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Label-free DNA-based detection of *Mycobacterium tuberculosis* and rifampicin resistance through hydration induced stress in microcantilevers

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ABSTRACT: We have developed a label-free assay for the genomic detection of *Mycobacterium tuberculosis* and rifampicin resistance. The method relies on the quantification of the hydration induced stress on microcantilever biosensors functionalized with oligonucleotide probes, before and after hybridization with specific targets. We have found a limit of detection of 10 fg/mL for PCR amplified products of 122 bp. Furthermore, the technique can successfully target genomic DNA (gDNA) fragments of length > 500 bp and it can successfully discriminate single mismatches. We have used both loci IS6110 and *rpoB* as targets to detect the mycobacteria and the rifampicin resistance from gDNA directly extracted from bacterial culture and without PCR amplification. We have been able to detect 2 pg/mL target concentration in samples with excess of interfering DNA and in a total analysis time of one hour and 30 minutes. The detection limit found demonstrates capability to develop direct assays without the need for long culture steps or PCR amplification. The methodology can be easily translated to different microbial targets and it is suitable for further development of miniaturized devices and multiplexed detection.

Tuberculosis (TB) is an infectious disease caused by the pathogen *Mycobacterium tuberculosis* (MTB). It seconds HIV in worldwide deaths due to a single infectious agent. According to the World Health Organization (WHO), around 8.6 million people developed TB and 1.3 million died from this illness in 2012.¹ Although healthy people infected with MTB usually do not show symptoms, since the immune system keeps the bacteria in a dormancy state, they may act as disease vectors. Around 20% of infected people develop symptoms due to eventual depression of the immune system several years after the initial infection. Moreover, the spread of multidrug-resistant (MDR) or extensively drug-resistant (XDR) strains is a growing problem, being recognized as a global threat since 2006.¹⁻³ In accordance with the WHO, fully successful diagnosis and treatment of multidrug-resistant TB (MDR-TB) is far from being achieved. In fact, less than 25% of the people estimated to have MDR-TB were detected in 2012.¹ Even though TB and drug-resistant TB are treatable, the diagnostic must be done quickly and effectively. Otherwise, the mortality, the risk of developing resistance to further drugs and the risk of generating outbreaks increase seriously.⁴ The development of simple and affordable point of care diagnostic tests is key to address the eradication of the tuberculosis epidemic. Here, we demonstrate the suitability of microcantilever biosensors to face this challenge.

The mycobacterial culture has been for decades, and still remains, the gold standard for the diagnosis of TB, but due to the slow growth rate of the microorganism, the diagnostic may delay several weeks. Sputum smear microscopy has been also widely used despite its limited sensitivity.⁵ Drug resistance has been detected traditionally by means of drug susceptibility testing on cultured specimens.^{1,6} Recently, molecular biology assays have proven fast and efficient for MTB and antibiotic resistance identification, with real-time PCR (RT-PCR) as the reference technology,^{1,4} being its sensitivity comparable to culture methods.⁴ Still, RT-PCR requires expensive and complex equipment, trained personnel and sustainable routine,⁵ which compromises its implementation in developing countries.

Novel methodologies are under research with the aim of reducing costs and analysis time, while increasing the sensitivity, portability and disposability.⁷⁻¹⁴ Still, PCR-free and label-free assays capable of detecting low concentrations of the target pathogen are scarce. Among these emerging technologies, microcantilevers have been proposed as highly sensitive label-free DNA-based biosensors,¹⁵⁻²² although application to bacteria detection has been limited.¹⁷⁻¹⁹

We demonstrate here the label-free detection of *M. tuberculosis* and the identification of rifampicin resistance using gDNA extracted from culture without PCR amplification. For such purpose, we apply a methodology already demonstrated with synthetic oligonucleotides. The method is based on the quantification of the hydration induced stress on DNA probe monolayers on gold coated microcantilevers, before and after hybridization with the complementary targets.²³⁻²⁵

Results and discussion

In order to identify the pathogen, we designed a probe (called here ISseq) that hybridizes with a 20 mer sequence contained within the IS6110 element, an insertion sequence

characteristic of the species belonging to the *M. tuberculosis* complex. This sequence successfully discriminates MTB isolates, being suggested as a standard tool for the diagnosis of tuberculosis.²⁶ We have also designed a probe (called here rpoB_mut531) to detect the antibiotic resistance. Resistance to rifampicin appears as a result of a single mutation in the gene coding for the β subunit of the bacterial RNA polymerase, *rpoB*. The value of detecting rifampicin resistance lies in the fact that it has been reported in >90% of the multidrug resistant MTB isolates,²⁷ serving therefore as a marker for MDR-TB. Our probe rpoB_mut531 detects specifically the mutation 531 of the *rpoB* gene. In order to increase the hybridization efficiency, the thiolated sequences have a tail of 5 thymines between the C₃H₆ spacer and the sequence of interest in order to keep the hybridization bases away from the gold surface, due to the low affinity for gold of thymine.²⁸ Table 1 describes the probe and control sequences used in this study.

Strand name	Type of sequence	Strand sequence
ISseq	Probe	5'-GGTCTGCTACCCACAGCCG GTTTT-C ₃ H ₆ -SH-3'
ControlOligo	Negative control	5'-GTCGGACTCAAGCTATCAC TTTTT-C ₃ H ₆ -SH-3'
rpoB_mut531	Probe	5'-GCCGACTGTTGGCGCTGGG TTTTT-C ₃ H ₆ -SH-3'
rpoB_wt	Negative control	5'-GCCGACTGTCGGCGCTGGG TTTTT-C ₃ H ₆ -SH-3'

Table 1. DNA probe and control sequences.

The identification of specific sequences is performed here by surface hybridization of the targets on commercial microcantilevers functionalized with the ssDNA probe on the upper gold-coated side using thiol chemistry,²³ while backside and voids are blocked (see Supporting Information for further details). Briefly, in-plane forces between the ssDNA strands anchored onto the cantilever, as well as perturbation of the electron distribution of the surface gold atoms, result into an effective change of the surface stress of the active face of the cantilever with respect to the passive one. This effect gives rise to a cantilever bending. The bending profile is optically detected by the scanning laser beam deflection method,^{23,24} that can track the bending of hundreds of microcantilevers in minutes by scanning a laser beam over the microcantilever surface. The interaction forces between the immobilized ssDNA strands critically depend on the conformation of the hydration layers. These interactions can be tuned by controlling the relative humidity, and thereby the dependence of the cantilever deflection on the humidity provides a signature of the conformation state of the immobilized nucleic acids. We previously demonstrated that the measurement of the cantilever bending with respect to the relative humidity (RH) can be used for detecting hybridization.^{23,24} Further details about the origin of such response can be found in references.^{23, 29-33}

In figure 1 we show the basic steps to run a full experiment. The microcantilever chips are attached to the PDMS covered bottom surface of a modified 96 well microplate. A first scan is needed to record the surface stress curve of the initial ssDNA monolayer for each individual microcantilever. We

term this pre-incubation curve. Surface stress is obtained through measurement of the bending as described in Domínguez et al.²³ We show a typical pre-incubation curve in figure 1c. Recorded curves are labelled by the microcantilever position in the array. Then, as depicted in figure 1b, the chips are incubated with the sample solution for 1 hour under agitation. Afterwards, the chips are dried under a stream of dry nitrogen and the surface stress with changing humidity is measured again. We observe that the non-hybridized cantilevers, previously functionalized with the control sequence, follow the same stress pattern, replicating the initial pre-incubation curve for the ssDNA monolayer. Conversely, microcantilevers that have undergone hybridization on its surface (formerly sensitized with the probe sequence) show a distinct stress pattern, clearly departing from the initial pre-incubation surface stress curve, as it is shown in figure 1d. In order to quantify the surface stress changes after hybridization, the sensor response is derived from comparison of the pre-incubation and post-incubation curves measured for the same microcantilever. We define our sensor response signal, ΔS , as the absolute value of the difference in surface stress at 30% RH for the swelling curve, before and after incubation with the target sample solution, $\Delta S = |\sigma_{30\%, \text{ssDNA}} - \sigma_{30\%, \text{dsDNA}}|$, (see ΔS in figure 1d). In this way, each microcantilever is referenced to the response of that same microcantilever before hybridization. This procedure filters out any spurious signals that may arise due to small differences in mechanical properties between different microcantilevers. Further details can be found in our previous work related to synthetic oligonucleotides detection.²³

Since this is the first time hydration induced stress on microcantilevers is used to detect naturally derived DNA, we tested first our sensors with a IS6110 PCR amplified product of 122 bp.³⁴ Detection microcantilevers were functionalized with a sequence contained within the insertion element IS6110, called here ISseq. Control cantilevers were functionalized with an unrelated sequence, called here ControlOligo (see Table 1). The PCR product was purified and used as target in the hybridization sample solution at concentrations ranging from 10 ng/mL to 1 fg/mL. Hybridization was performed through incubation during one hour, as described in detail in the Supporting Information. We have avoided longer incubation times given the need for a rapid analysis for the pathogen detection. In figure 2 we show the average value and standard deviation of the sensor response signal, ΔS , for the 96 cantilevers measured. By defining the detection threshold value for the sensor signal ΔS as the mean value of the controls plus twice the standard deviation, the rate of true positives in the assay (sensitivity) is of 0.95 and the rate of true negatives in the controls (specificity) is of 0.93. Sensitivity is defined here as the ratio between the cantilevers that being functionalized with the probe sequence showed a sensor signal, ΔS , larger than the detection threshold to the total number of probe microcantilevers. Specificity is defined as the ratio between the cantilevers that being functionalized with the negative control sequence gave a sensor signal response value, ΔS , below the detection threshold to the total number of control microcantilevers. We have included all of our measurements for the full concentration range used.

Figure 1. Representation of the basic steps followed in a multiplexed experiment. (a) Measurement of the surface stress response with humidity changes for each microcantilever and (b) incubation, washing and drying in microwells. (c) Example of a curve measured in microcantilevers labelled C1(0,0) and C1(0,1) by its position in the array. Curves obtained by scanning cantilevers C1(0,0) and C1(0,1) before (c) and after (d) incubation with the target DNA are shown. The difference of the surface stress value at 30% RH between the pre-incubation ssDNA swelling curve and the after hybridization curves defines the sensor signal, $\Delta S = |\sigma_{30\%, \text{ssDNA}} - \sigma_{30\%, \text{dsDNA}}|$.

Figure 2. Detection of *Mycobacterium tuberculosis* through locus IS6110 in PCR-amplified samples. Average and standard deviation of the sensor response signal for the 96 microcantilevers functionalized with ISseq, complementary to a region contained within IS6110 (solid blue columns), and ControlOligo, the negative control (patterned orange columns), incubated with a PCR product amplified from the insertion sequence IS6110. The limit of detection found is of 10 fg/mL.

Slight differences in the grafting density of the ssDNA monolayers,^{30,32} beyond control yet, give rise to slightly different hybridization kinetics and thus, to the variation in the sensor response signal, as seen from the error bars in figure 2. This effect was already discussed in Domínguez et al. for synthetic oligonucleotides.²³ From figure 2, the nanomechanical signal clearly evidences the detection events for concentrations higher than 10 fg/mL.

We thus proceeded to the direct detection of genomic DNA without prior amplification. In order to improve the accessibility of the targets to the surface tethered probes, the gDNA was partially digested with the restriction enzyme AluI (Thermo Scientific, USA) that in IS6110 leaves a 554 bp

fragment containing the region called here ISseq for the bacteria identification and in *rpoB* a 586 bp fragment containing the *rpoB_mut531* sequence, indicative of rifampicin resistance. Proteins from the digestion solution and the hybridization buffer and fragments of unrelated gDNA were present in the hybridization sample solution. In figure 3a we depict the average value and standard deviation of the sensor signal, ΔS , for 66 microcantilevers functionalized with ISseq or ControlOligo and incubated with the digested gDNA at concentrations ranging from 20 ng/mL to 200 fg/mL. The concentration of 2 pg/mL is the lowest we could detect. Figure 3b shows the mean and standard deviation found for the 56 microcantilevers sensitized with *rpo_mut531* (rifampicin resistance detection sequence) and *rpo_wt* (negative control). The gDNA concentrations tested ranged from 20 ng/mL to 200 fg/mL, and we found a limit of detection (LOD) of 2 pg/mL. Thus, the technique is sensitive enough to discriminate the presence of gDNA from *M. tuberculosis* in a proteinous sample with interfering DNA at concentrations of the target as low as 2 pg/mL. The technique can also discriminate the single mismatch involved in the rifampicin resistance. We can note from Fig. 3a the different trends with concentration for the perfect match (ISseq) and the unrelated sequence (ControlOligo). On the contrary, in Fig. 3b we observe similar trends with concentration for both probes *rpoB_mut531* and *rpoB_wt*. In this case, binding is specific for both probes, given there is only one mismatched base, and we can just expect a difference in binding affinity. This still provides a sensor signal that is large enough to allow discrimination of the single mismatch. The single mismatch discrimination might be improved further by increasing the hybridization temperature or using a buffer that further destabilizes mismatched DNA duplexes.³⁵

In table 2 we compare the methodology presented here to both well established and emerging technologies tackling the detection of bacteria. Comparison is made for analysis time, limit of detection and sample volume. Table 2 also identifies the methodologies already tested with the pathogen *Mycobacterium tuberculosis* and antibiotic resistance.

Technology	Limit of detection	Analysis time	Sample volume	Tested with MTB	Antibiotic resistance identification
Multi-channel series piezoelectric quartz crystal biosensor ¹²	10 CFU/mL	> 1 day	500 μ L	Yes	No
3D Immunomagnetic Flow Assay ¹⁴	10 CFU/mL	3 min	10 mL	No	No
Cepheid Gene XPERT ⁴	131 CFU/mL	2 h	2-3 mL	Yes	Yes
Quartz crystal microbalance ¹³	10 ⁵ CFU/mL	20 min	160 μ L	Yes	No
Diagnostic magnetic resonance biosensor ⁷	10 ³ CFU/mL	30 min	5 – 10 μ L	No	No
Surface plasmon resonance biosensor ¹¹	2x10 ⁶ CFU/mL	< 1 h	—	Yes	No
AuNp based electrochemical biosensor ¹⁰	3x10 ⁵ CFU/mL	> 3 h	—	Yes	No
Cantilever biosensor (dynamic mode) ^{17,18}	700 CFU/mL	1 – 2 h	2 mL	No	No
Microcantilever biosensor (hydration induced stress) ^{this work}	500 CFU/mL	1 h 30 min	50 μ L	Yes	Yes

Table 2. Comparative table of technologies for bacteria detection and antibiotic resistance identification.

Figure 3. Identification of *Mycobacterium tuberculosis* and rifampicin resistance from extracted, purified and enzymatically digested gDNA. Average and standard deviation of the sensor response signal for the microcantilevers functionalized with ISseq and rpoB_mut531 and controls. The sequences ISseq and rpoB_mut531 identify the MTB and rifampicin resistance, respectively. a) MTB detection. Measurements from 66 microcantilevers functionalized with ISseq (blue solid columns) and ControlOligo (orange patterned columns). The limit of detection is 2 pg/mL. b) Rifampicin resistance detection. Results from 56 cantilevers sensitized with the probe sequence rpoB_mut531 (blue solid columns) and the control sequence rpoB_wt (orange patterned columns). The limit of detection is 2 pg/mL. Rifampicin resistance detection demands the discrimination of a single mismatch.

Microncantilever sensors have the advantage that they demand of very low sample volume. Also, the ex-situ detection methodology presented here allows the microcantilever design to be kept very simple. The incubation steps with the fluid samples can be performed similarly to standard protocols in microwell plates, such as in widely used ELISA tests. All the basic elements in this technology are compatible with future full automatization and high throughput read-out at low cost in a point of care platform. Laser optics are accessible and the

optical read-out and alignment have already been automated by the laser scanning technique.^{36,37} Simple silicon cantilevers without integrated read-out can be mass produced at very low cost and the handling and washing steps can also be automated.^{22,38,39}

Conclusion

With this work we demonstrate that quantification of the hydration induced stress on microcantilevers provides a new approach to identify pathogens through conserved genes, as well as identifying antibiotic resistances such as rifampicin. We prove sensitivity to one single mismatch in a sample with 2 pg/mL target concentration and with excess of interfering gDNA. The detected gDNA target concentration is equivalent to 500 bacteria/mL. Detection of the pathogen can be performed in a total analysis time of 1 hour and 30 minutes. This work proves suitability of hydration induced stress measurements on microcantilevers for addressing fluid samples from patients with no need for previous culture steps or PCR amplification. The methodology can be easily translated to different microbial targets and it is suitable for further development of miniaturized devices and multiplexed detection. These results encourage further development into a fully automated diagnosis platform at the point of care. Such advancements will aid in the fast diagnosis of infections and its proper antibiotic treatment.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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