Tuberculosis 92 (2012) 38–47

Contents lists available at SciVerse ScienceDirect

Tuberculosis

journal homepage: http://intl.elsevierhealth.com/journals/tube

MOLECULAR ASPECTS

Understanding the interaction of Lipoarabinomannan with membrane mimetic architectures

Harshini Mukundan [a,*], Dominique N. Price [a], Matthew Goertz [b], Ramakrishnan Parthasarathi [e], Gabriel A. Montaño [c], Sandeep Kumar [a], Matthew R. Scholfield [a], Aaron S. Anderson [a], S. Gnanakaran [e], Srinivas Iyer [d], Jurgen Schmidt [a], Basil I. Swanson [a,**]

[a] Chemistry Division, Los Alamos National Laboratory, Los Alamos, NM 87545, USA
[b] Center for Integrated Nanotechnologies, Sandia National Laboratory, Albuquerque, NM 87185, USA
[c] Center for Integrated Nanotechnologies, Los Alamos National Laboratory, Los Alamos, NM 87545, USA
[d] Bioscience Division, Los Alamos National Laboratory, Los Alamos, NM 87545, USA
[e] Theoretical Division, Los Alamos National Laboratory, Los Alamos, NM 87545, USA

ARTICLE INFO

Article history:
Received 10 May 2011
Received in revised form
6 September 2011
Accepted 20 September 2011

Keywords:
Lipoarabinomannan
Amphiphiles
Diagnostics
Supported lipid bilayers
Biosensor
LAM atomistic model
Atomic force microscopy
Mass spectrometry

SUMMARY

Lipoarabinomannan (LAM) is a critical virulence factor in the pathogenesis of *Mycobacterium tuberculosis*, the causative agent of tuberculosis. LAM is secreted in urine and serum from infected patients and is being studied as a potential diagnostic indicator for the disease. Herein, we present a novel ultra-sensitive and specific detection strategy for monomeric LAM based on its amphiphilic nature and consequent interaction with supported lipid bilayers. Our strategy involves the capture of LAM on waveguides functionalized with membrane mimetic architectures, followed by detection with a fluorescently labeled polyclonal antibody. This approach offers ultra-sensitive detection of lipoarabinomannan (10 fM, within 15 min) and may be extended to other amphiphilic markers. We also show that chemical deacylation of LAM completely abrogates its association with the supported lipid bilayers. The loss of signal using the waveguide assay for deacylated LAM, as well as atomic force microscopy (AFM) images that show no change in height upon addition of deacylated LAM support this hypothesis. Mass spectrometry of chemically deacylated LAM indicates the presence of LAM-specific carbohydrate chains, which maintain antigenicity in immunoassays. Further, we have developed the first three-dimensional structural model of mannose-capped LAM that provides insights into the orientation of LAM on supported lipid bilayers.

1. Introduction

Lipoarabinomannan (LAM) is a cell wall lipoglycan and a key virulence factor for *Mycobacterium tuberculosis*.[1] Mannose-capped LAM is produced only by the slow-growing pathogenic mycobacteria such as *M. tuberculosis* and *Mycobacterium leprae*, suggesting a role in the pathology of tuberculosis (TB).[2] Indeed, the molecule has been shown to be critical in a number of functions including, but not limited to, phagocyte evasion,[3] inhibition of mycobacterial antigen processing by antigen presenting cells[4] and production of tumor necrosis factor.[5] For the purposes of this manuscript, the term LAM refers only to mannose-capped LAM produced by *M. tuberculosis*. The profound biological effects of LAM on the host cell have lead to its description as a mycobacterial endotoxin.[6] In addition, LAM has been shown to activate the innate immune response *via* toll-like receptors (TLRs), specifically TLR-2 and is therefore of interest in the design of vaccine and therapeutic strategies against TB.[7–9]

Several investigators have demonstrated the presence of LAM in urine[10] and serum[11] from TB patients. However, the application of this observation to disease detection or for tracking prognosis has been limited. The sensitivity and reliability of the only commercially available LAM assay for the diagnosis of TB (Clearview® TB ELISA, Inverness Medical Innovations, USA) remains controversial. Reither et al. reported poor sensitivity of this assay[12] whereas a more recent report[13] indicated a better sensitivity and potential application, especially in HIV-positive individuals. Availability of an

* Corresponding author. MS J567, C-PCS, Chemistry Division, Los Alamos National Laboratory, Los Alamos, NM 87545, USA. Tel.: +1 505 606 2122; fax: +1 505 667 0440.
** Corresponding author. MS J515, C-DO, Los Alamos National Laboratory, Los Alamos, New NM 87545, USA. Tel.: +1 505 667 5814; fax: +1 505 665 4355.
E-mail addresses: Harshini@lanl.gov (H. Mukundan), basil@lanl.gov (B.I. Swanson).

1472-9792/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tube.2011.09.006

ultra-sensitive and specific assay for LAM could prove invaluable in TB diagnosis and in the prediction of disease prognosis.

The sensor team at the Los Alamos National Laboratory has developed a waveguide-based optical biosensor platform for the rapid detection of biomarkers associated with disease.[14,15] Traditionally, we have used sandwich immunoassays on functionalized waveguides to detect pathogen biomarkers in complex background matrices. Herein, we demonstrate for the first time, a novel strategy for the detection of amphiphilic biomarkers (e.g. LAM) based on partitioning of the candidates into supported lipid bilayers. Detection of LAM using this approach is rapid (15 min), offers high sensitivity (less than 10 fM) using a polyclonal antibody, and most importantly, requires only one recognition ligand. Our experimental results, both using the optical biosensor and atomic force microscopy (AFM) clearly indicate that LAM partitions into the supported lipid bilayers by virtue of its acyl chain, and that this interaction is completely eliminated by chemical deacylation of LAM.

In addition to our experimental results, we have used molecular modeling approaches to develop the first three-dimensional structural model of LAM from *M. tuberculosis* and theoretically analyzed the interaction of LAM with supported lipid bilayers. Such a model is highly valuable to the interpretation of our experimental results and can facilitate understanding of the interaction of LAM or similar biomolecules with the host cell.

2. Materials and methods

2.1. Materials

The waveguide-based sensor was developed at the Los Alamos National laboratory.[16] LAM (14–19 Kda) from *M. tuberculosis* H37Rv culture, and the goat anti-rabbit polyclonal antibody and monoclonal antibodies for the antigen were procured by a materials contract from the Colorado State University (*via* BEI Resources). EZ-link Sulfo–NHS–LC-LC-Biotin and streptavidin were from Pierce. Alexa Fluor 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. All other assay reagents and MALDI matrices were from Sigma–Aldrich, unless otherwise specified. 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.[15] $SiON_x$ films have a thickness of $\sim$ 120 nm ($\pm$5 nm) and a refractive index of 1.80 $\pm$ 0.06. A thin $\sim$10 nm coating of SiO_2 is deposited on the active waveguide surface for functionalization with a lipid bilayer.

2.2. Waveguide-based detection of LAM

The novel supported lipid bilayer-based assay format is illustrated in Scheme 1. Rabbit polyclonal anti-LAM antibody was labeled with AF647 (succinimide ester) according to manufacturer's protocol (Invitrogen). Protein concentration of the labeled antibody was determined after purification on a Sepharose column, and the degree of labeling (i.e., moles of dye/mol of the protein) was calculated to be 4.5. The ability of the labeled antibody to effectively bind LAM was determined by immunoblot studies.

The essential components of the waveguide-based optical biosensor including the deposition of a supported phospholipid bilayer (waveguide functionalization) have been described before.[14,17] Briefly, single-mode planar optical waveguides were cleaned by sequential sonication in hexane, chloroform, and ethanol, followed by UV-ozone cleaning for 40 min, and assembly into a flow cell. DOPC vesicles were prepared by sonication, with or without 0.1% cap-biotinyl, and fused onto the cleaned waveguides, and allowed to stabilize for 12 h at room temperature. Functionalized waveguides were mounted on a flow cell holder and blocked for 30 min with phosphate buffered saline (PBS) containing 2% bovine serum albumin (BSA) as a blocking agent. After blocking, the waveguides were rinsed with PBS. In all experiments, the waveguide-associated background (intrinsic measure of impurities associated with the instrument itself) and coupling efficiency (typically 40–50%, incident power is 440 μW) were measured. After each addition, the flow cell was washed with PBS ($\sim$1.5 ml, 60X flow cell volume), unless otherwise specified.

Non-specific interactions 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, 5 min, RT). Specific detection of LAM was then measured by the addition of the antigen (spiked into bovine serum) to the flow cell (15 min, RT), followed by addition of the reporter antibody (100 nM, 5 min, RT). The fluorescence signal associated with the binding of LAM to the reporter was measured using the spectrometer of the biosensor platform. Concentration-dependent measurement of LAM in serum was evaluated by measuring varying concentrations of the antigen (i.e., 1, 10, 50 and 100 fM) spiked in bovine serum. These experiments were performed on separate waveguides, each of which was functionalized with DOPC bilayers.

2.3. Chemical deacylation of LAM

To determine the role of the acyl chain in the interaction of LAM with the lipid bilayer, LAM was chemically deacylated by the method of Brennan, Chatterjee *et al.*[2] LAM (1 mg/mL stock) was deacylated in 0.1 M NaOH (final concentration 20 μM in solution). Following 2.5 h incubation at 37 °C, acetic acid (1 M, 0.5 μL) was added to the tube to neutralize the pH to 7.0. The deacylated material was clarified by running on a hydrated Sepharose mini-G-25 column at 4500 rpm for 3 min. To remove the lipids from the deacylated material, a modified Folch extraction method was used. The deacylated material was diluted 20-fold with a 2:1 solution of chloroform: methanol and stirred (20 min, RT). Then, 0.2-fold volume of nanopure water was added and the mixture was agitated for 30 s, followed by centrifugation to allow separation of the layers. The top aqueous layer with the sugars was divided into two fractions. One fraction was dried under argon, dissolved in 1:1 acetic anhydride: pyridine, and heated at 100 °C for 4 h with stirring to achieve peracetylation of sugars. Both fractions were dried under argon, dissolved in acetonitrile, and analyzed by MALDI mass spectrometry.

MALDI-MS analysis of deacylated LAM was performed on a 4800 Plus MALDI-TOFTOF instrument (AB Sciex, Foster City, CA). The matrices used were either Indole acrylic acid (10^{-1} M solution in acetonitrile) or α-cyano-4-hydroxycinnamic acid (5 mg/mL in 50% acetonitrile, ammonium citrate was added to 10 mM final concentration). The sample was mixed with prepared matrix and spotted on stainless steel probes. Analysis was performed in both positive and negative ion reflectron modes at 0.81 detector voltage, and 600 total laser shots were averaged for each spectrum. Laser intensity was varied as necessary to optimize signal-to-noise ratios and resolution. When desired, MSMS was performed using 0.97 as the detector voltage multiplier, and 1500 total laser shots were averaged per spectrum.

The ability of the deacylated material to bind antibodies was evaluated by immunoassays. For this, intact and deacylated LAM was blotted on nitrocellulose membrane (3 μL, 1 mg/mL) and allowed to dry. The membrane was then blocked for 1 h with 2% BSA/1x PBS. The blots were probed with anti-LAM reporter