Journal of Microbiological Methods xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Detection of stealthy small amphiphilic biomarkers

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ARTICLE INFO

Article history:
Received 25 March 2014
Received in revised form 20 May 2014
Accepted 20 May 2014
Available online xxxx

Keywords:
Amphiphiles
Biomarkers
Phenolic glycolipid
Mycobactin
Lipid bilayer
Membrane insertion assay

ABSTRACT

Pathogen-specific biomarkers are secreted in the host during infection. Many important biomarkers are not proteins but rather small molecules that cannot be directly detected by conventional methods. However, these small molecule biomarkers, such as phenolic glycolipid-I (PGL-I) of *Mycobacterium leprae* and Mycobactin T (MbT) of *Mycobacterium tuberculosis*, are critical to the pathophysiology of infection, and may be important in the development of diagnostics, vaccines, and novel therapeutic strategies. Methods for the direct detection of these biomarkers may be of significance both for the diagnosis of infectious disease, and also for the laboratory study of such molecules. Herein, we present, for the first time, a transduction approach for the direct and rapid (30 min) detection of small amphiphilic biomarkers in complex samples (e.g. serum) using a single affinity reagent. To our knowledge, this is the first demonstration of an assay for the direct detection of PGL-I, and the first single-reporter assay for the detection of MbT. The assay format exploits the amphiphilic chemistry of the small molecule biomarkers, and is universally applicable to all amphiphiles. The assay is only the first step towards developing a robust system for the detection of amphiphilic biomarkers that are critical to infectious disease pathophysiology.

1. Introduction

Pathogens secrete characteristic molecules that may be useful for their growth, survival, and pathogenesis in an infected host, and are often signatures of infection (aka biomarkers). Many of these biomarkers, such as Lipoarabinomannan (LAM) from *Mycobacterium tuberculosis* (Kaur et al., 2009; Mishra et al., 2011), and lipopolysaccharide (LPS) from *Escherichia coli* (Arenas, 2012) (Fig. 1A), are not proteins, but are lipidated glycans (Ray et al., 2013) that are critical to bacterial virulence. Indeed, many such biomarkers are known innate immune agonists that are secreted and present in the infected host very early in infection, and thus are potential diagnostic targets for infectious diseases. Indeed, the direct detection of both LAM and LPS has been used for the diagnosis of tuberculosis (Minion et al., 2011) and food poisoning (De Boer and Heuvelink, 2000). The detection of said biomarkers directly in blood, however, has been elusive. As suggested by their biochemistry, many amphiphilic biomarkers do not occur in monomeric confirmation in the aqueous host vasculature, but are found in association with host carrier proteins. For instance, it has been demonstrated that LPS (Levine et al., 1993; Van Amersfoort et al., 2003) and LAM (Sakamuri et al., 2013a) associate with host high-density lipoprotein (HDL) in blood, and this interaction may play a critical role in the distribution, recognition, and clearance of these molecules. Understanding the interaction of amphiphilic virulence factors, and studying their distribution and expression in the infected host is therefore critical to efficient prevention, diagnosis, and treatment of infectious diseases.

Many amphiphilic virulence factors of relevance to bacterial pathogenesis are small molecules that cannot be efficiently studied by conventional methodologies because (1) conventional methods such as ELISA/lateral flow assays are associated with lower specificity and sensitivity, especially in complex biological matrices such as blood, and cannot be used to interrogate the very small circulating concentrations of bacterial biomarkers in the host; (2) for small molecules (e.g. phenolic glycolipids (PGL), of mycobacteria or bacterial siderophores; Ratledge, 2004; Spencer and Brennan, 2011), two recognition ligands that bind orthogonal epitopes are not available, precluding their detection by classic sandwich immunoassays. These biomarkers cannot be conjugated to classical surfaces (ELISA plates, nitrocellulose filter paper) because of their biochemical and solubility properties, making direct detection impossible. Beyond these, the use of conventional platforms (e.g. lateral flow, ELISA, and flow cytometry) developed for protein targets in the detection of lipidated sugars is challenging in itself. Because of these issues, direct detection of amphiphilic pathogen biomarkers, especially

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http://dx.doi.org/10.1016/j.mimet.2014.05.012
0167-7012/

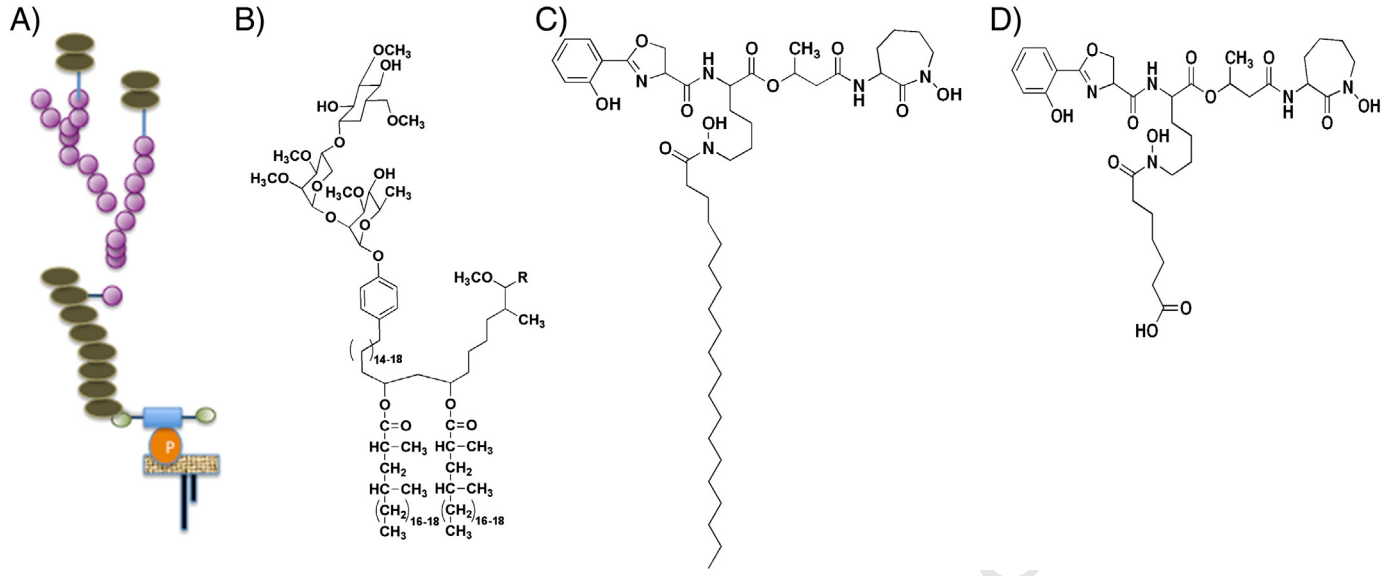

Fig. 1. Schematic illustration of the structure of (A) lipoarabinomannan, (C) MbT, (D) carboxymycobactin T from *Mycobacterium tuberculosis*, and (B) PGL-I from *Mycobacterium leprae* (not drawn to scale).

small molecules, is not extensively practiced, and biomarker-based detection of infectious diseases has largely relied on serological methods (Herrera et al., 2011). The development of effective strategies for the direct detection of small molecule non-protein biomarkers in a complex background can thus improve our understanding of bacterial pathogenesis, and identify novel diagnostic, vaccine, and therapeutic targets for infectious diseases.

As mentioned earlier, many biomarkers and virulence factors secreted by bacteria (Fig. 1) are amphiphilic, comprising hydrophobic (lipid) and hydrophilic (carbohydrate, peptide, or protein) moieties (Ray et al., 2013). Large amphiphilic biomarkers such as LPS, lipoteichoic acid (LTA, Gram-positive bacteria), and LAM (Fig. 1A) can be detected by sandwich immunoassays because antibodies recognizing orthogonal epitopes of the target are available (De Boer and Heuvelink, 2000; Minion et al., 2011), but such approaches cannot be used for the direct detection of small molecules such as phenolic glycolipid-I (PGL-I) of *M. leprae* and Mycobactin T (MbT) of *M. tuberculosis* (Fig. 1B and C). MbT is a siderophore secreted by *M. tuberculosis* for the sequestration of iron from the host. MbT and carboxy-MbT (Fig. 1C and D) are produced at high concentrations by mycobacteria *in vitro* during the logarithmic phase of growth under conditions of iron limitation (De Voss et al., 1999; Ratledge, 2004). PGLs are mycobacterial cell wall components and critical virulence factors. PGL in *M. tuberculosis* is speculated to be an indicator of hypervirulence (Onwueme et al., 2005; Reed et al., 2004). PGL-I in *M. leprae* is responsible for the neurotropism of the disease and is the basis for the serodiagnosis of leprosy (Cho et al., 1986; Moura et al., 2008; Spencer and Brennan, 2011; Young et al., 1985). This molecule has been found in large concentrations in tissues of experimentally infected nine-banded armadillos (Spencer and Brennan, 2011).

Herein we report a simple method, termed membrane insertion, to detect small molecule amphiphiles with a single recognition ligand by exploiting their association/interaction with a supported lipid bilayer. We have previously demonstrated the use of this technology for the detection of large molecules (amphiphiles and other), using a waveguide-based biosensor platform that was developed at Los Alamos Q2 National Laboratory (Mukundan et al., 2012a, b). The results reported herein represent the first step in translating this technology for the detection of small molecules that have a significant role to play in the pathophysiology of infectious diseases, but yet remain poorly studied to date. This is likely because of their complex biochemistry, and the fact that conventional assay technologies are largely tailored to suit proteins and nucleic acids, and not lipoglycans. The strategy presented in this manuscript is an assay transduction concept, and is not limited to any particular sensor platform for application, that applies to lapidated sugars of biological significance.

2. Materials and Methods

2.1. Materials

PGL-I and a rabbit polyclonal antibody for the biomarker were obtained from the Leprosy Materials Consortium at the Colorado State University (now BEI Resources). Mycobactin J was obtained from Allied Monitor. Human and bovine serum were purchased from Biomedical Technologies and Hyclone Laboratories, respectively. Alexa Fluor 647 (AF647) Protein Labeling kit was procured from Invitrogen. MbT and anti-mycobactin monoclonal antibodies were generated as previously described (Capek et al., submitted for publication).

2.2. Waveguide-based optical biosensor

Experiments were performed on a waveguide-based biosensor platform, which we have previously applied to the detection of biomarkers associated with breast cancer (Mukundan et al., 2009a,b,c), anthrax Q3 Q4 (Mukundan et al., 2010), influenza (Kale et al., 2008), and tuberculosis (Mukundan et al., 2012b) using either a sandwich immunoassay or membrane insertion assay as the transduction approach (Mukundan et al., 2012b; Mukundan et al., 2012a; Sakamuri et al., 2013b). Detection of biomarkers within the evanescent field of a planar optical waveguide enhances sensitivity, and minimizes background signal from excitation of fluorescence from impurities in complex biological samples (a result of the short penetration depth of the evanescent field into the sample above the waveguide) (Mukundan et al., 2009a,b,c). All measurements Q5 are made on an OceanOptics spectrometer interfaced with the instrument (Mukundan et al., 2009a,b,c). Q6

2.3. Preparation of fluorescently labeled antibodies

Antibodies specific to the biomarkers were labeled with fluorescent dyes (AF647), and characterized by indirect and competition immunoassays using established methods (Mukundan et al., 2009a,b,c). Anti- Q7 body concentrations and time of incubation were optimized using

Please cite this article as: Sakamuri, R.M., et al., Detection of stealthy small amphiphilic biomarkers, J. Microbiol. Methods (2014), http://dx.doi.org/10.1016/j.mimet.2014.05.012