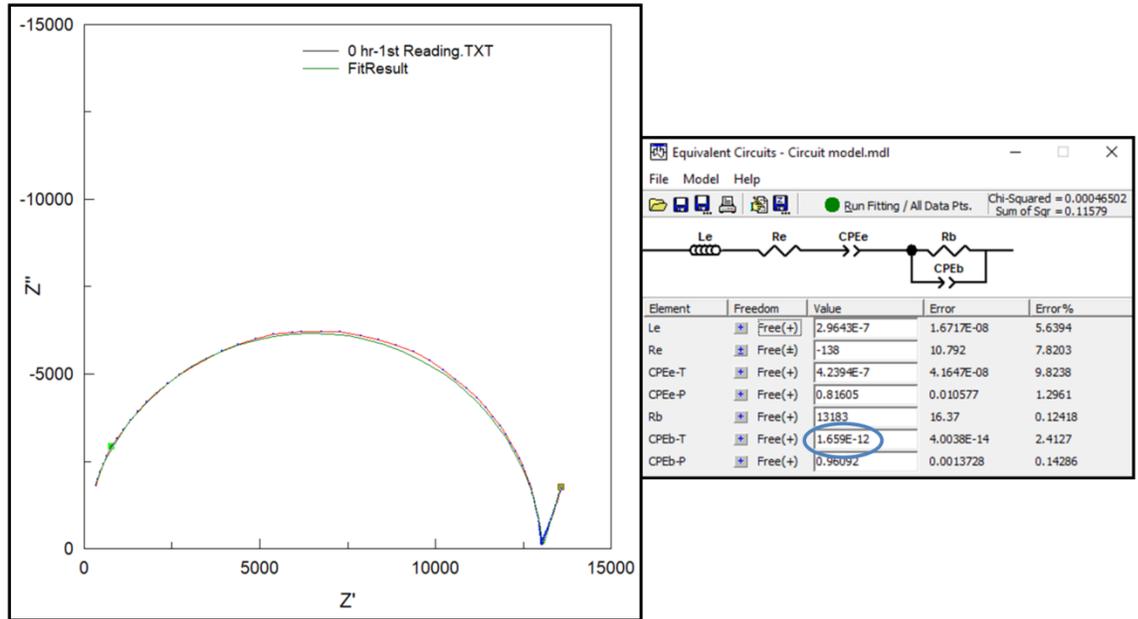


Figure 5. Data analysis using the program ZView®

The left side shows the graphical fit-line to the Resistance (Z') and Reactance (Z'') which is fit to the circuit of choice (top right). The fit results in an estimate of the bulk capacitance (circled, bottom right).

When trying to evaluate the efficacy of a candidate drug (whether the mycobacterium is able to grow in the presence of drug, is inhibited from growing, or is killed), our problem reduces to establishing whether one can ascertain in an unbiased manner if the value of the bulk capacitances increases, decreases or remains unchanged over the observed duration. To do so, we fit a linear equation to the data of (average) C_b vs. time using Microsoft Excel, and examine the slope. Excel also provides a 95% confidence interval for the calculated slope. The upper and lower bounds of this confidence interval are also recorded to interpret whether the C_b is increases, decreases or remains unchanged.

If the confidence interval encompasses zero (i.e. the upper bound of this confidence interval is positive, whereas the lower bound is negative), then one cannot assert with confidence that the C_b is either increasing or decreasing, and the sample is deemed to be static. On the other hand if both upper and lower bounds are positive, then one can assert that the C_b is increasing (and hence the microorganisms are proliferating) and if both the upper and lower bounds are negative, then one can assert that the C_b is decreasing, presumably because the microorganisms are being killed by the drug.

4. Results

4.1. Sample Preparation (Decontamination + Extraction)

As discussed earlier and illustrated in Figure 1, our method requires that most mycobacterium present in a patient sample (sputum) survive the decontamination process and be subsequently collected by the MNPs into a

“pellet” for resuspension into a small volume of DST media. The protocols adopted for decontamination and MNP-based isolation were also described earlier, along with the protocols adopted to evaluate the number of living bacteria of various types present in the sample of interest. Results (n=3 for *M. smegmatis*, n = 2 for *M. bovis BCG*) are presented in Table 2 and Figure 6.

Type	Sample	Initial Load (CFU)	Load Surviving Decontamination (CFU)	Load isolated by MNPs (CFU)
<i>M. smegmatis</i>	Mycobacteria	2,000 ± 0	1,413 ± 730	1,180 ± 326
	Contaminant	1,873 ± 532	11 ± 0	11 ± 2.82
<i>M. bovis BCG</i>	Mycobacteria	18,000 ± 0	10,200 ± 1800	12,500 ± 4500
	Contaminant	7,500 ± 1975	41 ± 26	41 ± 26

Table 2.

Loads of viable mycobacteria and “contaminant” bacteria present at each stage of the isolation process.

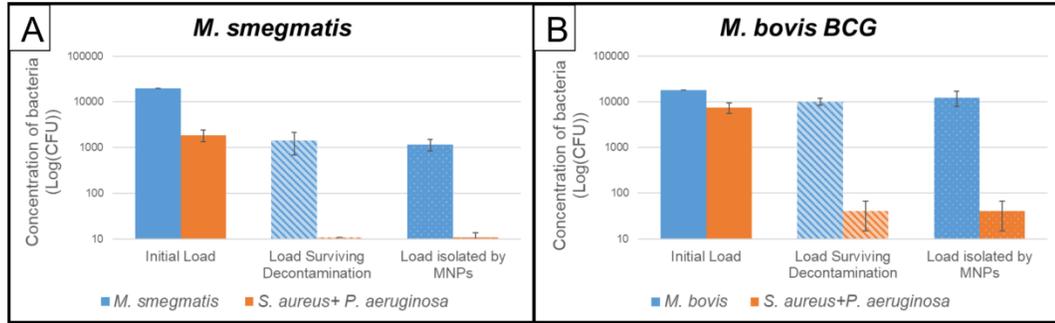


Figure 6. Decontamination and Extraction Results

Data showing decontamination and extraction of viable mycobacteria from synthetic sputum using RapiPREP-TB™ beads for (A) *M. smegmatis* (n=3) and (B) *M. bovis BCG* (n=2) (>95% of cells viable after de-contamination are collected to the pellet).

4.2. m-EIS assay of mycobacterial growth/death

As explained earlier, m-EIS experiments were conducted wherein candidate drugs at different concentrations were allowed to act on samples mimicking those that we expect to obtain after the sample-preparation process (in terms of loads of living mycobacteria and the MNPs present), and the measured bulk capacitance tracked over a period of time (3 to 5 hours for *M. smegmatis* and up to 3 days for *M. bovis BCG*). To verify that the MNPs do not affect either the behavior of the mycobacterial cells (with respect to their susceptibility to the drugs tested), or the m-EIS method to monitor the said growth or death, other experiments are run in parallel where conditions are similar to the first set of experiments, but where MNPs are not present. Results (values of bulk capacitance, scaled to the time $t=0$ “baseline” value) are shown in Figure 7 and Figure 8 for *M. smegmatis*, and *M. bovis BCG* respectively. Table 3 and Table 4 shows the inference one can draw regarding growth/death/stasis of the mycobacteria based on how these measured values of bulk capacitance change over time. The tables show the estimates of the slope of the straight line fitted to the average of the measured bulk capacitance vs. time data with a 95% confidence interval (CI). If upper and the lower bounds of the confidence interval lies entirely in the positive range, mycobacteria in the sample are deemed to be proliferating; if the CI is entirely within the negative range, mycobacteria are deemed to be dying, and if the CI overlaps zero, mycobacteria are deemed to be static under the conditions tested.

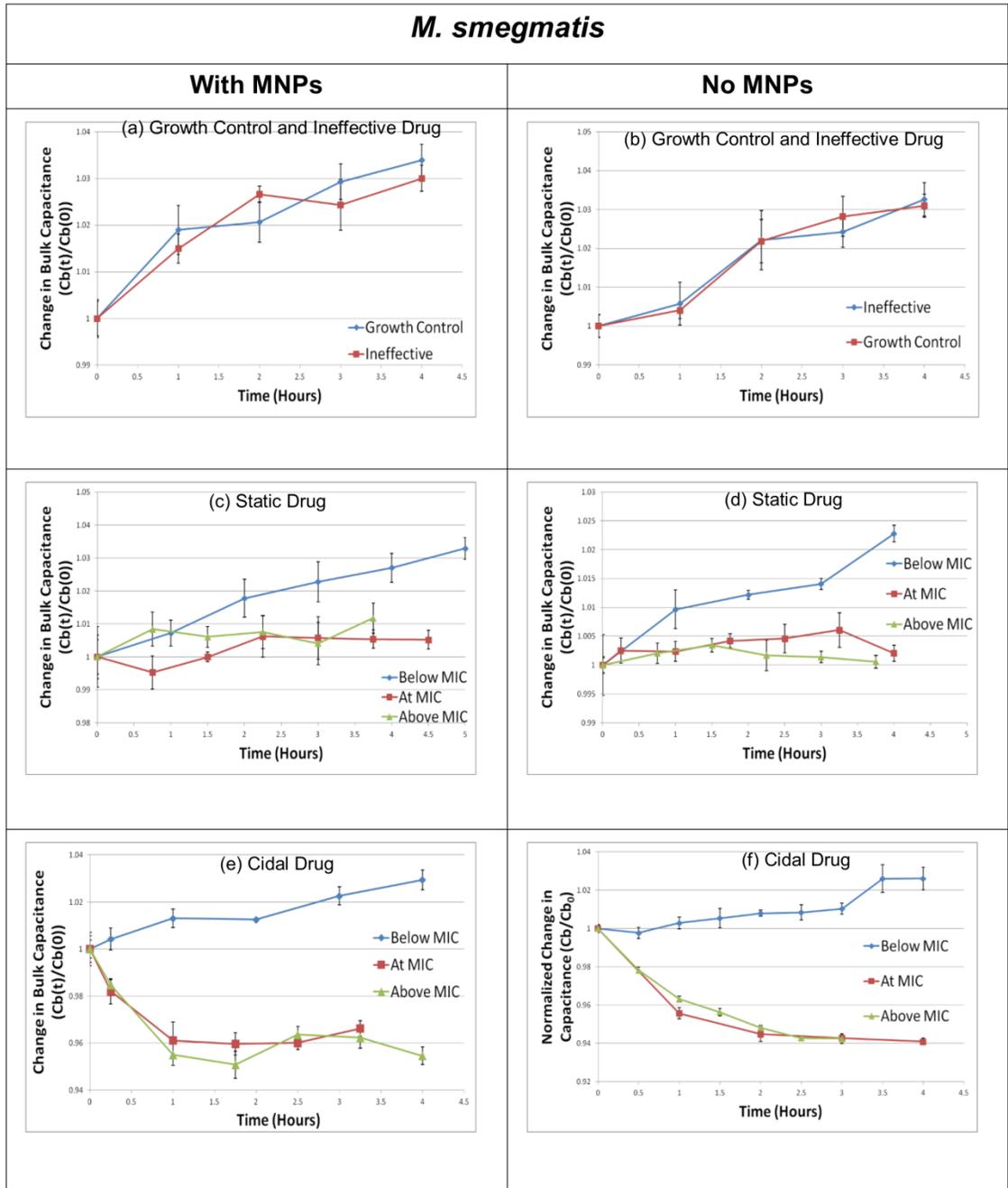


Figure 7. Bulk Capacitance Changes over time for *M. smegmatis*

*Change in the value of the measured bulk capacitance (C_b) as a function of time for *M. smegmatis* cultures with and without Magnetic Nano-particles (MNPs) when exposed to cidal, static, and ineffective antibiotics. All changes are normalized to the baseline (time $t=0$) value. ($n=5$ at each data point)*

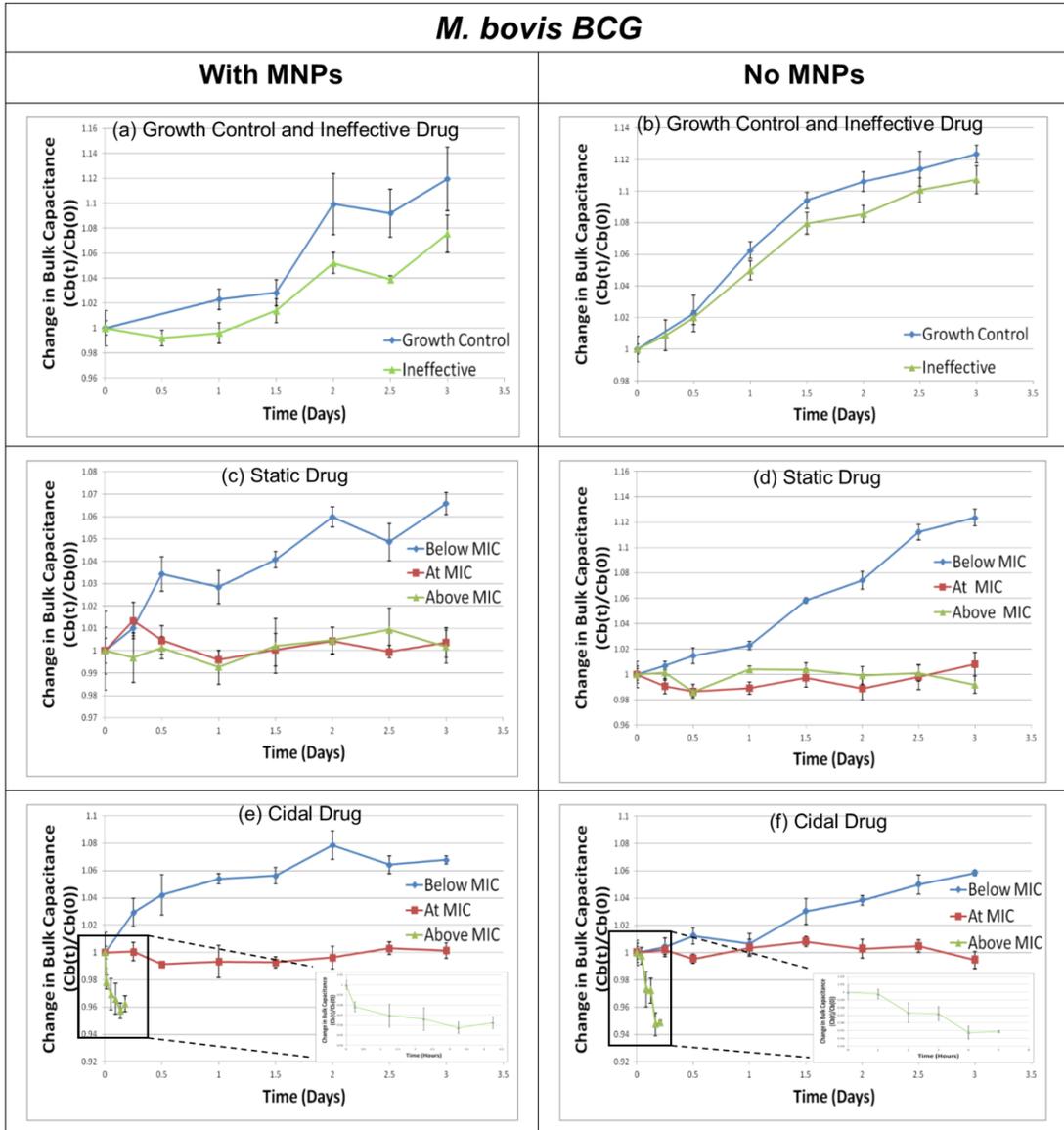


Figure 8. Bulk Capacitance Changes over time for *M. bovis* BCG

Change in the value of the measured bulk capacitance (C_b) as a function of time for *M. bovis* BCG cultures with and without Magnetic Nano-particles (MNPs) when exposed to cidal, static, and ineffective antibiotics. All changes are normalized to the baseline (time $t=0$) value. ($n=5$ at each data point).

<i>M. smegmatis</i>						
Sample Type	Antibiotic	Concentration of Antibiotic ($\mu\text{g/mL}$)	Estimate of Slope	95% CI Lower Bound	95% CI Upper Bound	Inference
With MNP	Growth Control	0	0.0078	0.0030	0.0127	Growth
	Rifampicin	16	0.0069	0.0010	0.0129	Growth
	Ethambutol	0.125	0.0065	0.0051	0.0079	Growth
		4	0.0014	-0.0002	0.0030	Stasis
		32	0.0018	-0.0013	0.0049	Stasis
	Amikacin	0.0625	0.0068	0.0045	0.0090	Growth
		1	-0.0091	-0.0215	0.0033	Stasis
		16	-0.0082	-0.0184	0.0019	Stasis
Without MNP	Growth Control	0	0.0086	0.0041	0.0131	Growth
	Rifampicin	16	0.0084	0.0048	0.0119	Growth
	Ethambutol	0.125	0.0050	0.0024	0.0076	Growth
		4	0.0007	-0.0005	0.0020	Stasis
		32	0.0000	-0.0012	0.0012	Stasis
	Amikacin	0.0625	0.0069	0.0043	0.0095	Growth
		1	-0.0131	-0.0289	0.0027	Stasis
		16	-0.0184	-0.0259	-0.0109	Death

Table 3.

*Estimates of slope of the straight line fitted to data consisting of measured bulk capacitance value (C_b) (dependent variable) as a function of time (t) (independent variable) for various conditions, along with the lower and upper bounds of the 95% confidence interval for *M. smegmatis*.*

<i>M. bovis</i> BCG						
Sample Type	Antibiotic	Concentration of Antibiotic ($\mu\text{g/mL}$)	Estimate of Slope	95% CI Lower Bound	95% CI Upper Bound	Inference
With MNP	Growth Control	0	0.0427	0.0209	0.0646	Growth
	Pyrazidamine	16	0.0269	0.0125	0.0413	Growth
	Ethambutol	0.125	0.0193	0.0111	0.0275	Growth
		4	-0.0011	-0.0057	0.0036	Stasis
		32	0.0026	-0.0011	0.0063	Stasis
	Amikacin	0.125	0.0192	0.0068	0.0316	Growth
		1	0.0013	-0.0025	0.0052	Stasis
16		-0.0075	-0.0146	-0.0003	Death	
Without MNP	Growth Control	0	0.0427	0.0289	0.0564	Growth
	Pyrazidamine	16	0.0426	0.0338	0.0514	Growth
	Ethambutol	0.125	0.0436	0.0368	0.0504	Growth
		4	0.0034	-0.0023	0.0091	Stasis
		32	-0.0004	-0.0061	0.0053	Stasis
	Amikacin	0.125	0.0200	0.0156	0.0244	Growth
		1	0.0000	-0.0041	0.0042	Stasis
16		-0.0116	-0.0163	-0.0069	Death	

Table 4.

*Estimates of slope of the straight line fitted to data consisting of measured bulk capacitance value (C_b) (dependent variable) as a function of time (t) (independent variable) for various conditions, along with the lower and upper bounds of the 95% confidence interval for *M. bovis* BCG.*

5. Discussion:

In this work, we try to demonstrate “proof of principle” that (a) live mycobacteria can be isolated from sputum using MNPs with high efficiency (almost all the bacteria that survive decontamination) and (b) that the efficacy of candidate drugs on the mycobacteria thus isolated (in suspensions containing MNPs) could be tested in real-time using m-EIS.

As can be seen from our isolation experiments (Table 2 and Figure 6), the decontamination protocol used resulted was largely successful in eliminating “contaminant” gram positive and gram negative species (less than 0.6% survival for both *M. smegmatis* and *M. bovis BCG*). However, this was accompanied by significant losses in the number of mycobacteria (only ~70% survival for *M. smegmatis* and ~56% survival for *M. bovis BCG*). However, it must be noted that the widely used protocol (50), from which our protocols were derived by adjusting the strength of chemicals and time of exposure, was formulated for *M. tuberculosis*, a mycobacterium that is hardier than the ones tested by us. Complete (> 5 logs) elimination of contaminant bacteria and > 90% survival (<1 log elimination) of the *M. tuberculosis* have been reported for this protocol. (51).

More interestingly, virtually all bacteria (both mycobacteria other bacteria) that survive decontamination seem to be collected by the MNPs, with virtually no difference in the loads estimated after decontamination, and after collection using MNPs. It may be noted that these beads were designed to collect *M. tuberculosis* cells, and the near-100% collection of other mycobacteria is a better-than-expected outcome. Thus, in a real-world situation (where the decontamination

process is likely to lead to complete elimination of contaminants and high survival rates for the mycobacteria), it is very likely that our process is will allow us to collect virtually all the living mycobacteria originally present in the sample into a “pellet”, which can then be re-suspended into a small volume of DST buffer.

As can be seen from our m-EIS results (Figures 7 and 8, and Tables 3 and 4), all the results (both with and without MNPs) are consistent with our expectations in the sense that (a) electrical signatures of growth (increase in bulk capacitance) are observed for controls (no drugs), for drugs known to be ineffective, and for bacteriostatic and bactericidal drugs present at concentrations below MIC, (b) electrical signatures of death (decrease in bulk capacitance) are seen for cidal drugs at concentration at and/or above MIC, and (c) electrical signatures corresponding to bacterial stasis (no significant change in bulk capacitance over time) can be seen for bacteriostatic drugs at and above the MIC (and for the bactericidal drug at MIC in the case of *M bovis BCG*). Moreover, similar samples with and without MNPs display similar behavior, thereby laying to rest our apprehensions regarding the potential interference with either the bacterial behavior or the electrical readings.

One major limitation of the current study is that while the two parts of the study (isolation using MNPs and m-EIS) were conducted independently, although care was taken to ensure that the composition of the suspensions subjected to m-EIS was similar to those obtained at the end of the isolation protocol. Future work will incorporate (a) real world sample (human sputum with *M. tuberculosis* cells), (b) self-contained and sealable microfluidic cassettes with multiple wells,

each containing chosen amounts of candidate drugs pre-loaded into 10 μ L “wells”, into which suspensions containing growth media, mycobacteria and MNPs can be loaded, and (c) integrated electronics to keep track of multiple wells in parallel. We have previously demonstrated the use of m-EIS to detect mycobacteria in decontaminated sputum samples: both on the basis of detecting their proliferation (a process taking ~36 hours for *M. bovis BCG*)(52), and a novel “detection by death” approach (53). Micro-wells dedicated to implementing these approaches may be incorporated into the same microfluidic cassette to bring together a portable, sensitive, and affordable device to detect *M. tuberculosis* in sputum samples, and simultaneously determine the multidrug resistance profile of the pathogen, all within 3 days of sputum collection.

It may be noted that the m-EIS data presented earlier was obtained using suspensions containing mycobacteria at concentrations of $\sim 10^5$ CFU/ml loaded into microchannels in which the volumes of the region between the electrodes was $\sim 10\mu$ L. Thus, this implies that 1 condition (candidate drug at a chosen concentration) can be tested with only about 1000 CFUs of mycobacteria. Phenotypic DST for *M. tuberculosis* is often performed by testing similar concentrations of mycobacteria ($\sim 10^5$ CFU/ml) against certain “cutoff” concentrations of candidate drugs (54). Thus, using only 5000 – 10,000 cells isolated from a single (or small number of) sputum samples, one can potentially conduct phenotypic drug susceptibility tests against a number of drugs within 3 days (compared to 4 to 6 weeks with current technology). Moreover, given that in the final product, the readings would be obtained electronically from a sealed

microfluidic cassette, and the assessment of mycobacterial behavior (growth / death / stasis) can be made without user judgment, this product should be able to overcome the drawback of the MODS technique, and provide a rapid, reliable and relatively inexpensive alternative to current instruments / methods available for phenotypic DST.

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