Sensitive Serologic Detection of Pathogenic *Mycoplasma* via Electrochemical Biosensor

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Abstract

Mycoplasma pneumonia infects 2 million people every year and is responsible for upper respiratory infections and "walking pneumonia." Here, we describe the creation of a novel electrochemical biosensor capable of detecting pathogenic *Mycoplasma* for use in academic, research and clinical applications. Current diagnostics of *Mycoplasma*, such as molecular-based assays, PCR and serological analysis, are time consuming, expensive and not particularly accurate. Serological analysis is our main focus, not only because it is not available in the United States, but due to the fact that current "serological kits" do not measure the presence of the microorganism, but instead measure the host immune response, creating the possibility of a false negative result for most infected individuals. In response, our biosensor is designed for rapid, reliable, and reagentless serological detection of several common *Mycoplasma* strains. We are able to detect this subgroup of *Mollicute* bacteria by detecting a protein that is commonly secreted by many pathogenic mycoplasmas, P48 protein. A modified aptamer against P48 was incorporated into a custom oligonucleotide scaffold and is used in a gold-electrode-bound fashion to give a robust, dose-dependent electrochemical signal change upon binding the secreted P48 target. Ultimately, this biosensor should bring improvements to diagnosis and thus treatment of *Mycoplasma* in patients who present a proposed infection.

Introduction

Mycoplasma Characteristics

- Uniquely have small genomes and no cell walls.
- Wholly dependent on host for survival.
- Most common form is respiratory infection, but some strains are implicated in bacterial vaginosis and rheumatoid arthritis.[2]

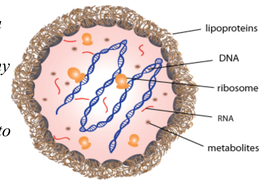


Figure 1: *This genus of bacteria lack a cell wall. Without a cell wall, they are unaffected by many common antibiotics, such as penicillin, that target cell wall synthesis, and are very difficult to detect.*[1]

- There are 16 known species isolated from humans. Out of the 16 species, six are known to cause diseases: *M. fermentans, M. hominis, M.genitalium, M. pneumoniae, Ureoplasma urealyticum*, and *U. parvum.*[1]
- *M. pneumoniae* and *M. genitalium* are most common in Humans.[1]

M. Pneumoniae

- Transmitted through airborne droplets from person-to-person.[2]
- If not detected early, endocytosis in the host cells occurs which aids in the establishment of a latent or chronic disease state, immune response evasion, and decreased drug therapy effectiveness.[2]
- Limited diagnostic methods are available. Efficient initial diagnosis is the key element to eradication.[2]

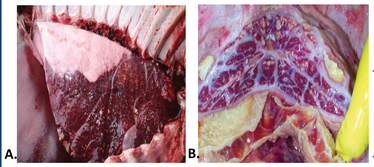


Figure 2: A. *Lungs of a calf that died from Mycoplasma pneumonia. Microabscesses are seen throughout the lung tissue.*[3] **B.** *Cross section of lung tissue with Mycoplasma pneumonia abscesses.*[3]

M. Genitalium

- An emerging cause of STIs and implicated in urogenital infections of men and women worldwide.[4]
- *M. genitalium* is an important etiologic agent of acute and persistent male NGU and is responsible for approximately 20-35 percent of non-chlamydial NGU cases.[5]
- It is a slow-growing organism. Culture can take up to 6 months, and only a few laboratories in the world are able to recover clinical isolates.[6]
- NAAT tests (polymerase chain reaction or transcription mediated amplification) for *M. genitalium* are available in some large medical centers and commercial laboratories.[6]
- There is no diagnostic test for *M. genitalium* that is cleared by the FDA for use in the United States.[6]

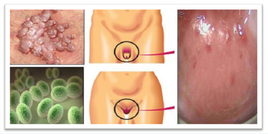


Figure 3: *Mycoplasma genitalium present in males and females.*[2]

Materials & Methods

Mycoplasma detection

Main detection techniques are PCR, Serology and Immunoglobulin M assays. Physicians must use a combination of serology and PCR to provide rapid, reliable, and accurate diagnosis of *M. pneumoniae* infections.[7]

"No available diagnostic test reliably and rapidly detects *M. pneumoniae*." *The Infectious Disease Society of America*

PCR
Advantages: Consistent results and high specificity.[7]
Disadvantages: Lack of commercial kits and false-negative result.[7]

Serology
Advantages: more rapid and accurate.[7]
Disadvantages: Not time efficient, reliable, or specific, and may render false positive results after about 7 days following the onset of disease.[7]

Immunoglobulin M assays
Advantages: Most sensitive testing method.[7]
Disadvantages: IgM response may by nonspecific or absent, particularly in adults.[8]

Biosensor
Advantages: Rapid, efficient diagnostic tool that can detect a specific desired molecular target, can function in complex media, and provide quantitative detection in minutes.[9]

Through **Biosensors**, we can streamline *Mycoplasma* testing into one procedure.

P48 Selection Criteria

- *Mycoplasma* is a difficult organism to detect. Through extensive literature reviews and BLAST analysis, we found a secreted outer membrane lipoprotein that is produced by most pathogenic *Mycoplasma* strains.
- P48 is an invariable, constantly expressed, immunodominant, surface lipoprotein homologue.[10]
- P48 homologs were found in the complete genome sequences from *M. pneumonia* and *M. genitalium.*[10]

P48 Biosensor Design

- An existing aptamer (a target-binding DNA sequence) was modified against P48 protein, discovered by China Agricultural University in 2014[11], into a sensitive electrochemical biosensor **Figure 4**.
- This aptamer was designed using the computational analysis program, Quikfold, in which the sequence was slightly modified to create both an On and Off folding-state at a 1-to-1 ratio.
- The On and Off state refer to the protein being bound (On) or unbound (Off), allowing for accurate, efficient electrochemical determination of P48 proteins' presence **Figure 4**.

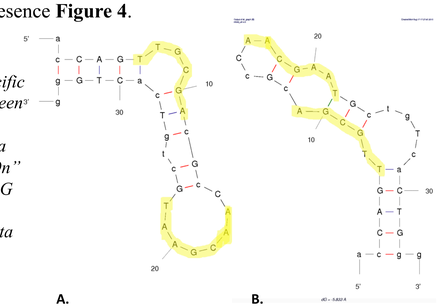


Figure 4: *P48 specific aptamer that has been engineered to sensitively bind to a given target.* **A.** *"On" bound state, Delta G of -5.5 A.* **B.** *"Off" unbound state, Delta G of -5.833 A.*

Procedure

Preparation of P48 for Sensor Design

Initial Plasmid

- Growing *Mycoplasma* in a laboratory setting is impractical. The contamination of cell cultures by mycoplasmas remains a major problem. Mycoplasmas can produce a number of unwanted effects in cultures/environments they infect and they are resistant to most antibiotics.
- P48 protein, which was expressed using pGEX-2T vector in E. coli, was obtained from a collaborator in Italy.
- Unfortunately, this plasmid did not grow correctly, likely due to mutation in the resistance gene. To correct this, we used PCR, restriction digest, and ligation to move to a new plasmid.

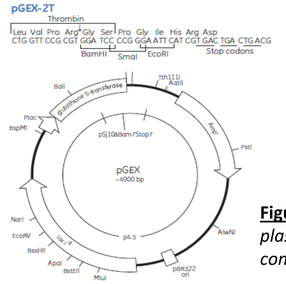


Figure 5: *pGEX-2T plasmid vector containing P48*

New plasmid utilized was pET22b. PET vectors have a high level of expression in a convenient fusion tagged system.

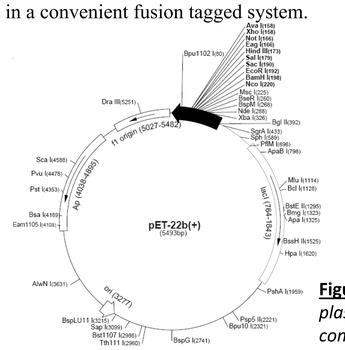


Figure 6: *pET22b plasmid vector containing P48*

P48 Induction Gel Electrophoresis

We see expression of P48 at 45kDA in the induced BL21 cells.

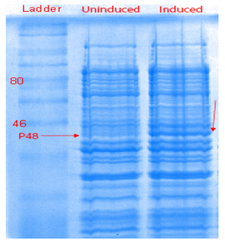


Figure 7: *PCR gel with P48 expression in induced cells*

Future Work

P48 Sensor Utilization

The biosensor uses a change in folded conformation to report target binding. Methylene blue was attached to a thymine predicted to be significantly different in its flexibility and distance from the electrode surface. This ensures a robust difference in electrical signal between the two folded states. In absence of target, the "non-binding" state, lowers the efficiency of electron transfer from a methylene blue (MB) reporter. In the presence of the target, the "binding" state, efficiently transfers electrons to the gold surface, and is gradually favored.[12]

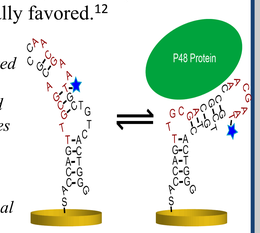
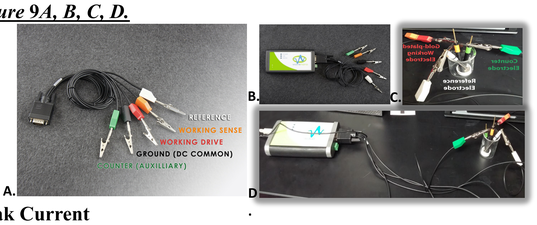


Figure 8: *Schematic of aptamer scaffold electrochemical DNA (E-DNA) biosensor directed against P48. A specific target-binding aptamer (red) is inserted into an oligonucleotide scaffold (black). A thiol group (S) on the scaffold attaches it to the gold electrode surface (yellow). The methylene blue molecule (blue star, attached to the scaffold) changes in response to aptamer-target binding, producing a measurable electrical current change.*[16]

P48 Diagnostic Testing

Purification and Isolation

High levels of this protein are tested for real world diagnostic performance. The affinity and detection limit of each biosensor will be tested via square wave voltammetric scanning of equilibrated solutions containing P48 protein. Instruments utilized can be viewed below in ***Figure 9A, B, C, D.***



Peak Current

Peak current from methylene blue electron transfer will be analyzed using custom peak fitting software.[12] E-DNA biosensors display equilibrium signal change in response to target concentration which yields an apparent dissociation constant (KD) value after the data is fitted with a custom peak-fitting algorithm in response to the concentration of the target.[12]

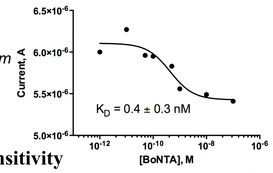


Figure 10: *Example of Fitted Data from E-DNA Biosensor for the Detection of Botulinum Neurotoxin A.*[12]

Determination of Biosensor Sensitivity

The number of mycoplasmas are expressed in terms of colony-forming units (CFUs), color-changing units (CCUs), or copy numbers. One CCU is estimated to contain 10–100 organisms. Between 102 and 104 CCU/mL are present in respiratory secretions during acute infection. We will use these values to assess the sensitivity of the Biosensor and ensure that this sensor can be used in whole blood.

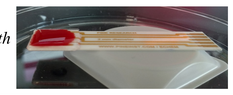


Figure 11: *Ceramic patterned microelectrode with protective acrylamide hydrogel isolating whole bovine blood from sensor surface*

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