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DIAGNOSTICS

Rapid detection of *Mycobacterium tuberculosis* biomarkers in a sandwich immunoassay format using a waveguide-based optical biosensor

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SUMMARY

Early diagnosis of active tuberculosis (TB) remains an elusive challenge, especially in individuals with disseminated TB and HIV co-infection. Recent studies have shown a promise for the direct detection of pathogen-specific biomarkers such as lipoarabinomannan (LAM) for the diagnosis of TB in HIV-positive individuals. Currently, traditional immunoassay platforms that suffer from poor sensitivity and high non-specific interactions are used for the detection of such biomarkers. In this manuscript, we demonstrate the development of sandwich immunoassays for the direct detection of three TB-specific biomarkers, namely LAM, early secretory antigenic target 6 (ESAT6) and antigen 85 complex (Ag85), using a waveguide-based optical biosensor platform. Combining detection within the evanescent field of a planar optical waveguide with functional surfaces that reduce non-specific interactions allows for the ultra-sensitive and quantitative detection of biomarkers (an order of magnitude enhanced sensitivity, as compared to plate-based ELISA) in complex patient samples (urine, serum) within a short time. We also demonstrate the detection of LAM in urine from a small sample of subjects being treated for TB using this approach with excellent sensitivity and 100% corroboration with disease status. These results suggest that pathogen-specific biomarkers can be applied for the rapid and effective diagnosis of disease. It is likely that detection of a combination of biomarkers offers greater reliability of diagnosis, rather than detection of any single pathogen biomarker. NCT00341601.

1. Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is a major global health threat that was responsible for 9.4 million incident cases worldwide in 2009, resulting in 1.6 million deaths.[1] Increased incidence of drug resistant strains of *M. tuberculosis* and HIV co-infection, as well as the continued migration of individuals from areas where TB is endemic to more industrialized countries, have all made TB a global medical emergency.[2] Current diagnostic strategies such as sputum-smear microscopy are unreliable, especially in individuals with HIV co-infection.[3] It is widely accepted that the development of rapid and accurate new diagnostic tools is essential to improve TB control in developing countries, and its consequent dissemination to the industrialized world.

Use of pathogen-specific biomarkers for the diagnosis of TB has been controversial. Several pathogen-specific biomarkers of TB have been identified. Noteworthy among them are the early secretory antigenic target 6 (ESAT6), antigen 85 complex (Ag85) and the cell wall glycolipid lipoarabinomannan (LAM). ESAT6 is the basis for the diagnosis of TB using the interferon-γ release assays.[4] Talaat et al.[5] have developed immunoassays for the rapid detection of ESAT6 antibodies. An ELISA-based assay for the detection of

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ESAT6 antibodies in TB sera was associated with 97.6% sensitivity and 75% specificity in the diagnosis of TB infection. However, discriminating exposed individuals in an endemic background using antibody-detection strategies is difficult. This is because of one of many reasons: an individual may be vaccinated against said disease resulting in a cross-reactive response with antibody-based diagnostics, or may have been exposed to the pathogen or its near neighbors without actually being afflicted by the disease, resulting in an enhanced antibody response. Antibodies to ESAT6 are also a sensitive strategy for the detection of early bovine TB infection.[6] Ag85 has been of interest in the diagnosis of tuberculosis-associated meningitis. Kashyap et al.[7] have shown that 89% of the population evaluated in their study of tuberculous meningitis (71 of 80 enrolled patients) were positive for Ag85 complex. The protein has also been reported to be present in the blood of patients with central nervous system TB.[8,9] LAM is a prominent lipoglycan component of the cell wall of *M. tuberculosis*. Of late, the direct detection of LAM has garnered interest for the diagnosis of TB, especially in HIV-positive individuals.[10,11] Reither et al., evaluated a plate-based sandwich ELISA immunoassay for LAM for the diagnosis of TB in 291 Tanzanian patients with pulmonary tuberculosis.[12] They demonstrated that the assay had a high sensitivity (87.8%) for detection of TB and also that the specificity of the test was higher in women (66.7%), and in patients with HIV co-infection (62%) when compared to sputum-smear microscopy. Shah et al.[10,13] conducted a nested prospective cohort study in three South African hospitals to evaluate the accuracy of semi-quantitative urinary detection of LAM using the commercially available Clearview TB ELISA (Inverness Medical Innovations, Waltham, MA). The authors concluded that the LAM test detected a subset of HIV-positive individuals with severe TB whose sputum-smear microscopy results were sub-optimal. Further, these studies indicate that urinary LAM correlates with bacillary burden in microbiologically confirmed TB. These studies suggest that urinary detection of biomarkers such as LAM might be of value in the detection of TB, especially in HIV-positive individuals. However, the current assays for LAM detection in urine, such as the Chemogen Inverness Dipstick and ELISA assays, are semi-quantitative. Further, lateral flow immunoassays and plate-based colorimetric assays have poor sensitivity as a consequence of the high non-specific interactions in complex biological samples.[14] Indeed, Reither et al. reported that although the LAM ELISA was effective in diagnosis of TB in HIV-positive populations, the sensitivity of the assay could be significantly improved.[12]

Accordingly, the development of ultra-sensitive strategies for the detection of the low concentrations of pathogen-specific biomarkers in patients may permit the sensitive, specific and rapid diagnosis of active TB infection, with low false negatives. As described above, this strategy may be effective in the diagnosis of early TB (ESAT6), disseminated and pulmonary TB (Ag85) and TB in children and HIV-positive individuals (LAM).[15,16]

Our team has developed a waveguide-based optical biosensor for the rapid, sensitive and specific detection of biomarkers associated with pathogens and effectively demonstrated it for the detection of biomarkers associated with influenza,[17] anthrax,[18] breast cancer[19] and tuberculosis.[20] We have also developed novel surface functionalization chemistry, self-assembled monolayers (SAMs), for the minimization of non-specific interactions associated with complex biological samples, a strategy that effectively enhances the signal over noise ratio, thereby improving assay sensitivity.[21,22] Herein, we demonstrate the application of our sensor platform to the detection of three TB-specific biomarkers, LAM, ESAT6 and Ag85, in a sandwich immunoassay format, and compare them with the sensitivity achieved by traditional plate-based ELISAs. We also demonstrate, in a blinded study, the effectiveness of this strategy for the sensitive and quantitative detection of LAM in urine and ESAT6 in serum, from a small set of human samples collected from a study of TB infected persons.

2. Materials and methods

2.1. Materials

The waveguide-based sensor was developed at the Los Alamos National laboratory.[18] Silicon oxynitride ($SiON_x$) planar optical waveguides were fabricated at nGimat (Atlanta) and have been effectively used with the waveguide-based biosensor platform for over a decade.[23] $SiON_x$ films have a thickness of ~120 nm (±5 nm) and a refractive index of 1.80 ± 0.06. A thin ~10 nm coating of SiO_2 is deposited on the active waveguide surface for functionalization. Materials required for waveguide functionalization with self-assembled monolayers (SAMs) are described elsewhere.[21] LAM (14–19 kDa), ESAT6, and Ag85 complex from *M. tuberculosis* H37Rv culture and, the rabbit polyclonal antibody and monoclonal antibodies and cell lines for the above antigens were procured by a materials contract from the Colorado State University (*via* BEI Resources). Monoclonal anti-ESAT6 antibodies (HYB 076-08) and EasyLink Streptavidin conjugation kit were purchased from AbCam. EZ-link Sulfo–NHS–LC–LC–Biotin and streptavidin were from Pierce. AlexaFluor 647 (AF647) labeling kit was from Invitrogen. 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine-N-(cap biotinyl) (cap-biotin-PE) were from Avanti Polar Lipids, Inc. Miniature G-25 sephadex columns were acquired from Harvard Apparatus and bovine serum was purchased from Hyclone Laboratories. Human Serum was obtained from Biomedical Technologies Inc. Nunc Immunosorp plates for use in immunoassays were purchased from Nalgene Nunc Inc., (Milwaukee, WI). Secondary antibodies were purchased from Jackson ImmunoResearch, West Grove, PA and Southern Biotech Ltd, Birmingham, Alabama. All other immunoassay and protein estimation reagents were purchased from Pierce Biologicals Ltd., Rockford, Illinois or Sigma Aldrich.

2.2. Humans subjects

Korean Ministry of Health Welfare and Family and NIAID co-sponsored a Natural History study conducted the National Masan Tuberculosis Hospital (NMTH) to investigate occurrence of multi-drug resistant tuberculosis (MDR-TB) and the rate of relapse in Korean subjects undergoing treatment for tuberculosis for the first time or those that were being retreated (http://clinicaltrials.gov/NCT00341601). An initial blood and periodic sputum samples were collected from all subjects during treatment and subjects who had taken 7 days or less of anti-tubercular therapy were asked to provide a urine sample for analysis. Other objectives of the study included the investigation of possible biomarkers of disease and thus age and gender matched control subjects were recruited from the surrounding province and given a physical exam, chest X-ray, and sputum exam to exclude active TB. The blood of control subjects was also tested with the QuantiFERON-T Gold assay (QFT-G; Cellestis Limited, Carnegie, Victoria, Australia) to determine their exposure status to *M. tb*. The NMTH and NIAID institutional review boards approved the study protocol and all subjects provided written informed consent for the protocol.

2.3. Collection of urine samples

Biomarkers of interest (LAM, ESAT6 and Ag85) were spiked into control human urine and subjected to a variety of treatments to design the optimal procedure for their collection. The procedures

evaluated were: stability at room temperature (up to 4 h), with protease inhibitor treatment, pH correction, upon dialysis, filtration and centrifugal clarification. It was determined that ESAT6 and LAM were stable in spiked urine for 2 h, after which their stability deteriorated. We also observed that low-speed centrifugation to clarify the sample, performed immediately after sample collection did not compromise biomarker stability in urine. However, this method did not offer any advantage to sample stability or non-specific binding either. All other methods were, in fact, detrimental, for the stability of LAM in spiked urine. Based on these observations, urine samples were collected from the patients, aliquoted (1 mL each) and snap-frozen in liquid nitrogen within 60 min of collection. Urine was collected from HIV-free, TB-infected subjects and control subjects, coded, tested for the absence of culturable *M. tb*, and shipped to LANL where they were frozen at −80 °C until use.

2.4. Detection of LAM, Ag85 and ESAT6 by traditional plate-based ELISA

Microtiter plates from Dynatech were used for LAM and Ag85 ELISAs, whereas Nunc MaxiSorp flat-bottom 96 well plates were used for ESAT6.PBS containing 2% BSA was used for blocking (37 °C for 2 h). The washing protocol comprised of PBS-0.1% tween 20 (3×), followed by PBS (3×). Horseradish peroxidase (HRP) conjugated reporter antibody was used in all cases (37 °C for 1 h). After washing, 100 μl of substrate solution, H_2O_2–tetramethylbenzidine (TMB), was added to the wells, and incubated for 30 min, RT. The reaction was stopped with 100 μl of 2.5 N H_2SO_4, and the absorbance was read at 450 nm on a Molecular Devices Absorbance Plate Reader. Each test was performed in triplicate, and the mean absorbance of control wells was subtracted from that of wells with LAM before analysis.

For the LAM assay, Polyclonal LAM antibody (20 mg/ml) was diluted (1:500) in carbonate buffer (pH 9.6) and incubated overnight at 4 °C. After washing and blocking, either mannose capped LAM, or an unknown clinical sample was added to the wells (2 h 37 °C). After washing HRP-conjugated reporter antibody was added, and the specific reaction measured as described above.

For Ag85 complex, 100 μl of CS90 (150 nM) was coated on the plates (2 h RT, followed by 4 °C overnight). After washing and blocking, Ag85 (diluted in serum) was added at varying concentrations. Following incubation (37 °C, 2 h) and washing, reporter antibody was added and plates developed with TMB substrate.

For ESAT6, wells were coated with anti-ESAT6 polyclonal antibody in PBS (100 μl, 200 nM, overnight at 4 °C). After washing and blocking, varying concentrations of ESAT6 in PBS-0.5% BSA were added, and incubated (37 °C for 2 h). After washing, 15 nM anti-ESAT6 monoclonal antibody (2 h, 37 °C) was added. After washing, HRP-conjugated goat-anti mouse IgG polyclonal antibody was added (1 h at 37 °C). Plates were washed, and developed with TMB substrate.

2.5. Preparation of antibodies for fluorescence assays on the waveguide-based biosensor

We evaluated several available antibodies from the BEI resources, for use in the assay. The choice of capture and reporter antibodies and their effective concentrations in the assays were determined by conventional plate-based assays, using checkerboard titration. Another major deciding factor in the choice of the antibodies was the relative stability of the labeled antibody at 4 °C for several weeks. Capture antibodies (CS40 for LAM, monoclonal anti-ESAT6 antibody for ESAT6, CS90 for Ag85) were biotinylated using Sulfo-NHS biotin (20-fold molar excess), as described in detail elsewhere.[24] The reporter antibodies (rabbit polyclonal antibodies against LAM, ESAT6 and Ag85) were labeled with AF647 using a fluorescence labeling kit from Invitrogen as per the manufacturer's instructions. In both cases, labeled protein was separated from unlabeled by gel filtration followed by exhaustive dialysis (if applicable) in PBS. Labeled proteins were tested for binding activity (immunoblot) and the extent of biotinylation (4-hydroxy azo-benzene-2-carboxylic acid analysis) and fluorescence labeling (UV–Vis spectroscopy). Detailed methods for labeling and characterization have been presented elsewhere.[24] Only material with optimal protein recovery and degree of labeling was used for the assays described below.

2.6. Cleaning and functionalization of waveguides

Single mode planar optical waveguides consisting of a 10 nm coating of SiO_2 for the functionalization of the waveguide surface were used. Waveguides and glass coverslips were cleaned by sonication in chloroform and ethanol, and water (5 min each), followed by exposure to UV-light and ozone (40 min) and subsequently functionalized using either lipid bilayers or SAMs as sensing films. Waveguides were functionalized for bioassays with either unilamellar bilayers or self-assembled monolayers. The preparation of biotinylated (1%) unilamellar vesicles by sonication has been previously described.[19,24] Briefly, thin films of DOPC (5 mM stock in $CHCl_3$, 60 μl) with 1% cap biotinyl (v/v, sodium salt, 5 mM stock in $CHCl_3$, 0.6 μl) were deposited on test tubes by evaporation of chloroform under argon and then, re-hydrated in buffer (PBS, 0.01 M, 60 min). They were then subjected to eight freeze–thaw cycles (frozen in liquid nitrogen and thawed in warm water) and sonicated with a probe tip sonicator (Branson, 50% duty cycle) for 5 min to form uniform vesicles.

Waveguides and coverslips were assembled into a flow cell using a gasket to form a tight seal between them as has been outlined earlier.[19,23] A supported bilayer was formed on the waveguide surface by injecting the vesicle solution into the flow cell, and allowing it to stabilize (12–24 h). The functionalized surface was blocked with PBS containing 2% BSA for 1 h before use. These bilayer-coated waveguides remained stable for a maximum of 1 week under refrigeration.

Preparation of SAMs has also been described previously.[21] Briefly, waveguide and coverslip surfaces were cleaned as described above, and rinsed in water, blown dry, and placed in a vacuum desiccators with a small volume (~50 μl) of 1-(3-aminopropyl)-1-methyldiethoxy silane (APMDES), which was held at static vacuum (200 torr) for 2 h. The coated substrates were annealed (110 °C, 30 min), allowed to cool in a drying desiccator, rinsed with ethanol, blown dry, and analyzed (contact angle). Silanized slides were immersed in a solution containing a mixture of carboxylic acid-terminated polyethylene glycol (PEG) reagents with either a methoxy group (99–99.99% of the total PEG volume) or a Fmoc-protected amine (0.01–1% of the PEG volume) with standard peptide coupling reagents. After confirmation of coupling by contact angle, protecting groups were removed (20% piperidine/NMP, 2 × 15 min), and biotin was attached to the resulting free-amine *via* its commercially available *N*-hydroxysuccinimide ester. All steps after amino-silanization were carried out in N-methyl-pyrrolidinone (NMP) as the solvent. The slides were rinsed thoroughly between steps; ethanol was used after silanization, while NMP, acetone, and ethanol were used subsequently. After biotinylation, the slides were rinsed and dried under a stream of argon.

2.7. Sandwich immunoassays for biomarkers on the waveguide-based optical biosensor

The general scheme for the detection of TB biomarkers on functionalized waveguides by sandwich immunoassay format is described in Scheme 1. This description applies to all three biomarkers for which sandwich immunoassays were developed

(LAM, Ag85 and ESAT6). Differences in incubation time and antibody concentrations are specifically mentioned.

In all experiments, functionalized waveguides (either SAMs or lipid bilayers, depending on the experiment) were mounted in the flow cell and blocked with 2% BSA in PBS for 60 min, room temperature and waveguide-associated background (intrinsic measure of impurities associated with the instrument) and coupling efficiency (typically 40–50%, incident power 440 μW), measured. After each addition, the flow cell was washed with PBS (~1.5 ml, 60× flow cell volume), unless otherwise specified. Then, fluorescently labeled streptavidin (Strep-AF647, 50 pM) was injected into the flow cell (5 min, RT), and washed and fluorescence due to the binding of streptavidin to the biotinylated functional surface measured to confirm integrity of the functionalization process.

Unlabeled streptavidin (10 nM) was then added (5 min, RT) to saturate the functional surface, followed by addition of the biotinylated capture antibody specific to the biomarker being detected. For ESAT6, streptavidin conjugated capture antibody was added directly to the biotin-functionalized waveguide surface. The concentrations and incubation times were different for the biomarkers (125 nM of anti-LAM CS40 for 15 min, 150 nM of anti-Ag85 CS90 for 30 min, 110 nM of anti-ESAT6 monoclonal for 60 min). Non-specific signal (NS) were determined in each experiment by the addition of control bovine serum (1:10 dilution, 15 min, RT), followed by the reporter antibody (anti-LAM-AF647 (100 nM), anti-ESAT6 (40 nM), anti-Ag85 (75 nM), 15 min, RT). Specific detection of the biomarker was measured by the addition of the antigen (spiked in bovine serum) to the flow cell (30–60 min, RT, depending on the antigen), followed by addition of the reporter antibody (15 min, RT). The fluorescence signal associated with the binding of the biomarker to the reporter was measured using a spectrometer interface. Standard curves for the concentration-dependent detection of the biomarkers were generated on different waveguides.

2.8. Measurement of LAM and ESAT6 in patient samples

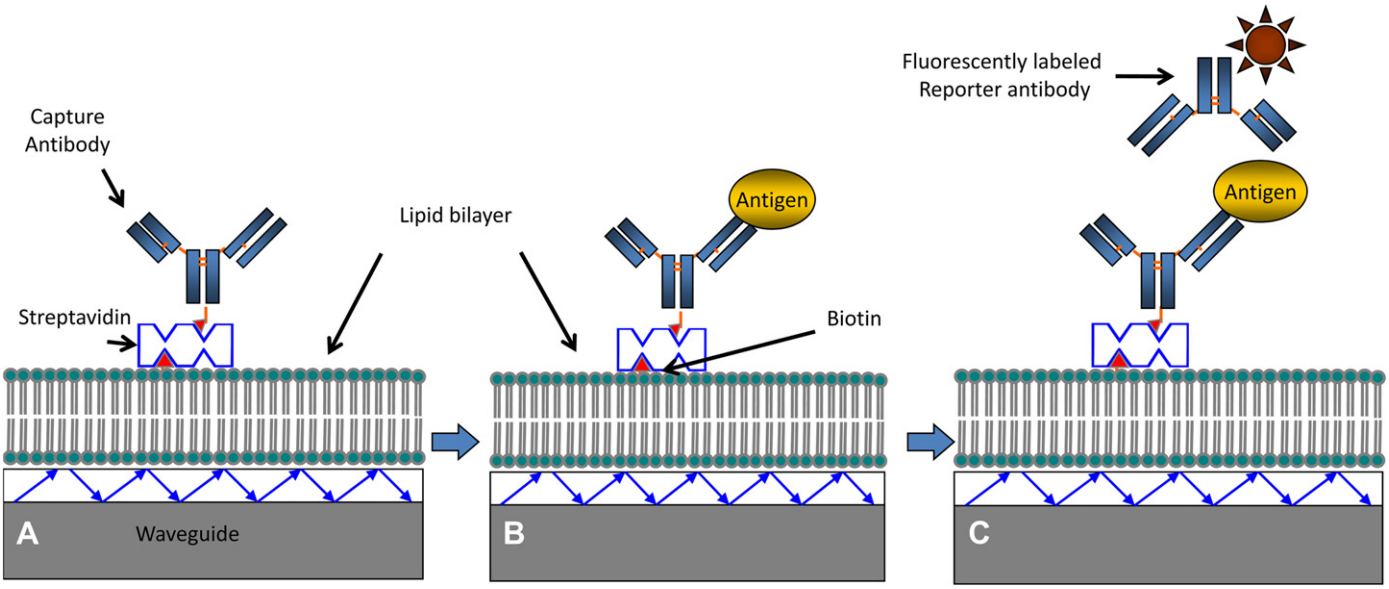
As indicated in Section 2.3, urine was collected in S. Korea and, evaluated for *M. tb* sterility and shipped frozen to LANL. Aliquots were thawed and used in the assay within 30 min. In all experiments, a standard measurement of a known concentration of LAM was performed on functionalized waveguides (Section 2.5) to serve as an internal standard for quantitation of the LAM concentration in the unknown sample. The fluorescent signal associated with this measurement was photobleached back to baseline before subsequent measurements. Patient urine, diluted 10× in PBS, was then added to the flow cell and incubated for 60 min, RT. After washing with PBS, the reporter antibody was added and the specific signal, indicating LAM in the patient urine, was measured. The signal associated with the interaction of the reporter antibody with control serum, in the absence of the antigen (either patient sample or standard LAM) is the non-specific signal (NS). This measurement also encompasses the waveguide-background. The signal measured with the interaction of the reporter antibody with the antigen is the specific signal. In all cases, the non-specific signal was subtracted from the specific signal. Extrapolation of the corrected specific signal against the standard measurement made on the same waveguide with a known concentration of LAM provides for quantitation of LAM concentration in urine.

In all experiments, a second standard LAM measurement was made after the measurement of the unknown sample. This measurement confirms the reproducibility of the signal on the same waveguide, the availability of sufficient capture sites to assure reliable multiple measurements on the same waveguide and the stability of optical parameters during the experiment.

3. Results

3.1. Detection of biomarkers by conventional immunoassays

We evaluated several available antibodies from BEI Resources before deciding on the antibodies used in the waveguide-assays. For LAM, we evaluated CS35 and CS40 as possible monoclonal antibodies for capture of the antigen. However, CS35 also binds to arabinose-LAM as observed by immunoblot analysis (data not shown), leading to the choice of CS40 as the capture antibody in our assay.



Scheme 1. Illustration of the sandwich immunoassay on a functionalized waveguide. A) waveguides functionalized with a lipid bilayer are prepared by binding of capture antibody using biotin–avidin chemistry. B) Addition of the sample containing the antigen results in specific binding. C) Finally, addition of a fluorescently labeled reporter antibody results in a specific signal measured *via* a spectrometer interface. Schematic not drawn to scale, and represents process for all three biomarkers investigated in this report.

Immunoblots were performed to determine the stability of the three biomarkers in urine after different treatments (data not shown). Biomarker stability and appropriate sample collection protocols are essential to the accurate determination of the success of any diagnostic/detection assay. Therefore, the stability of the spiked biomarkers in serum and/or urine was determined under a variety of conditions as described earlier. It was determined that snap freezing urine in liquid nitrogen causes no compromise in sensitivity of detection of LAM whereas treatment of urine with protease inhibitor cocktail, pH correction with PBS tablets (pH 7.4, 1 M final concentration), dialysis, centrifugal clarification and filtration, compromise sensitivity or completely inhibit detection. Similar stability studies were performed for ESAT6 and Ag85 in spiked plasma and urine. It was found that none of the treatments inhibits detection of these antigens nor does it significantly enhance it. Therefore, snap-frozen urine samples and frozen serum samples from both patients and controls were used for all measurements. Also, stability of LAM, as evaluated by binding to the antibody CS40, in urine decreases after 2 h, RT, and is undetectable by 3 h (data not shown).

We evaluated the sensitivity and specificity of detection of the three biomarkers: LAM, ESAT6 and Ag85, using conventional plate-based ELISAs using the same sample collection protocol and antibodies as used in the waveguide-based assays (Section 3.2) to provide an accurate comparison of the performance of the two assay platforms. Table 1 summarizes the data from standard plate-based ELISAs for the detection of LAM and ESAT6 and Ag85 (average of three measurements) in spiked samples (urine for LAM, serum for ESAT6 and Ag85). The limit of detection (LoD) for LAM using this approach is 78 nM (1.48 mg/L) in urine (LoD = signal * 3(standard deviation)), whereas the LoD for ESAT6 is 125 nM and that for Ag85 complex, is 183 nM, both in spiked serum. The table compares the LoD with the measurements made on the waveguide-platform, described in Section 3.2.

3.2. Detection of biomarkers in spiked samples using the waveguide-based biosensor

To accurately evaluate assay sensitivity and specificity, concentration-dependent measurements of the biomarkers were performed on the waveguide-based platform. Figure 1 demonstrates typical spectra measured with the detection of LAM (A), ESAT6 (B) and Ag85 (C) using the spectrometer interface of the waveguide-based platform. For LAM (Figure 1A), the waveguide-associated background and NS binding associated are minimal (<150 relative fluorescence units, RFU). The specific signal associated with the detection of 100 pM of LAM spiked in control urine is 1420 RFU. For 200 pM of ESAT6 (Figure 1B) in 50% human serum (conditions similar to those for patient serum samples), the specific signal (850 RFU) is significantly higher than waveguide-associated background and NS binding (160 RFU). For Ag85 (Figure 1C), a specific signal of 5020 RFU is shown over a minimal NS binding of 350 RFU, resulting in a phenomenal signal/background of 1673 for 500 pM Ag85 in 50% serum. However, further experiments were not performed for Ag85 owing to the highly unstable nature (<1 week) of the fluorescently labeled polyclonal reporter antibody, stressing the significance of reliable recognition ligands for immunoassays.

Table 1
Summary of plate-based ELISA measurements for *M. tb*. biomarkers LAM, ESAT6, and Ag85 complex. Optical density values measured at 450 nm absorbance were used to calculate concentrations reported. The NS binding and the signal measured at the LoD are shown for all three biomarkers, when diluted in serum/urine. The LoD measured on the waveguide-based platform is also indicated.

Sample	Biomarker	Optical density		Limit of detection (pM)	
		Control	Positive	ELISA	Waveguide
Serum	*Ag85*	0.06 ± 0.01	0.24 ± 0.01	183,000	0.5*
Serum	*ESAT6*	0.31 ± 0.1	0.9 ± 0.4	125,000	100
Urine	*LAM*	0.13 ± 0.005	0.18 ± 0.006	78,000	1

A complete standard curve was not generated because of poor antibody stability.
* LoD fog Ag85 is derived from two measurements.

3.3. Standard curves for the biomarkers in spiked samples

The average of three such measurements were made on different waveguides and used for the construction of the standard curve after correction for the waveguide-associated background and NS binding

For LAM (Figure 2A), The NS binding associated with the interaction of the reporter antibody in control urine is 122 RFU, and is considered the baseline value (0 pM). LoD is 1 pM in urine (19 ng/mL, 164 RFU) with r^2 of 0.817, which is reasonable given the intrinsic variability associated with measurements made on different waveguides. For ESAT6 in 50% serum (Figure 2B), NS binding is 247 RFU. LoD is 100 pM of ESAT6 with an r^2 of 0.99.

3.4. Detection of LAM in subject urine

To evaluate the potential use of biomarkers for the accurate detection or diagnosis of TB, the LAM assay was performed on 14 blinded urine samples collected from HIV-negative TB patients and healthy controls as per the protocol described in Section 2.2. For e.g.; for sample #6, the specific signal is 254 RFU (after non-specific signal correction, but without dilution correction), whereas the standard measurement for 10 pM LAM on the same waveguide is 415 RFU. After extrapolation and dilution correction, this corresponds to 24.5 pM LAM (465 ng/mL) in urine for sample #6, as plotted in Figure 3. This small cohort was simply tested to demonstrate the feasibility of this approach to the sensitive detection of pathogen biomarkers, and not as a clinical trial to establish the diagnostic reliability of said biomarkers. Figure 3 demonstrates that LAM concentrations were significantly higher (student *T*-test, Figure 3) in 10 of 14 samples with a concentration range of 17–338 pM, based on internal standards. 9 of these samples (1–9) were from subjects with a chest X-ray suggestive of active TB, sputum smears positive for acid-fast bacilli, and positive cultures on Lowenstein Jensen medium. A final sample (sample 14) was from a subject with a negative chest X-ray, sputum and physical examination, but was later found to have a positive Quantiferon–Gold test (9.28 IU/mL) suggestive of latent TB. This sample presented with 17 pM LAM (323 ng/mL). Four samples (10–13) gave signals below NS background, and were all determined to be negative for LAM. All these samples were from individuals recruited as controls, with a clean chest X-ray and physical exam and a negative Quantiferon–Gold test, suggesting the subjects did not have active or latent TB.

3.5. Detection of ESAT6 in serum

The sandwich assay for ESAT6 was performed in six blinded plasma samples also obtained from NMTH (Figure 4). ESAT6 was detected in three of the six samples (1–3) but not detected above NS levels in the other three samples 4–6. Samples 1–3 were collected from individuals with confirmed active TB (see above), and also tested positive for LAM. Samples 4–6 were found to be from the control TB-negative group.

4. Discussion

Despite being one of the oldest diseases known to mankind, current methods for the accurate diagnosis of active TB are

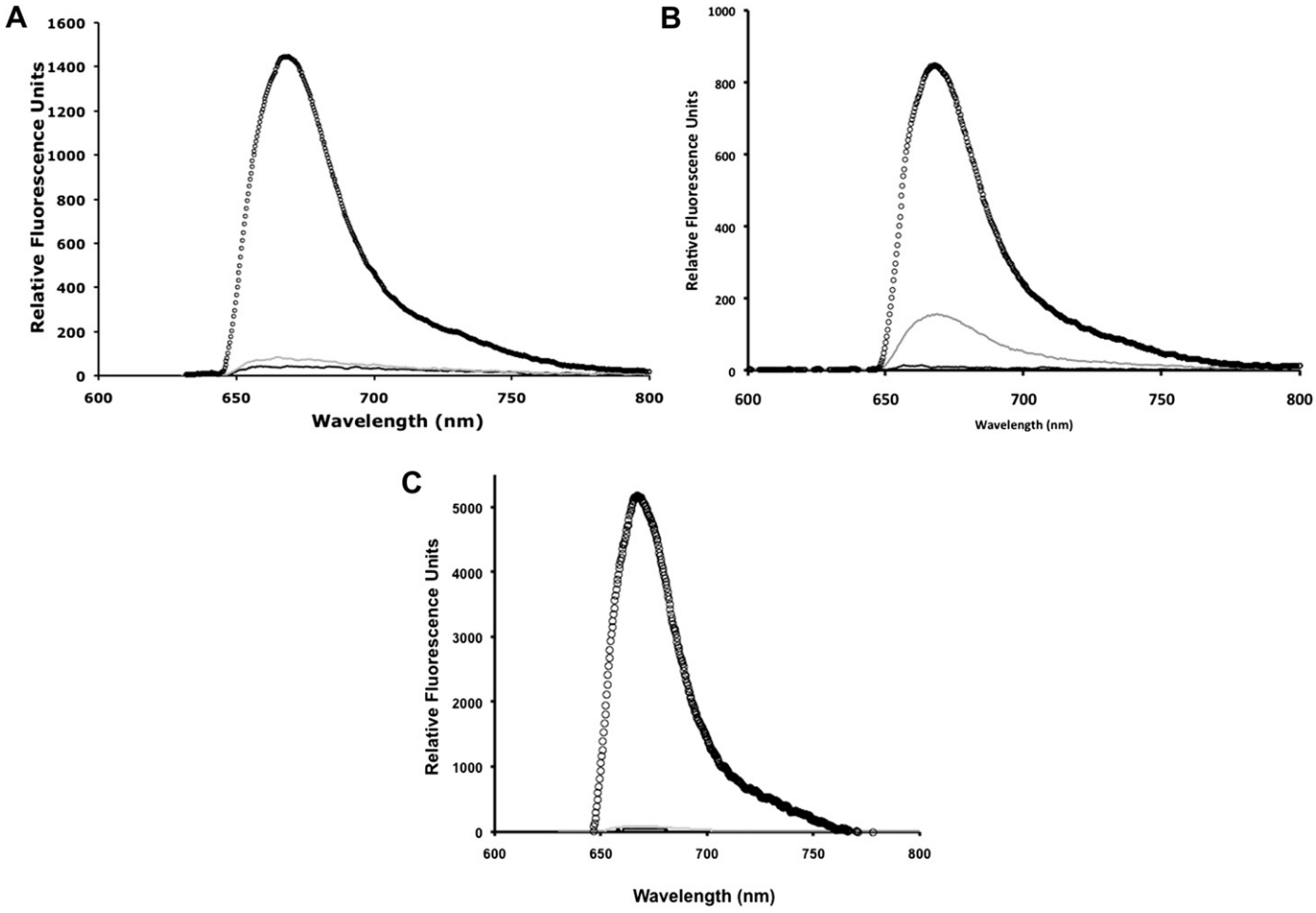


Figure 1. Representative curves for biomarker detection on the waveguide-based platform. A) LAM (100 pM), B) ESAT6 (200 pM) and C) Ag85 complex (500 pM). RFU measured is plotted as function of wavelength. Black lines indicate waveguide-associated background. Gray lines represent NS-binding associated with binding of reporter antibody to a control sample. Black circles indicate specific signal measured for the corresponding biomarker.

inadequate. Sputum-smear microscopy, which is the most common method for diagnosis used in countries with an active vaccination program (where the disease is endemic), is increasingly unreliable especially in individuals with co-infection with HIV[1]. There is thus a need for rapid, reliable and sensitive detection of active TB.

Infection in the human host results in the secretion of pathogen-specific biomarkers that are immediately recognized by the host innate immune receptors, resulting in an immune response that can culminate in the elimination of the pathogen, if successful. Mimicking innate immune recognition of pathogen biomarkers *in vitro* can allow for a rapid, early, sensitive and reliable method of diagnosis of infectious disease. However, this approach has several limitations: 1) most of the current methods of detection are not capable of quantitative detection of the very low concentrations of the pathogen biomarkers in the human host, 2) sensitive and specific recognition ligands are not available for many pathogen biomarkers, 3) most pathogen biomarkers are not secreted during the entire course of the disease, and information regarding biomarker expression as a function of disease progression is limited and 4) very little information is available regarding appropriate sample collection and processing strategies to preserve the bioactive properties of pathogen biomarkers.

In this manuscript, we have attempted to evaluate the use of direct detection of *M. tb*-specific biomarkers for the diagnosis of active infection. The novel findings in this manuscript are:

1. Lipidated biomarkers like LAM do not tolerate sample processing such as filtration, dialysis and pH correction. Snap freezing urine in liquid nitrogen as soon as it is collected is the most efficient method for preserving urinary LAM. Protein biomarkers like ESAT6 and Ag85 are stable after said sample processing methods in spiked samples. Improper sample processing may explain some of the discrepancies in existing data regarding the detection of urinary LAM. This is, to our knowledge, the first comprehensive demonstration of the effective of multiple sample processing methods on the stability of LAM in urine. However, this study did not control for the time of the day in which urine samples were collected. Samples collected in the morning are typically more concentrated, and hence could present with a greater concentration of the antigen. We propose to explore this hypothesis in future clinical evaluations of our assay.
2. We have demonstrated sensitive sandwich immunoassays for the detection of urinary LAM (LoD 1 pM, 19 ng/mL) and serum ESAT6 (LoD 100 pM) and Ag85 complex (500 fM) on our waveguide-based optical biosensor. Compared to commercial immunoassays such as the Alere® assay or the Inverness® lateral flow urinary LAM assay (LoD 0.1–1 μM),[10,13] or to conventional plate-based ELISAs in our laboratory using the same antibodies (LoD 183,000 pM, Table 1), the waveguide-based LAM assay offers at least an order of magnitude greater

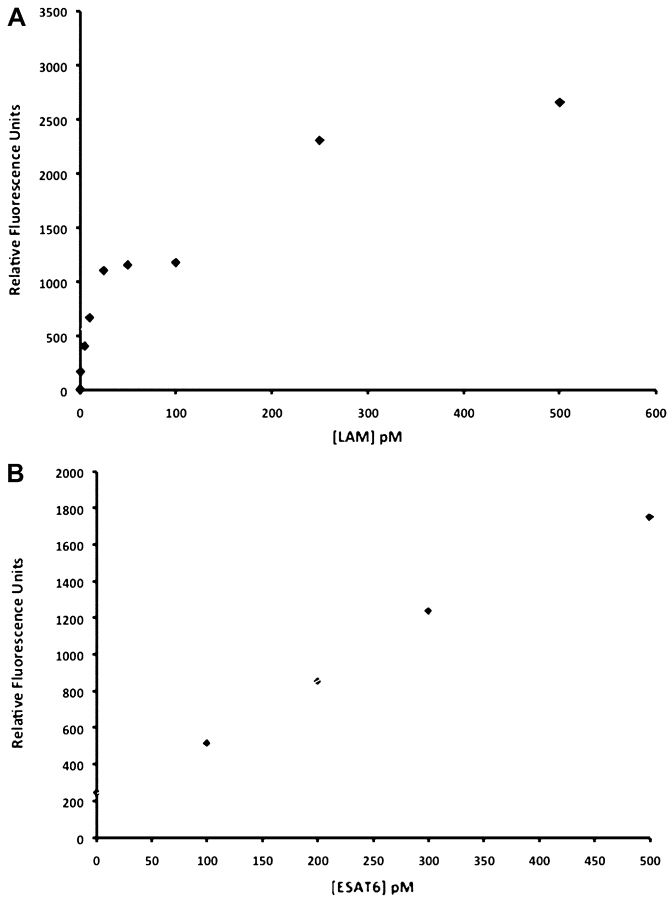
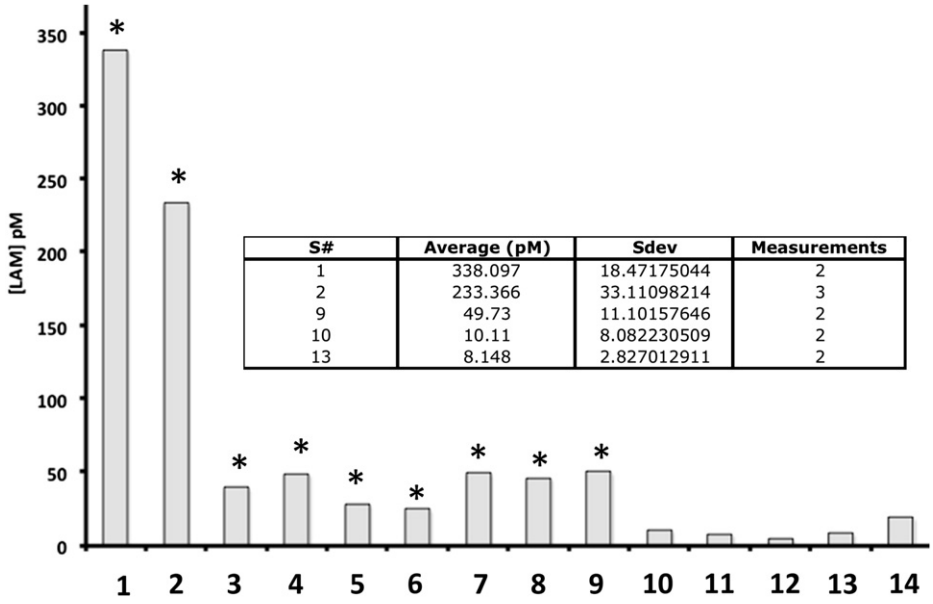
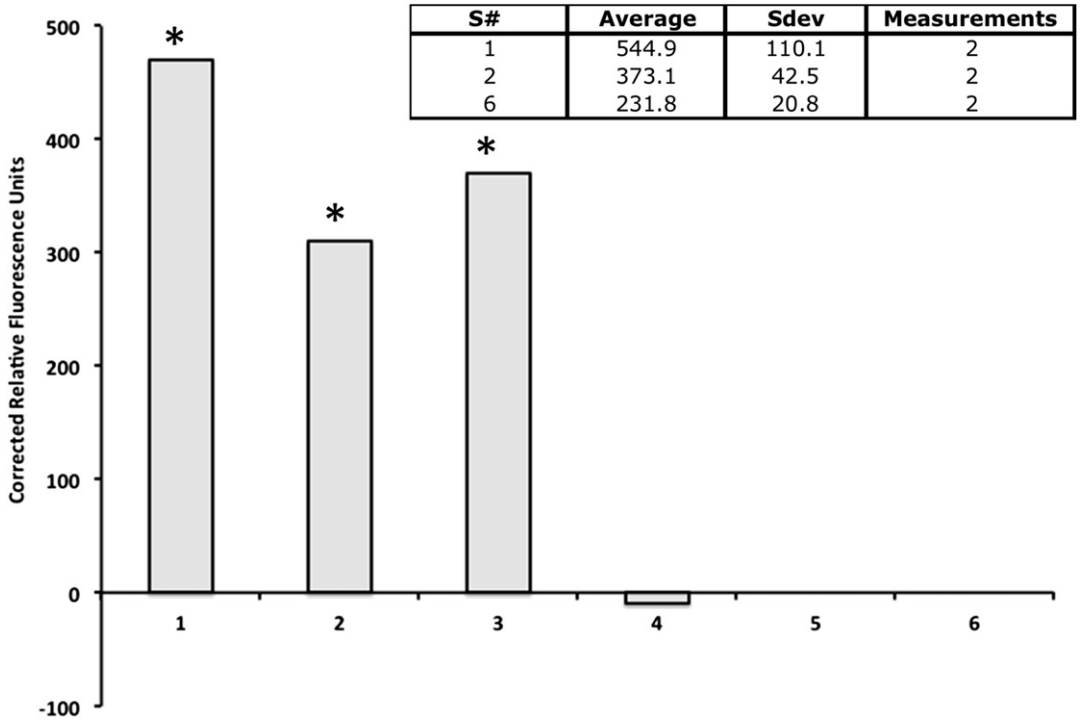


Figure 2. Standard curves constructed for LAM spiked in urine (A) and ESAT6 spiked in serum (B) on the waveguide-based platform. RFU is plotted as a function of concentration for each biomarker. Measurements ($n = 3$/concentration) were made on different waveguides.



S#	Average (pM)	Sdev	Measurements
1	338.097	18.47175044	2
2	233.366	33.11098214	3
9	49.73	11.10157646	2
10	10.11	8.082230509	2
13	8.148	2.827012911	2

Figure 3. Quantitative measurement of urinary LAM in a small cohort of TB patients without HIV co-infection. LAM concentration is plotted as a function of patient ID. LAM was detected only in patients with active TB (1–9, s#14) and not in QFG-negative controls (10–13). Measurements made on individual samples in a single readout are indicated in the graph. Only some measurements were repeated for validation. Inset shows measurements that were repeated 2× on the same waveguide, and associated standard deviation.



S#	Average	Sdev	Measurements
1	544.9	110.1	2
2	373.1	42.5	2
6	231.8	20.8	2

Figure 4. Quantitative measurement of serum ESAT6 in a small cohort of TB patients without HIV co-infection. ESAT6 concentration is plotted as a function of patient ID. The control sample is an average of measurements from three different samples. * indicates significant difference from control. Only measurements from individual samples are indicated in the graph. Some measurements were repeated for validation. Inset shows measurements that were repeated 2× on the same waveguide and associated average and standard deviation.

sensitivity (LoD 1 pM). The assays are rapid (15–60 min), quantitative, robust and can be integrated into a multiplex format.[25] This is, to our knowledge, the first quantitative estimation of LAM in a sandwich immunoassay format with a LoD of 1 pM.

3. We have demonstrated the quantitative detection of LAM in a small cohort of TB patients without HIV infection for the first time. This study does not establish the use of LAM as a diagnostic marker, but simply establishes the feasibility of using quantitative measurements of pathogen biomarkers as indicators of infection. We are currently exploring the use of a larger clinical cohort for the determination of diagnostic accuracy of LAM in individuals with and without HIV.
4. We have demonstrated, for the first time, the direct detection of ESAT6 in three plasma samples from patients with active TB.

The current study demonstrates the feasibility of development of sensitive and specific sandwich immunoassays for the direct detection of pathogen biomarkers in patients. The small sample set is simply aimed to demonstrate feasibility of biomarker detection in patients, rather than function as a clinical cohort. Indeed, a larger cohort of samples have to be evaluated to address precision metrics (false positives and negatives) with accuracy. Further, LAM, Ag85 and ESAT6 are, by no means, the most suitable biomarkers for the diagnosis of active TB infection. Indeed, no pathogen biomarker is likely expressed during the entire course of the disease, and measurement of a limited suite of such biomarkers may be required for accurate diagnosis of infection. Simultaneous measurement of other biomarkers like ESAT6 and Ag85, to name a few, can increase confidence in biomarker measurements and prevent some false positives in the diagnosis of tuberculosis. For the effective application of pathogen biomarkers to disease diagnosis, expression profiles of these biomarkers as a course of disease progression should be assessed. For e.g.; sample 17 (Figure 3) presented with a relatively high concentration of LAM (17 pM) but was negative for active tuberculosis by physical examination and sputum microscopy. However, subsequently, it was discovered that this individual was positive for the Quantiferon Gold test but did not have clinical signs of disease. This individual may thus be indicative of very early onset of active disease. Assessing expression profiles of pathogen biomarkers during disease progression can allow us to establish reliable thresholds and identify suitable biomarkers for the detection of early disease. We are currently initiating studies to determine expression profiles of LAM in samples collected longitudinally as a function of infection. Similar studies on other biomarkers will help identify a limited suite of these biomolecules that can inform on infection irrespective of the state of disease or treatment.

Our studies show that LAM is undetectable in urine using an immunoblot after 2 h storage at RT using CS40 as the capture antibody. This might simply be a consequence of the fact that the epitope that CS40 binds to is modified or damaged at this time point. Exploring the binding of LAM in urine to a suite of monoclonal antibodies may better inform on the stability of the different carbohydrate moieties in urine. Indeed, chemical reactions in the urine may affect the stability of sugars such as LAM, a phenomena that remains poorly understood. Urea was known to form acid-catalyzed condensation products with aldoses in the early 1900s[26,27] and crystalline condensation of sugars with urea has been extensively reported.[28] Moreover, interaction of glucose with urea to form glucose ureide has been reported. This process results in the release of ammonia, which may significantly alter the pH of urine and result in addition of ammonia to sugars followed by the Amadori rearrangement. Chemistry associated with urea can also directly affect the antibodies used in detection. Depending on the pH, temperature and time of exposure urea can decompose into isocyanates that will rapidly react with all exposed amines on the antibodies, possibly interfering with their recognition and binding affinities for carbohydrate markers like

LAM. Although we are unsure of the exact biochemical process occurring in urine, and the actual impact it might have on antibody binding, we suggest immediate freezing of urine after collection to prevent any degradation or alteration that might adversely affect assay performance.

Lack of effective and stable recognition ligands is another major limitation to the effective use of pathogen biomarkers for disease diagnosis and detection. Whereas we were able to accomplish the sensitive detection of Ag85 complex (LoD, 500 fM) in serum, we were unable to construct an effective standard curve or evaluate expression of the antigen in patients primarily because of the poor stability of the AlexaFluor-647 labeled polyclonal reporter antibody (less than a week at 4 °C). Availability of better, more stable and sensitive, recognition ligands is critical to understanding pathogen biomarker expression in the host.

Another major limitation is the detection sensitivity of target antigens in a sandwich immunoassay format. Indeed, we have previously demonstrated detection of LAM with femtomolar sensitivity using a membrane insertion transduction on our optical biosensor (LoD ~10 fM) within 15 min,[20] compared to the 1 pM achieved herein by sandwich immunoassay. Evaluating alternative, more sensitive transduction schemes such as membrane insertion can further improve assay resolution, which may be critical in the detection of very low concentrations of pathogen biomarkers in the host.

Pathogen biomarkers may not only prove valuable for the early diagnosis of tuberculosis, but may also indicate relapse, prognosis and response to treatment. Future controlled large cohort studies will explore the efficacy of pathogen biomarkers to reliably inform on these features. Determination of accurate quantitative expression profiles of pathogen biomarkers is critical to their use in diagnostics. We speculate that perhaps a limited suite of such biomarkers is required to achieve accurate diagnosis irrespective of the stage of infection.

The waveguide-based biosensor can be used for the detection of biomarkers at very low concentrations in complex samples within minutes, which can be applied to rapid diagnosis of diseases in the future.[23] Our team has been working on the alternate transduction strategies that do not require a capture affinity reagent,[20] development of robust functional surfaces[21] and recognition ligands[17] and fieldable inexpensive chips (unpublished data form our laboratory) and miniaturized sensors[29] for the transition of this platform to the field in the future. However, the assays presented here are not limited to our sensor platform, but can be used in concert with other ultra-sensitive detection platforms. For e.g., mBio Diagnostics Inc. (Colorado) has developed an ultra-sensitive portable biosensor that is based on evanescent-field detection. Our assays can be readily adapted to such an existing platform to be of immediate value to the research and diagnostics community. The use of sensitive sensor systems such as that describe here as well as MBio's platform could be used to obtain the expression profiles of biomarkers (e.g., LAM, ESAT-6 and Ag85C) for *M. tb* infected patients as a function of treatment. Such expression profiles may prove to be useful not only in diagnosing active infection but also in tracking bacterial load and in the early recognition of relapse.

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