Enhancement of Lateral Flow Assay for the Detection of Whole Viral Particle and Chlamydial Elementary Bodies

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Enhancement of Lateral Flow Assay for the Detection of Whole Viral Particle and Chlamydial Elementary Bodies

A Thesis Presented

by

JEFFREY M. GRIMES

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

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Enhancement of Lateral Flow Assay for the Detection of Whole Viral Particle and Chlamydia Elementary Bodies

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ABSTRACT

ENHANCEMENT OF LATERAL FLOW ASSAY FOR THE DETECTION OF
WHOLE VIRAL PARTICLE AND CHLAMYDIAL ELEMENTARY BODIES

FEBRUARY 2014

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*Chlamydia trachomatis* accounts for 3.6% of blindness worldwide, and is the leading cause of bacterial-induced blindness in the world. With the subtle initial presentation of the disease and the difficulty in clearing the infection without the aid of antibiotics, *C. trachomatis* can spread rapidly following introduction into a population. This problem is further compounded in resource limited areas due to the lack of trained personnel (i.e. Medical Doctors, Nurses), equipment, and finances to test and treat large portions of the population. A testing method that is both cheap and easy to interpret is necessary. Lateral flow assays (LFA) have been used for years to evaluate pregnancy status in the developed world, and their low cost, ease of use and disposable nature make them a worthwhile candidate, but the current use of visual reporters (i.e. gold or latex nanoparticles) does not allow for adequate sensitivity for true clinical use. Fluorescent reporters, particularly fluorescent nanoparticles, would lower the limit of detection (LOD) and allow for the detection of acute and subclinical infections, which would allow for an effective and objective screening method for trachoma and many other diseases. An effective, rapid, and disposable test would allow for mass screening to be
implemented which, in turn, would allow for rapid and targeted treatment. The results in this study show that the use of fluorescent-based reporters greatly improve the LOD of the LFA, with both FITC and RuSNP reporters showing a reduction in the LOD by 1 and 2.5 logs respectively when compared to traditional colorimetric reporters. This substantial improvement in the LOD of the LFA allows for the tests to be used to detect relevant levels of viral pathogens. A similar improvement in the LOD was seen when using FITC-labeled antibodies which improved the sensitivity of LFAs with regards to the detection of *C. trachomatis*. The use of fluorescent-based reporters in LFAs greatly improves the LOD for both viruses and bacteria, allowing for their detection at clinically relevant levels.
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LIST OF ABBREVIATIONS

Acquired Immunodeficiency Syndrome – AIDS

Actin related protein 2 and 3 - Arp 2/3

Antibodies – Abs

Corneal Opacification – CO

Cysteine-rich proteins – CRPs

Elementary body – EB

Enzyme-linked Immunosorbant assay – ELISA

Follicular trachoma – TF

Lateral Flow assay - LFA

Light Emitting Diodes - LED

Major outer membrane protein – MOMP

Mass drug administration - MDA

Penicillin binding proteins – Pbp

Peptidoglycan – PG

Point of care – POC
CHAPTER 1

INTRODUCTION

Diagnostics play a critical role in health care worldwide, allowing for the verification of the integrity of the food/water supply and allowing for effective monitoring and timely treatment of diseases. The benefits of a wide-spread and comprehensive diagnostic monitoring program for endemic disease can be best exemplified in the decrease and resurgence of African sleeping sickness, which reached historic lows (< 2000 reported new cases) in the 1960’s predominantly due to wide-spread screening, but steadily rose as funding for the monitoring and control measures fell off [1-3]. While the necessity for diagnostics is ubiquitous, the political, social, and health care infrastructures that are paramount to the introduction of effective health care vary greatly between countries [4–6]. Further complicating the issue, the current gold standard diagnostic tools (i.e. Polymerase Chain Reaction (PCR), culture) are too costly for a comprehensive screening of an entire population [6].

While the burden of disease in the developed world is predominantly lifestyle related, the developing world suffers greatly from communicable diseases and, increasingly, lifestyle diseases as the average life span continues to increase [5, 7]. The current cost and limited availability of trained personnel capable of employing modern diagnostic tools leads to a large population which is left undiagnosed and untreated for serious illness [7-9]. The development, and therefore the availability, of cheap diagnostics has lagged behind, mostly due to funding being predominantly focused
towards the development of new antibiotics and vaccines. Recent developments such as
the rise of antibiotic resistance, the threat of pandemics due to globalization, and the
spread of disease with no current cure (i.e. Human Immunodeficiency virus (HIV)) have
led to increases in funding for Point of care (POC) diagnostics. Increased funding for
low-cost POC diagnostics, coupled with the advancement of lab-on-chip and smart phone
technology, is allowing for a greater degree of sensitivity and accuracy, generating results
that are nearing the sensitivity of PCR [2–5]. The currently deployed POC diagnostic
tests have a high rate of error (19%-96% accuracy) however, as they depend heavily on
the end user’s ability to follow and execute instructions [7-13]. As the optimal test would
be simple for a lay person to use, greater sensitivity and fidelity is needed to compensate
for end-user error, while keeping the test simple and inexpensive.

1.1 Chlamydia trachomatis

*Chlamydia trachomatis* is an obligate intracellular Gram-negative bacterium,
belonging to the family Chlamydiaceae and the genus *Chlamydia*. There are nine species
in the genus *Chlamydia*, with *C. trachomatis* and *C. pneumoniae* being prominent human
pathogens [14]. *C. trachomatis* is the causative agent of trachoma, a disease that is
responsible for 3% (~39 million cases worldwide) of reported cases of blindness,
predominantly in tropical developing countries with limited resources. *C. trachomatis* is
also a prominent causative agent of several sexually transmitted diseases (STDs). With
more than 92 million new cases occurring worldwide each year, *C. trachomatis* is the
largest single cause of bacterial-induced STDs and blindness globally [15-19]. Due to the
ability of *C. trachomatis* to invade numerous tissues, this one bacterium can elicit a wide range of STDs, including ophthalmia neonatorum and pneumonitis in infants, pelvic inflammatory disease (PID), proctitis, urethritis, cervicitis, inguinal lymphadenitis and reactive arthritis in adolescents and adults. *C. trachomatis* has also been correlated to squamous-cell carcinoma of the uterine cervix and is a complicating and enhancing factor in HIV-1 infection and transmission [20-24]. Issues stemming from *Chlamydia* are compounded by the fact that large percentages of the infected population are asymptomatic for urogenital infections, meaning that ~ 80% of women and ~ 50% of men are left untreated. This wide-spread sub-clinical population has the potential to cause numerous new cases of *C. trachomatis* infections and acts as a large reservoir for the disease, which perpetuates several STDs [25-27]. Another complicating factor is that protective immunity appears to be transient. This is due to *C. trachomatis* actively down-regulating MHC class I and class II antigen presenting pathways, which is a contributing factor to the large number of asymptomatic infections [28].

The development of an inexpensive, rapid, accurate, and simple test is needed for wide-spread screening of *C. trachomatis* to be a viable option. The currently deployed methods for screening for *C. trachomatis* are not viable for a large population, making the development of a cost effective screening method invaluable in decreasing the morbidity from *C. trachomatis* infections [29, 30]. Furthermore, while many *C. trachomatis* infections are thought to be effectively treated by antibiotics, follow-up studies have revealed that numerous patients develop re-infection, treatment failure or *C. trachomatis* persistence [31-37].
1.2 Trachoma

1.2.1 Trachoma Pathology and Clinical Features

The causative agent of endemic trachoma are four serovars (A, B, Ba, and C) of *C. trachomatis*, and while other serovars can causes conjunctiva these are isolated incidents, and do not lead to blinding. The first acquisition of trachoma likely occurs at a young age (1-10 years old), although the age of initial infection varies depending on the prevailing level of infection of the region, with hyper-endemic levels leading to infections in infants [37, 38].

Following initial infection, *C. trachomatis* appears to spread only to surrounding epithelium and does not penetrate into deeper tissues. The clinical signs of active trachoma are a result of the host immune system reacting to the infection, with typical signs including marked inflammatory cell infiltrate and the release of pro-inflammatory cytokines in the conjunctiva [39-41]. The clinical symptoms are papillary with or without follicular inflammation of the tarsal conjunctiva. As the infection is resolved the visible inflammation gradually subsides. There is a large correlation between age and active trachoma infections, with the prevalence and duration of the infection decreasing with age, implying that a maturation of the immune response is occurring [42, 43]. Yet, the development of an effective vaccine has proven difficult, as the immunity acquired appears to be transient and strain specific, leading to repeated infections in endemic environments [44, 45]. The reoccurring infections lead to chronic inflammation, causing
scar tissue to build within in the conjunctiva over time [46, 47]. Following sufficient accumulation, the scar tissue will contract, causing the eyelids to roll inwards towards the eye (entopion) and allowing the eyelashes to scratch the cornea (trichiasis). The most debilitating disease from trachoma is blindness due to corneal opacification (CO), which is severe corneal damage due in part to trichiasis. Trichiasis alone is not able to cause CO, however, and a number of other factors contribute, such as chronic inflammation of the conjunctiva and additional bacterial infections [48, 49].

1.2.2 Epidemiology

While trachoma affects large portions of the globe (see figure 1), with blinding trachoma endemic in 56 countries, Sub-Saharan Africa has the highest prevalence of active trachoma and trichiasis [49-51]. In 2012, the World Health Organization (WHO) reported ~22 million cases of active trachoma and ~7.2 million cases with trichiasis. Prior to that, WHO’s comprehensive burden of disease study, released in 2004, suggested that the prevalence of disease was ~40 million people with active trachoma and ~8.2 million with trichiasis [53]. These current estimates represent a substantial decrease, as the previous study estimated the current burden of active trachoma at ~84 million [54]. The substantial decrease in estimated cases has been linked in part to over-estimations of the burden of active trachoma in India and China. In the past, trachoma had a far wider distribution, affecting both Europe and North America. As sanitation and living conditions improved, manifestations of trachoma declined, and currently trachoma is not a health risk in Europe or North America, despite a large population infected with C. trachomatis [55].
While clinical manifestations of trachoma vary with age, young children act as the main reservoirs for active trachoma infections, and as they age the frequency and duration of the infection decreases [56, 57]. The conjunctival scarring that occurs accumulates with age and repeated infections, and does not typically become evident until the second or third decade of life [58,59]. The more severe repercussions of trachoma, such as Entropion, trichiasis, and CO, tend to develop later in life, but in hyper-endemic regions blinding can occur in children [60]. Women have been found to suffer trichiasis and CO at a higher rate than men, likely due to women having closer contact and greater interaction with children, the main reservoir of disease [59,61].

Risk factors for trachoma are typically related to increasing the transmission of *C. trachomatis* from infected to non-infected individuals [62]. Copious secretions found around the eyes have consistently been associated with active trachoma, and close contact with these secretions promotes transmission and attracts flies, which act as vectors for transmission. Another risk factor is the scarcity of water and its related cleanliness, as face washing reduces the transmission of *C. trachomatis*. An additional compounding factor is the lack of latrines, as fecal contamination provides breeding material for the flies [63]. Crowded living conditions and limited resources are additional factors that promote transmission. In resource-limited areas, the slow development of changes needed to reduce the burden of trachoma, coupled with current demographic trends and the lack of effective control programs, predicts an increase in the total number of people blinded by trachoma [64].
Figure 1. Global Distribution of Trachoma. Countries colored deep orange are those with endemic trachoma while those colored light blue are those being monitored for trachoma. The uncolored countries have no reported incidence of trachoma. These data are based on WHO’s Global Study of Disease 2008 [54]. The map of the earth is open source from http://misterjogja.com/wp-content/uploads/2013/04/blank-world-map-qh51jnu.jpg and it was hand-colored by the author.

1.2.3 Burden of Trachoma

The global burden due to trachoma is not heavily influenced by mortality, but rather morbidity, mainly due to visual impairment and trichiasis. The visual impairment is divided into two categories: Blindness and Low Vision. Blindness, as defined by WHO, is visual acuity of less than 3/60 (20/400, ICD-10 visual impairment categories 3, 4, and 5). Low Vision, as defined by WHO, is visual acuity of less than 6/18 (20/60, ICD-10 visual impairment categories 1 and 2) [65]. The visual impairment or blindness has a profound effect beyond the individual, as the person’s ability to function and provide for his/her family is severely reduced, often creating a greater burden on the family and
community. This in turn helps to perpetuate the poverty, shown to be a contributing factor in contracting trachoma. Trichiasis, while not immediately inducing blindness, has a major impact on quality of life and can affect functionality in everyday life, typically by causing pain and photophobia. A study of women from Tanzania showed that sufferers of trichiasis without visual impairment sustained comparable disability to those individuals with visual impairment induced from sources other than trachoma [66].

The difficulty in estimating the burden of disease induced by trachoma is that it does not cause mortality rapidly or directly, but rather induces morbidity, and so new measures were developed to evaluate the burden caused by trachoma. These measures were developed in the Global Burden of Disease study (GBD) [67]. The GBD study developed a new measure of the burden of disease: Disability-Adjusted Life Years (DALYs). This measures the gap in healthy life lost between an ‘ideal’ healthy population and the reality caused by a specific disease in terms of premature mortality and disability in a population. DALYs are calculated based on two major components: premature mortality, or years of life lost (YLL), and the years of healthy life lost due to a disability caused by a disease, years of life lived with a disability (YLD). YLD is a weighted measurement based on the number of people dying or disabled from a disease in a given population which allows for the severity of the disability to be taken into account - the more severe the disability, the greater the number of YLDs that are lost [67].
The GBD study estimated the burden of trachoma to be about 1.3 million DALYs annually, due mostly to YLD. This estimate used WHO figures for the number of people affected by trachoma at that time. Another estimate was made by Frick and colleagues in 2000, but in their calculation they only used YLD, as they considered YLL estimates to be unreliable [68]. They then re-estimated the number of people with blindness from trachoma worldwide based on the reassessment of survey data collected since 1980. Following that, based upon previously published data, they estimated that for each person blinded by trachoma there were an additional 1.4 affected with low vision. These changes lead to an estimation of the annual DALYs of 3.6 million, with sub-Saharan Africa constituting the bulk of those affected globally at 72% [68].

Although the estimates of the burden of trachoma are a useful tool for measuring the affect of trachoma, they are skewed due to several factors. The most significant issue is that the calculations rely on data gathered on the prevalence of endemic trachoma, and while the most recent estimates have become more accurate, an accurate accounting of the global prevalence of trachoma is still difficult to obtain. Secondly, trachoma’s affect on DALYs is predominantly measured in YLD, as there is a lack of comprehensive data relating to the degree in which trachoma-related blindness affects premature mortality. One study has investigated the correlation between visual impairment and increases in mortality in rural communities located in sub-Saharan Africa. They found that blind people had increased rates in mortality compared to sighted controls [69]. Additional studies are needed to investigate this question for trachoma. Finally, there is some debate over the inclusion of trichiasis as a disabling disease sequel, independent of visual
impairment. Frick and colleges reported that trichiasis, independent of visual impairment, increased the burden of disease caused by trachoma by as much as 50% [70].

1.2.4 Current Methods of Treatment, Control, and its Cost-Effectiveness

The current and most widely deployed method for the treatment and control of trachoma is called SAFE: surgery for trichiasis, antibiotics for infection, facial cleanliness, and environmental improvements to reduce transmission. The SAFE strategy was developed and promoted by WHO and GET2020. GET2020 was established following the 1998 World Health Assembly, which resolved to eliminate blinding trachoma by the year 2020, and is a collaboration between the WHO, national trachoma control organizations, industry, non-governmental organizations, and academic institutions [71, 72].

The current body of data supports the conclusion that the SAFE strategy is clinically effective [73]. Trichiasis surgery has been shown to effectively prevent additional damage to the eye and prevents the onset of blindness while increasing quality of life. However, there are several problems with both the availability of the surgery and a patient’s willingness to undergo surgery. Even when the surgery cost is covered, patients are at times reluctant to undergo corrective surgery due to several factors: burden of household tasks, indirect cost of surgery, lack of companion, and fear of surgery. Additionally, as the traveling distance increased to greater than one hour, the likelihood that suffers of trichiasis would seek corrective surgery decreased [74]. While corrective
surgery is effective in the short term, one study showed that surgery alone is not effective for combating blinding from trachoma in the long term, due to a relatively high rate of reoccurrence of trichiasis in countries with endemic trachoma [75,76].

Antibiotic treatment reduces the risk of active trachoma and ocular chlamydial infection in people infected with \textit{C. trachomatis}, and the antibiotics regiment currently recommended by the WHO is oral azithromycin, single dose, or topical tetracycline, twice daily for 6 wk. In clinical trials, both of these antibiotics have been shown to be effective at reducing the prevalence of both active trachoma and \textit{C. trachomatis} infection [77, 78]. However the clinical signs of active trachoma are subtle, and at times it is difficult to identify a \textit{C. trachomatis} infection. Compounding this issue is \textit{C. trachomatis’} ability to rapidly reemerge in populations where some infected cases are left untreated. Systematic and opportunistic screening has been purposed as a possible method to identify and more effectively treat chlamydial infections, but currently deployed diagnostic methods would make such an endeavor cost prohibitive [79]. Therefore, the current recommendation is for mass drug administration (MDA) of entire communities in endemic regions, which are conducted annually for several years, until the prevalence of follicular trachoma (TF) in children ages 1–9 years drops below 5% [80]. Studies show that when yearly treatment is halted TF resurgence is observed. This was seen in two regions of Mali, where following the cessation of a 3 year MDA program rates of TF prevalence in children ages 1-10 years increased from ~2% to ~8% [81]. Additionally, since MDA calls for treatment until the prevalence of TF drops below 5%, large portions
of the population are being treated with antibiotics without need, which has led to increases in antibiotic resistant bacteria [82].

The last two components of the SAFE strategy, face washing and environmental interventions, have mixed but limited published data supporting or refuting their effectiveness [83]. One study showed that when health education was given, the incidence of active trachoma was lower in the village given health education than in the control village at six months [84]. However, a study preformed in 2010 on the effect of health education and a clean water supply on the rate of trachoma found no statistically significant difference between the control villages and the intervention villages [85]. Similarly, contradictory reports on the effectiveness of fly control through the use of insecticides have been reported on. One study showed a reduction of the prevalence of active trachoma by 55.8% while another report showed no statistically significant change in rates of trachoma between test and control populations, 43% versus 44%, following one year of insecticide use [85, 86]. While conclusive data is difficult to attain, historical and observational studies strongly support the view that general improvements in hygiene and the control of disease vectors can have a profound long-term effect on this disease [55].

Several estimates have been made for the cost-effectiveness of the SAFE strategy. For trichiasis surgery, the cost, varying by region, is estimated to be about 19 International Dollars (I$) per case in Africa, with a comparable cost in other resource
limited regions. Surgery was found to be very cost-effective, with estimates ranging from I$13 to I$78 per DALY, depending on the region [87, 88]. In contrast, the cost-effectiveness of antibiotic treatment is more debatable, with estimates ranging from I$9,000 to I$65,000 per DALY prevented. MDA does become more cost effective if the antibiotics are either donated or sold at a reduced cost, however [87]. Several investigators have produced estimates of the cost-effectiveness of trachoma control programs or individual components of the SAFE strategy. A comprehensive analysis of the entire SAFE strategy has yet to be performed.

1.3 Current Methods of Diagnostics

1.3.1 Polymerase Chain Reaction

Since PCR’s development in 1983 by Karry Mullis, it has become an indispensable tool in the diagnosis of a wide array of disease, along with numerous other uses [88]. PCR functions based upon the use of a thermally stable DNA polymerase, allowing for repeated annealing and melting of the primer and template following each round of amplification. This allows for PCR to amplify the amount of target DNA present by several million-fold following 25 cycles. This in turn allows for a very high degree of sensitivity, and theoretically allows for the detection of a single copy of the target DNA [89].

For the detection of *C. trachomatis*, several types of PCR assays are widely employed: ligase chain reaction and strand displacement amplification assays, both of
which target nucleotide sequences on the cryptic plasmid, and transcription-mediated amplification reaction, which targets ribosomal RNA. Both of these targets have multiple copies, allowing for greater sensitivity. As there are multiple copies of the target sites, PCR is theoretically capable of detecting less than one elementary body (EB), and has been shown to do so in purified suspensions of Chlamydia [90]. Evaluations of these amplification methods have demonstrated that PCR has a higher degree of sensitivity than culture, microscopy, or current immunoassays [91, 92].

While the theoretical LOD is one copy, the actual sensitivity with clinical specimens is lower, mostly due to sampling variability and the inhibition of the amplification reaction from various factors in the specimens [93]. While PCR assays are highly accurate and have a great degree of sensitivity, they all are limited in their application as a POC diagnostic for several reasons. First, the process of PCR is easily contaminated, and stringent handling procedures are needed in order to prevent contamination. Secondly, while not as time intensive as culture based diagnostic methods, the amount of time needed to properly prepare samples, perform the assay, and interpret the results is still substantial [94]. Finally, the cost of the required equipment and highly trained personnel to perform the PCR excludes it as a viable diagnostic test for wide-spread use in developing countries and limited resource settings [93-95].
1.3.2 Culture Based Diagnostics

While there have been great advances in molecular diagnostics, predominantly due to real time PCR, the growth of *Chlamydia* (or any pathogen of interest) in culture is still considered, by some, to be the gold standard for diagnostics [96, 97]. The ability to grow *Chlamydia* in a cell line allows for very high degree of specificity, potentially able to detect a single viable EB. It has further benefit over molecular diagnostic techniques in that it will only detect infectious *Chlamydia*, as PCR and immune diagnostics will detect live or dead cells. Also, all serovars of *Chlamydia* appear to be able to grow in cell culture if the inoculum is centrifuged onto preformed, pretreated cell monolayers [98]. Culture based diagnostic methods are highly dependent upon the person performing the cell culture, however, and inexperience can lead to poor results. The relatively long period of time required (48-72 hours) and the necessity for highly skilled personnel to gather the sample, perform the subsequent preparation, culturing, and microscopy of *Chlamydia* make this method unsuitable for POC diagnostics [96-99]. Also, as *Chlamydia* are obligate intracellular pathogens, they must be grown in a host cell, adding an additional cost and complication to the procedure.

1.3.3 Direct Cytological Microscopy

While there are numerous molecular tools available for diagnostics, microscopy still remains an invaluable method for diagnosing a number of diseases in the developing and developed world. It is still considered one of the most common and reliable methods for diagnosing two of the most prevalent disease in the developing world: tuberculosis
and malaria [99-103]. Trachoma is also diagnosable by direct cytological examination via the observation of typical intracytoplasmic inclusions. This type of screening is most effective in detecting acute inclusion conjunctivitis in newborns, and it compares favorably to culture based methods with a sensitivity of ~90% [100]. However, this form of screening is relatively insensitive in regards to diagnosing conjunctivitis in adults. One of the greatest difficulties in using microscopy in the diagnosis of trachoma is the expense and limited availability of microscopes. Several recent developments have made the use of microscopy more appealing for POC diagnostic purposes, however, including light emitting diodes (LEDs) and the use of smart phones in place of a fluorescent microscope. LEDs have many advantages over traditional mercury vapour fluorescent bulbs: they are able to run on batteries, can be manufactured to produce a specific wave length of light, negating the need for a filter, and they perform equally well without a dark room [100–102]. In light of this, the WHO has recommended a shift away from traditional fluorescent microscopy bulbs to LEDs [103]. The ability of a smart phone to replace and function as a fluorescent microscope is a recent innovation. The Ozcan Group at UCLA has created programs and minor attachments for smart phones which allow them to read fluorescent signals and function as a microscope at a fraction of the cost of traditional microscopy equipment [104]. While these advances have made microscopes more readily available, difficulties in the wide-spread use of this method still remain, as taking a proper image is only a portion of the problem. Trained personnel capable of properly preparing, staining, and then analyzing and imaging the samples are still required, representing the most significant barrier to the use of microscopy as a POC diagnostic test.
1.3.4 Electrochemical Detection

Electrochemical detection methods are used in several POC diagnostic devices, most widely in the blood glucose assay. The modern glucose test meter functions based on reading electrochemical signals generated by either glucose oxidase or glucose dehydrogenase [105-107]. A blood sample is placed on a test strip and is then driven up the strip due to capillary action. Glucose in the blood reacts with an enzyme on the electrode containing glucose oxidase (or dehydrogenase). The enzyme is then reoxidized with an excess of a mediator reagent, which is in turn reoxidised by the reaction occurring at the electrode, generating a current which is then measured in order to calculate the amount of glucose present [108]. While this works highly efficiently with detecting the glucose levels in blood, many of the desired analytes lack glucose or electroactive molecules. To overcome this, several studies have used Abs tagged with either an electroactive molecule or an enzyme that converts an electrochemically silent species into an active one, allowing for a signal to be generated [109, 110]. Several publications have shown that this technique has a very low LOD, with limits below 1 pM. One study showed that Staphylococcal enterotoxin B could be captured with specific antibodies (Abs) that were attached near the electrode, followed by the attachment of a secondary Ab which was tagged with horseradish peroxidase in the presence of a set amount of reactant, allowing them to measure and quantify very minute amounts of the Staphylococcal enterotoxin B. [111-113].
1.3.5 Enzyme-linked Immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) is the product of a series of developments pioneered in the 1970’s which allowed for a highly sensitive test that is relatively easy to perform when compared to culture-based methods. The ELISA functions by using specific Abs against a desired antigen which are absorbed onto a surface, typically a 96 well plate. This is a version of an ELISA known as a sandwich ELISA, in which the antigen is then placed in the well with an additional primary Ab which targets a separate antigen from the Abs bound to the well. Following a washing and blocking procedure to inhibit and remove non-specific binding, a secondary Ab is used which is conjugated to an enzyme (i.e. peroxidase) which will catalyze a color change, denoting the presence or absence of the antigen [114].

There are currently several commercially available ELISAs for the detection of Chlamydia which are relatively sensitive, with accuracies ranging from 62% to 98% and specificities ranging from 92% to 100% depending on the sampling location and the gender that the sample was derived from [115, 116]. While the specificity is high, it can be jeopardized by cross-reactivity with other organisms. This cross-reactivity makes it necessary, at times, to confirm a positive result by testing a sample via immunofluorescence with a major outer membrane protein (MOMP)-specific monoclonal Ab, or by blocking suspected antigens causing the false positive, a process which is commonly included in some ELISA kits. Detecting false negative results presents an additional problem, as the implausibility of checking all negative results leads to insensitivity in the assay. While these problems do not prohibit ELISAs from being
employed as a diagnostic method, they do make the test rather unsuited for POC diagnostics in resource limited settings, as the test needs both a clean environment in order to prevent contamination and trained personal to properly employ the test.

1.4 The “Optimal” POC Assay

There has been a substantial upsurge in both the development and the availability of POC diagnostic tests for several diseases and medical conditions. The general push in diagnostics has been for the creation of tests that are rapid and intuitive, most notably in resource limited areas. While several different health and drug evaluation organizations have created desired criteria for what could be described as the “optimal” POC diagnostic assay, there are no rigid standards in the field. In 1988, a law entitled Recommendations for Clinical Laboratory Improvement Amendments was passed by Congress, which strictly regulates and monitors laboratory work that is considered complex and has a high potential for error. It also includes a set of criteria by which a test may be waived from these requirements if it met the U.S. Food and Drug Administration’s (FDA) definition of a simple device. The FDA defines a simple device as: fully automated, unitized or self-contained tests, using direct unprocessed samples, requiring only simple non-technique dependent manipulation of sample or reagents, needing no operator analysis or intervention, and having a simple to read and understand output requiring no calculations. While these criteria were designed for simplicity in clinic diagnostic settings, they also meet the desired parameters for a diagnostic test to be used in resource limited settings [117]. The WHO has also created criteria for a POC test: a POC diagnostic test should be
affordable by the population at risk, sensitive (>43%), specific (98%), easy to use and interrupt, equipment-free, and accessible to the population which requires it [111]. These two standards are an excellent place to start when looking to define the requirements for an effective POC test for resource limited settings, in that it be simple to use and interrupt by the lay person using it, with a high degree of specificity, is inexpensive relative to the end user, and the sample used is easy to acquire and needs little to no processing.

1.5 Currently Available POC Diagnostics

1.5.1 Lateral Flow Assay

The LFA is a common tool for at-home and POC diagnostics, with commercially available LFAs quickly becoming more prominent. LFAs function through the use of Ab-antigen interactions which target a specific antigen, followed by the use of a colorimetric indicator, usually either gold colloidal or latex nanoparticles (np), to represent a positive result [13]. The LOD and the sensitivity of these tests are limited, however, with 19%-96% accuracy when compared to current detection methods such as PCR or culture techniques. These tests are further limited by the use of the human eye and subjective judgment to determine whether the test line has sufficient intensity to be considered positive [13, 53, 54, 118-121].

LFAs have been used for decades in at-home pregnancy tests, and more recently at-home tests for Acquired Immunodeficiency Syndrome (AIDS) and Chlamydia have
become commercially available [121]. The principle of a LFA is based on two commonly used molecular tools: immuno-labeling and -precipitation. In a commercially available LFA, the sample is placed on a sample pad which, while porous, is used to exclude larger particles that could block the capillaries in the porous membrane of the test strip. The sample pad transports the sample into the conjugate pad. The conjugate pad contains an amount of dried colloidal gold/latex-Ab conjugate, which is resolubilized when the sample reaches it. Abs conjugated to the gold/latex form a complex with the target antigen, if present in the sample. Capillary action drives the solution through the conjugate pad and to the test strip, which contains the test and control line. When the solution reaches the test line, which is comprised of a primary Ab for the target antigen, the colloidal gold/latex–Ab-antigen complexes will be immobilized and concentrated, representing a positive result (Figure 2a). Alternatively, if the antigen is absent or not present in sufficient amounts, the colloidal gold-Ab conjugates will not be immobilized in sufficient quantities to form a band, indicating a negative result (Figure 2b). The validity of the test is affirmed by a control line placed past the test line which contains either anti-IgG or a high concentration of the targeted antigen [121]. Previous results have shown that the LOD of viruses with traditional colorimetric indicators is in the range of $1 \times 10^6$ to $1 \times 10^7$ pfu/ml, while bacteria have a LOD between 200 to 5000 cells per test, and depends on several factors: the antibodies chosen and whether they are competitive, the purity of the sample used, and the rate at which the fluid moves through the test strip [122,123].
Figure 2. Schematic representation of a lateral flow assay (LFA) using the sandwich mechanism. (a) Represents a positive result, and (b) a negative result. The sample to be tested is premixed with an excess of reporter-antibody conjugate. If the desired antigen is present in the sample, the conjugates will bind to it, creating a reporter-antibody-antigen complex. Following the mixing of the sample, a LFA is dipped sample pad (colored green)-first into the experimental or unknown sample to be tested. Capillary action drives the sample solution through the sample pad and onto the nitrocellulose (shown in grey). As the experimental or unknown sample moves past the ‘test line’, an area of immobilized antibodies specific for the target antigen adsorbed to the nitrocellulose, the reporter-antibody-antigen complexes in the sample are captured by the antibody. If enough complexes are present, the reporter will become sufficiently concentrated in the ‘test line’ area to cause a visible color change on the test strip. Excess reporter-antibody conjugates in the remaining solution will continue to move up the nitrocellulose by capillary action (i.e. lateral flow), eventually encountering a ‘control line’ composed of immobilized target antigen or anti-IgG. This ‘control line’ is designed to capture all remaining conjugates in the sample, allowing the test to be validated through a forced concentration of the reporter. Capillary action continues to drive the excess sample up the nitrocellulose, where it is absorbed by the absorbent pad (yellow square).
1.5.2 At-home Chlamydia Test Kit

There are now several commercially available POC tests for *C. trachomatis*, due to rising demand for more rapid diagnostics to prevent and treat the spread of chlamydial infections. However, it should be noted that these tests are designed primarily to diagnose the sexually transmitted disease (STD), and the sampling materials provided in the kits reflect this. The QuickVue® Chlamydia Test is a typical LFA, using colloidal gold and latex np for its reporter, leading to a colorimetric indication. In a clinical study conducted by the manufactures of the QuickVue® test, 723 women who were already attending a STD clinic were tested. Of the 723 endocervical samples taken, 73 were positive for *C. trachomatis* by culture and 67 were positive by the QuickVue® test, leading to an accuracy of 81%, although there were 6 false positives. An additional study looking at 500 patients showed that the test had a sensitivity of 82.5% and specificity of 99%. However, it should be noted that while the tests were performed by a random group of people of varied education and background, the samples themselves were taken by trained medical personnel at OBGYN and STD clinics, which likely skewed the numbers in favor of greater sensitivity [124]. Furthermore, while the study checked potential false positives in the case of the LFA showing a positive result while a culture method returned negative via PCR, culture negative samples with a negative QuickVue® result were not retested in this manner [124, 125].

Several publications evaluating the QuickVue® test and several other commercially available tests lend credence to this assertion [126-129]. A study published in 2002 used PCR as the standard for confirming or refuting the results of the
QuickVue® test. The study involved two groups of 100 women each: one group was considered a high-risk population while the other was considered low-risk [128]. In the high-risk population, the QuickVue® test had a sensitivity of 65% and a specificity of 100%. The sensitivity in the low-risk population was substantially lower at only 25%. When both populations are taken into account, the sensitivity and specificity of the QuickVue® test according to this study is 55% and 100% respectively, which is well below the sensitivity reported by the manufactures on their website. These results are further supported by a study published in 2010, in which three POC test kits which are commercially available in the Netherlands, QuickVue®, Biorapid CHLAMYDIA Ag test, and Handlab- C, were compared to PCR using a population of 763 people. All three of these tests fell well below their advertised sensitivity, with QuickVue® reporting a sensitivity of 25% and specificity of 99.7%. The Biorapid test had a sensitivity of 17% and specificity of 93.7%. The Handlab- C test has a sensitivity of 22.5% and specify of 88.9%. While all three of the tests had a high degree of specificity the overall sensitivity was poor, especially when compared to the advertised sensitivity of all three assays which between the three claim sensitivity between 82% and 98% [130].
CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals and Materials

Tetraethyl orthosilicate, tris(2,2'-bipyridyl)dichlororuthenium(II) hexahydrate (Ru(bpy)$_3^{2+}$), and L-lysine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Streptavidin was obtained from Southern Biotech (Birmingham, AL, USA). 3-glycidoxypropyl trimethoxysilane (GOPTMS) was purchased from AcrosOrganics (NJ, USA). Absolute ethanol, acetone, methanol, toluene, hydrochloric acid, sodium chloride, sodium hydroxide, Triton X-100, Tween-20, Tris, and BSA were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Sodium phosphate dibasic, Potassium phosphate monobasic, NS borax (sodium tetraborate) were obtained from Sigma-Aldrich Chemical Company (Milwaukee, WI, USA). All chemicals used in this study were analytical reagent grade. Nitrocellulose membrane, and absorbent pad were purchased from GE Healthcare Bio-Sciences Corp. (Piscataway, NJ, USA). Chlamydia antibodies were purchased from Fitzgerald Industrial International (Acton, MA, USA). T7-tag biotinylated monoclonal antibodies and T7 antibodies were purchased from Novagen®, an affiliate of Merck (San Diego, CA, USA). Anti-IgG antibodies were purchased from ABcam (Cambridge, MA, USA). All solutions and buffers were prepared using ultrapure water from Barnstead Nanopure Infinity™ ultrapure water system (Thermo Scientific, Dubuque, IA, USA). *E. coli* BL21 stock was purchased from EMD Millipore(Billerica,
Massachusetts) and wild-type bacteriophage T7 stock was purchased directly from ATCC (BAA-1025-B2, Manassas, Virginia).

2.2 Apparatus

A multi-mode microplate reader (Synergy™ 2) was obtained from BioTek Instruments, Inc. (Winooski, VT, USA). A dispenser (Linomat IV) was purchased from American Laboratory Trading Inc. (East Lyme, CT, USA).

2.3 T7 Bacteriophage Working Stock Preparation

*E. coli* BL21 culture was grown overnight in 50 ml of Luria Broth (LB), pH 7.5, at 37 °C with shaking. 1 ml of overnight culture was then added to 200 ml of fresh LB and incubated at 37 °C with shaking until an optical density (OD) of at least .6 at 600 nm was reached (an OD of .627 was reached after 3.5 hours). Then 20 µl of T7 stock was added to the *E. coli* BL21 culture and allowed to incubate, with shaking, at 37 °C for 1.5 hours. 5 g of NaCl was added to the 200 ml culture. The culture was then split into six 35 ml tubes and spun at 8,000 RPM on a Fiberlite F21-8x50y fixed angle rotor (Thermoscientific, Waltham, MA) for 10 minutes at room temperature. Supernatant was collected and filtered through .22 µm SCFA filter (Corning Life Science, Corning, NY). 40 ml of this filtered culture was divided into four 13.5 ml ultracentrifuge tubes and spun at 35,000 RPM for 2 hours on a Fiberlite F65L-6X13.5 fixed angle rotor (Thermoscientific) at room temperature. Supernatant was removed and pellets in each tube were resuspended in 1 ml of 25 mM MES buffer, pH 6.0, and then combined for a
total of 4 ml of purified T7 working stock. The titer (4.4 X 10^{11} pfu/ml) of this purified T7 working stock was determined following the double agar overlay plaque assay as outlined.

2.4 Preparation and Assembly of Lateral Flow Assay

Plastic transparency with dimensions of 75mm x 215 mm was used as the backing for the LFA. “Sticky” tape from 3M with a width of 38 mm was purchased and used as the adhesive to bind the nitrocellulose to the plastic backing. The “sticky” tape was applied to the plastic transparency between the 10mm and 48mm position vertically on the plastic.

Nitrocellulose type AE 98 was purchased from Whatman. A 38 mm x 215 mm x 5 µm piece of nitrocellulose was used in the production of each set of LFAs. The nitrocellulose was handled with gloves to prevent contamination, and was placed flush with the adhesive in order to prevent air bubbles from forming.

Following the addition of plastic backing to the nitrocellulose, the test and control lines were added. The lines were placed on the nitrocellulose uniformly through the use of a Linomat IV. The amount of Ab applied to each line was 1.9 µg/cm. The test line was applied at 29 mm and the control line was placed at 38 mm to allow for proper alignment for fluorescent readings. Following application of each line, the Linomate was sterilized with 10% bleach followed by three washes with DI water to prevent
contamination between the test and control line Abs. After both the test and control line were applied, the backed nitrocellulose was allowed to dry in a vacuum oven at 28° C and 16.8 pka for 2 hours.

Following drying, the nitrocellulose was blocked in a buffer of 1% BSA, 10 mM Tris, and 100 mM NaCl for 1 hour at pH 7.4. The assay was then removed and excess liquid was blotted away with a chemwipe. The sample was then washed 5 times in 0.1% Tween-20, 10 mM Tris, and 100 mM NaCl for 5 min to remove excess BSA. After the washing process the LFA was then dried overnight at 28° C and 16.8 pka.
Place Adhesive

Place Nitrocellulose

Spray Test and Control Line

Overnight Drying

Block

Blot then Wash

Overnight Drying, Cut, and add Adhesive

Add Sample and Absorbent Pad
Figure 3. Manufacture of a Lateral Flow Assay (LFA) test strip. Plastic backing (solid, white) is cut into a 75 x 215 mm sheet. A 38 x 215 mm piece (gray, spotted) of adhesive is then placed between 10 mm and 48 mm. A 38 x 215 mm piece of nitrocellulose (colored light grey) is then firmly applied to the adhesive. The test line (blue, line), which is composed of IgG that targets the primary antigen, is then aspirated by the Linomate IV onto the nitrocellulose. The control line (red, line), which is comprised of anti-IgG or the target antigen, is then aspirated onto the nitrocellulose. The sheet with the applied test and control lines is then dried overnight at low humidity. The sheet is then placed in a blocking solution [(light blue), 1% BSA, 10 mM Tris, and 100 mM NaCl at pH 7.4] for 1 hour. Following the blocking, the sheet is blotted dry and placed in a wash solution (0.1% Tween-20, 10 mM Tris, and 100 mM NaCl at pH 7.4) for 5 min, after which it is blotted dry again. The previous step is repeated 4 additional times. Following the washing, the sample is then dried overnight in low humidity. The sheet is then cut into 4 mm strips, and adhesive is applied above and below the nitrocellulose. An absorbent pad (colored yellow) and sample pad (colored green) are then adhered to the test strip.
Figure 4. Determination of blocking time. The nitrocellulose represented here is the same as was used in our LFAs and was blocked for variable amounts of time as follows: (a) 20 sec, (b) 1 min, (c) 10 min, (d) 30 min, and (e) 1 hr. The base (bracketed area) of the test strips labeled ‘a’ through ‘d’ display nonspecific binding (as revealed by the red coloration), while test strip labeled ‘e’ displays no nonspecific binding at the base (no red color). Determined that blocking must be conducted for at least 1 hour.

2.5 Conjugation of Gold Nanoparticles with Streptavidine to Biotinylated T7-tagged Abs

250 µl of gold np bound to streptavidin was added to 1375 µl of HPLC water. The OD was then checked at 520 nm to confirm it equaled one. 2.83 µg of biotinylated Ab was then added to the gold np solution. The solution was then placed on a shaker, at low
speed, and incubated at room temperature for 45 min. Following shaking, 170 µl of 10% BSA at pH 9 was added to halt the reaction. This was followed by incubation at room temperature for 20 min shaking gently. Following the 20 min incubation, the sample was centrifuged at 5000 g at 4°C for 20 min. The supernatant was then removed and the precipitate was re-suspended in 2 mM Borax at pH 9. The OD at 520 nm was then taken.

### 2.6 Conjugation of Naked Gold Nanoparticles to Chlamydial Abs

A kit was purchased from BioAssay Works which provided naked gold np and the necessary buffers to perform the reaction. 0.5 ml of naked gold np at OD 15 was placed into a clean micro centrifuge tube. 6 µl of Buffer A, provided, and 4 µl of Buffer B, provided, were added to bring the reaction mixture to pH 7.3. Following, 4.2 µl of anti-chlamydia polyclonal Abs at 5 mg/ml, suspended in 1x PBS, were added to the reaction mixture. The sample was incubated at room temperature for 30 minutes, shaking gently. The reaction was halted by the addition of 50 µl of BSA Blocking Stabilizer Solution, provided.

### 2.7 Test Conditions for Gold Nanoparticles in a LFA for T7 Bacteriophage

Serial dilations were performed from 1x 10^{11} to 1x 10^{3} pfu/ml at 1 log intervals, with the negative control consisting of 1x PBS. Each sample was tested three independent times. 30 µl of a sample was placed in a 1.5 ml microcentrifuge tube and to it was added 20 µl of a dynamic blocking buffer (0.2% BSA, 0.3% Tween-20, 0.1% polyethylene
glycol, 0.2% NaN₃, 100 mM Tris pH 8) and 15 µl of gold np-Ab conjugate. It was incubated at room temperature on a shaker at medium speed for 10 min and then the mixed sample was placed into a larger reservoir. The LFA was placed into the reservoir and the sample was allowed to run up the test. After 30 min the LFA was transferred to a new sample well which was loaded with 80 µl of washing buffer (0.1% Tween-20, 100mM NaCl, 10mM Tris pH 7.4) to remove any nonspecific binding. The LFA was removed 2 hours later, and an image taken of the results immediately, as excessive time between completion of the assay and reading can lead to false positives.

2.8 Running Conditions for Gold Nanoparticles in Chlamydia LFAs

Serial dilutions were performed (1x PBS with 10mM EDTA pH 7.4) from 1x 10⁴ to 3.33x 10¹ pfu/ml at 1/10 log intervals with the negative control sample consisting of 1x PBS. Each sample was tested three independent times. 30 µl of a sample was placed in a 1.5 ml microcentrifuge tube and to it was added 20 µl of a dynamic blocking buffer (0.2% BSA, 0.3% Tween-20, 0.1% polyethylene glycol, 0.2% NaN₃, 100 mM Tris pH 8) and 5 µl of gold np-Ab conjugate. It was incubated at room temperature on a shaker at medium speed for 10 min and then the mixed sample was placed into a larger reservoir. The LFA was placed into the reservoir and the sample was allowed to run up the test. After 30 min the LFA was transferred to a new sample well which was loaded with 80 µl of washing buffer (0.1% Tween-20, 100mM NaCl, 10 mM Tris pH 7.4) to remove any nonspecific binding. The LFA was removed 2 hours later, and an image taken of the results immediately, as excessive time between completion of the assay and reading can lead to false positives.
2.9 Preparation of Ru(bpy)$_3^{2+}$-doped Silica Nanoparticles

RuSNP were prepared in aqueous medium by using a basic amino acid catalysis method [132]. Briefly, Ru(bpy)$_3^{2+}$ and tetraethyl orthosilicate (TEOS) were added to a reaction system by using three steps in order to control the size of the nanoparticles and the amount of the dye Ru(bpy)$_3^{2+}$ incorporated inside each nanoparticle. First, 36.1 mg of L-lysine was added as a basic catalyst to 36 ml of deionized water in a polytetrafluoroethylene (PTFE) bottle reactor. After the L-lysine was completely dissolved, 0.04 M Ru(bpy)$_3^{2+}$ solution with the designated volume was added under the agitation to the reactor. Next, TEOS used as a silica source with designated volume was added and mixed in the reactor. Finally, the reactor was put into a 90 °C silicone bath while stirring at 500 rpm. The Ru(bpy)$_3^{2+}$ solution and the TEOS were added to the system three times in intervals of 24 hours. In a representative case, the volumes of the Ru(bpy)$_3^{2+}$ solution/TEOS added were 50 μl/2.7 ml, 400 μl/5.4 ml, and 400 μl/5.4 ml, respectively. After the hydrolysis of TEOS was completed, orange-colored RuSNP solution was dialyzed in deionized water for 48 hours in order to remove the non-incorporated dye and L-lysine in the solution. The deionized water was changed three times during the dialysis. The particles were then concentrated by centrifugation (30,000 x g at 4°C, 60 minutes). Finally, the RuSNP were washed twice in absolute ethanol and dried in a vacuum oven (16.8 kPa, 40 °C).
2.10 Surface modification of Ru(bpy)$_3^{2+}$-doped Silica Nanoparticles

A portion (2.3 mg) of dried RuSNP were re-suspended in 4.5 mL dry toluene in a 20 mL glass vial and sonicated at room temperature for one hour. Then, 0.5 mL of GOPTMS was added to the vial which was placed into a 65 °C water bath while shaking at 200 rpm for 2 hours. The pellet was collected following centrifugation (10,000 x g at 4 °C, 30 minutes), and then sequentially washed in dry toluene, acetone, and ethanol, and dried in a vacuum oven overnight (16.8 kPa, 25 °C). The GOPTMS-modified RuSNP (RuSNP-GOPTMS) were immediately used or stored at 4 °C for later usage.

2.11 Conjugation of Fluorescent Nanoparticles to Abs

280 µl of PBS (40 mM NaCl, 50 mM sodium phosphate buffer, pH 9) was added to 0.4 mg of RuSNP-GOPTMs in a 1.5 ml centrifuge tube and sonicated for 5 min. Following, 150 µg of Ab was added to the solution which was inverted at room temperature for one hour. Following the one hour incubation at room temperature, the solution was placed on a shaker at medium speed at 4° C for 48 hours. 280 µl of blocking buffer(100 mM Tris, 40 mM NaCl, 1% BSA pH 7.4) was added to halt the reaction, and allowed to incubate overnight at 4° C, shaking.
2.12 Running Conditions for Fluorescent Nanoparticles with T7

Bacteriophage

Serial dilutions were performed from $1 \times 10^{11}$ too $1 \times 10^3$ pfu/ ml at 1 log intervals with the negative control consisting of 25 mM MES. Each sample was tested three independent times. 30 µl of a sample was placed in a 1.5 ml microcentrifuge tube and to it was added 20 µl of a dynamic blocking buffer (0.2% BSA, 0.3% Tween-20, 0.1% polyethylene glycol, 0.2% NaN₃, 100 mM Tris pH 8) and 15 µl of fluorescent np-Ab conjugate. It was incubated at room temperature on a shaker at medium speed for 10 min and then the mixed sample was placed into a larger reservoir. The LFA was placed into the reservoir and the sample was allowed to run up the test. After 30 min the LFA was transferred to a new sample well which was loaded with 60 µl of washing buffer (0.1%
Tween-20, 100 mM NaCl, 10 mM Tris pH 7.4) to remove any nonspecific binding. The LFA was removed 1 hour later, and loaded into the fluorescent plate reader and the results were recorded.

Figure 6. Running a lateral flow assay (LFA) test strip. (a) The sample is added to the reaction mixture (reporter-Ab conjugate and flow buffer) and (b) incubated for 10 min at 27 °C with agitation. (c) Following the incubation, the LFA is placed sample pad-first into the sample mixture. (d) The sample mixture is then driven by capillary action up the LFA. Following a 30 min incubation, a wash buffer (0.1% Tween-20, 100 mM NaCl, 10 mM Tris pH 7.4) is added in order to (e) remove unbound reporter-Ab conjugate. Following the 1 hr incubation, the LFA is (f) removed from the test tube. Images are taken of LFAs that use colorimetric reporters, while fluorescent-based LFAs are loaded into the (g) cassette. It is then placed into a fluorometer and the fluorescent signal is (h) read and recorded.

2.13 Fluorescent Running of Chlamydia

Serial dilutions were preformed (1x PBS with 10 mM EDTA pH 7.4) from 1x 10⁴ to 3.33x 10¹ pfu/ ml at 1/10 log intervals with the negative control sample consisting of 1x PBS and 10 mM EDTA. Each sample was tested three independent times. 30 µl of a
sample was placed in a 1.5 ml microcentrifuge tube and to it was added 20 µl of a dynamic blocking buffer (0.2% BSA, 0.3% Tween-20, 0.1% polyethylene glycol, 0.2% NaN₃, 100 mM Tris pH 8) and 5 µl of fluorescent np-Ab conjugate. It was incubated at room temperature on a shaker at medium speed for 10 min and then the mixed sample was placed into a larger reservoir. The LFA was placed into the reservoir and the sample was allowed to run up the test. After 30 min the LFA was transferred to a new sample well which was loaded with 80 µl of washing buffer (0.1% Tween-20, 100 mM NaCl, 10 mM Tris pH 7.4) to remove any nonspecific binding. The LFA was removed 2 hours later, and loaded into the fluorescent plate reader and the results were recorded.
CHAPTER 3

RESULTS

3.1 The LOD of Traditional Colorimetric Reporters Used in LFAs is Insufficient to Detect Medically Relevant Viral Loads

T7 bacteriophage was chosen as the antigen for the proof-of-concept testing for several reasons. First, it is a well understood virus with a wide variety of both polyclonal and monoclonal Abs available. It is also easy to maintain, handle, and propagate a large stock of T7 bacteriophage, due in part to its short life cycle (17 min at 37°C), which allows for rapid growth of the viral population. Furthermore, its host cell, *E. coli*, is also very easy to maintain and propagate. T7 also has the added benefit of not being a human pathogen, which allows for safer and easier handling.

Previous reports have shown that current colorimetric reporters are incapable of detecting viruses at medically relevant levels. However, LFA sensitivity varies depending upon the Abs used and the exact manufacturing process. A baseline was therefore needed in order to validate the LOD of the LFAs used in this study, and to confirm that any improvement in LOD is due to the use fluorescent reporters and not due to a difference in design.

A traditional LFA was performed with colloidal gold np acting as the reporter. A 30 µl sample was used, leading to an observed LOD of $1 \times 10^6$ pfu/ml (*Figure 7*), which is
within range of previously reported LOD for LFAs designed to detect viruses [1-7, 13]. While these results are similar to previously reported findings, they are of insufficient sensitivity to detect acute and subclinical infections, or even serious infection in many cases [100-103]. People infected with HIV, for instance, typically do not have a viral load above 10,000 copies/ml in the early stages of the disease. In fact, the amount of HIV needed to determine if the patient is likely to develop AIDS is a viral load >100,000 copies/ml within six months of seroconversion [100,102]. The current LOD for LFAs using colloidal gold is 2 logs below the LOD required to properly diagnose HIV.

**Figure 7. A lateral flow assay (LFA) using a colorimetric reporter to detect T7 bacteriophage.** The ‘+ Control’ is the area of the test strip (i.e. LFA) that will indicate whether a valid (red line present) or invalid (no line present) test occurred. The ‘Test line’ is an area of immobilized antibodies specific for the target antigen adsorbed to the nitrocellulose. The reporter-antibody-antigen complexes in the sample are captured by the antibody, and if enough complexes are present, the reporter will become sufficiently concentrated in the ‘test line’ area to cause a visible color change on the test strip (red line present). However, if no antigen is present, the reporter-antibody conjugates will not
be captured, indicating a negative result (no line present) of a given sample run through the test strip. The negative control without any T7 is shown in panel (a). The remaining solutions contain T7 at concentrations of (b) 9x10^5, (c) 1x10^6, (d) 1x10^7, (e) 1x10^8, or (f) 1x10^9 PFU/mL. This experiment was repeated 3 times, with a representative shown here. In this experiment a concentration of 1 x 10^6 PFU/mL was needed to display a positive result, which was also seen in the other 2 experiments.

3.2 Fluorescent Reporters are Capable of Detecting Viral Loads at Medically Relevant Levels

Since it has been demonstrated that current colorimetric reporters are insufficient for detecting viral loads at medically relevant levels, a change in reporter is needed in order for LFAs to become a valid option. Fluorescent- based reporters offer the potential to greatly improve the LOD of LFAs due to several factors. First, a fluorescent- based assay is read by a fluorometer, which provides a quantitative numerical value. By comparison, colorimetric reporters are qualitative, requiring end-user subjective judgment to determine a positive or negative result. Furthermore, the fluorometer has substantially greater acuity than the human eye and is capable of detecting fluorescent concentrations as low as one part per trillion. These factors should allow for a fluorescent-based reporter to greatly improve the LOD of a LFA.

Two distinct fluorophores were used in our assays: fluorescein isothiocyanate (FITC) and Ru(bpy)^32+-doped silica np (RuSNP). FITC was chosen as a baseline fluorescent molecule for several reasons: it is a commonly used in immuno-fluorescent staining, fluorescence-activated cell sorting, and is a well understood fluorophore. RuSNP was chosen due to its distinctive optical properties [131, 132]. Several of the
properties that make it an optimal fluorophore are its large Stokes Shift (an excitation of 460 nm and an emission of 620 nm), and its comparatively long excitation state (650 ns in aqueous solutions). It is therefore likely that RuSNP-based reporters will allow for LFAs to detect viral concentrations at medically relevant concentrations.

The fluorescent signal of FITC (dotted line, Figure 8) and fluorescent np (solid line, Figure 8) increased in a concentration-dependent manner and reached a signal to background ratio (S/B) of 1.65 and 2, respectively, at the viral concentration of 1x10^6 PFU/mL. The LOD was determined for the FITC labeled experiments to be 5x10^4 PFU/mL. This value was based on the S/B being three standard deviations above the background noise, with the S/B of FITC at 5x10^4 PFU/mL being 1.18. The fluorescent np LOD was determined to be 5x10^3 PFU/mL, with a S/B of 1.26. Statistical analysis was performed on the reported values, and the p-value for FITC at 5x10^4 PFU/mL was 0.003 with a standard deviation of ~2%, representing a high degree of both significance and reproducibility. For the fluorescent np at 5x10^3 PFU/mL, the p-value was <0.0001 with a standard deviation of ~4%, also representing a high degree of significance and reproducibility.

These results show that fluorescent based reporters, particularly RuSNP, are capable of detecting viral levels at medically relevant concentrations, as the LOD of RuSNP was determined to be 5,000 PFU/mL, which is below the average viral
Figure 8. Fluorescent reporters improve the limit of detection (LOD) of a lateral flow assay (LFA). The ratios of signal to background (S/B) in the presence of different concentrations of T7 bacteriophage are shown. The S/B is determined by dividing the signal from a test with varying concentrations of T7 bacteriophage by the signal of a negative control without T7 (background). Data shown are the mean of five independent measurements presented with their standard deviation. The fluorescent signal of FITC is represented by a dotted line and the signal from fluorescent np is represented by a solid line. (*) The LOD for RuSNP was determined to be $5 \times 10^3$ PFU/mL because the S/B was more than three standard deviations above 1 (background). The signal was 1.26 with a standard deviation of $\sim 4\%$. The signal values were compared to the background values via the one tailed unpaired t-test, with a p-value <0.0001. (**) The LOD for FITC was determined to be $5 \times 10^4$ PFU/mL. This was determined by the S/B being greater than three standard deviations above 1 (background). The signal was 1.18 with a standard deviation of $\sim 2\%$. The signal values were compared to the background values via the one tailed unpaired t-test, with a p-value of 0.003. RuSNP is a substantial improvement over colorimetric based reporters in current LFAs.

concentration found in HIV patients. RuSNP is a substantial improvement over colorimetric based reporters in current LFAs.
3.3 The LOD of Traditional Colorimetric Reporters Used in LFAs is Insufficient to Reliably Detect *C. trachomatis* EBs

It has been previously shown that colorimetric reporters do not have a sufficiently low LOD for the detection of viral loads at concentrations typically found in patients. Furthermore, previous reports have shown that current colorimetric reporters used in LFAs are incapable of reliably detecting Chlamydial EBs at medically relevant levels. Several studies have shown that the sensitivity of the LFAs currently available for the detection of *C. trachomatis* have a sensitivity between 17-25% and have a LOD between 200 to 5000 cells per test [122, 123]. However, a single *C. trachomatis* EB is capable of causing an infection, so the optimal assay would be able to reliably detect a single EB. The variations inherent in using a different set of Abs and assembly processes required that a baseline be performed in order to validate the LOD of the LFAs used in this study, and to confirm that any improvement in LOD is due to the use of fluorescent reporters and not a difference in design and test running process.

3.3.1 Fouling in a LFA Designed to Detect *C. trachomatis* Elementary Bodies

In this experiment fouling was observed, indicating that the particle size of the sample is larger than expected, as chlamydial elementary bodies are 300nm and are small enough to flow through the pores in the nitrocellulose.
Figure 9. Fouling in a lateral flow assay (LFA) using a colorimetric reporter to detect *C. trachomatis* elementary bodies. The ‘+ Control’ is the area of the test strip (i.e. LFA) that will indicate whether a valid (red line present) or invalid (no line present) test occurred. The ‘Test line’ is an area of immobilized antibodies specific for the target antigen adsorbed to the nitrocellulose. The reporter-antibody-antigen complexes in the sample are captured by the antibody, and if enough complexes are present, the reporter will become sufficiently concentrated in the ‘test line’ area to cause a visible color change on the test strip (red line present). However, if no antigen is present, the reporter-antibody conjugates will not be captured, indicating a negative result (no line present) of a given sample run through the test strip. The arrow is indicating fouling, which occurs due to the particle size of the sample being larger than the pore size found in the nitrocellulose. The negative control without any *C. trachomatis* elementary bodies is shown in panel (a). The remaining solution contains *C. trachomatis* elementary bodies at a concentration of (b) 1x10⁴ IFU/mL. This experiment was repeated 2 times, with a representative shown here.

Fouling was observed at the base of the LFA (Figure 9b, arrow). As the negative control (Figure 9a) did not exhibit any signs of fouling and the positive controls
on the tests were observed as valid (red line visible), the issue is unlikely to be due to a lack of blocking. Several potential problems could be responsible for the fouling issue. The first possibility is that the reporter-Ab conjugate could be acting as a cross-linker between multiple EBs, leading to a larger particle size. These cross-linked particles would then be large enough to block the pores in the nitrocellulose membrane, preventing additional material from flowing up the strip. The second possibility is that the EBs are aggregating due to excess Ca$^{2+}$ and Mg$^{2+}$ ions, which are present in large amounts due to the nature of the sample used, which is a cell lysate mixture chosen to mimic a sample taken from a patient.

3.3.2 The Fouling is not Due to Cross-Linking of the Reporter-Ab Conjugate to Multiple EBs

In order to test if the reporter-Ab conjugates are acting as a cross-linker, an additional series of LFA were run with several changes in methodology. The C. trachomatis sample was not incubated with the reporter-Ab conjugate. Instead, the sample was run directly up the test strip using a wash buffer to push excess sample through the test strip. After the sample had fully run up the strip, the reporter-Ab conjugate was added and run up the sample, and subsequently followed by a wash buffer to remove any nonspecific binding of the reporter-Ab conjugate. The changes in
Figure 10. Cross-linking is not the cause of fouling in a lateral flow assay (LFA) using a colorimetric reporter to detect *C. trachomatis* elementary bodies. The ‘+ Control’ is the area of the test strip (i.e. LFA) that will indicate whether a valid (red line present) or invalid (no line present) test occurred. The ‘Test line’ is an area of immobilized antibodies specific for the target antigen adsorbed to the nitrocellulose. The reporter-antibody-antigen complexes in the sample are captured by the antibody, and if enough complexes are present, the reporter will become sufficiently concentrated in the ‘test line’ area to cause a visible color change on the test strip (red line present). However, if no antigen is present, the reporter-antibody conjugates will not be captured, indicating a negative result (no line present) of a given sample run through the test strip. The arrow is indicating fouling, which occurs due to the particle size of the sample being larger the pore size found in the nitrocellulose. The negative control without any *C. trachomatis* elementary bodies is shown in panel (a). The remaining solution contains *C. trachomatis* elementary bodies at a concentration of (b) $1 \times 10^4$ IFU/mL. This experiment was repeated 2 times and a representative is shown here. Fouling was still observed despite running the reporter antibody conjugate up the test strip (i.e. LFA) following the *C. trachomatis* sample, ruling out cross linking as a possible cause of the fouling.
methodology allow for the Abs to interact with their antigens for the same amount of
time, but prevent cross-linking from occurring in the early portions of the test stip.

Fouling was still observed at the base of the LFA (Figure 10b, arrow). As seen
previously, the negative control (Figure 10a) did not exhibit any signs of fouling and the
positive controls on the tests were observed as valid (red line visible). Therefore, the
cross-linking of multiple reporter-Ab conjugates and EBs is not the underlying cause for
the fouling that is occurring in the nitrocellulose membrane.

3.3.3 The Fouling is Due to Excess Ca^{2+} and Mg^{2+} Ions Leading to EB Aggregation

In order to determine if the EBs were aggregating due to excess Ca^{2+} and Mg^{2+}
ions in the raw lysate sample, a zeta-potential and particle size analyzer was used to
measure the size of the particles in two differing samples. One sample was cell lysate
diluted in PBS to a concentration of 1x10^4 IFU/mL, while the second sample was
prepared with a PBS buffer with the addition of 5 mM of EDTA, as EDTA acts as a
chelating agent and will scavenge the Ca^{2+} and Mg^{2+} ions. With the excess positively
charged ions inter-chelated, the negatively charged outer membrane of the Chlamydial
EBs would prevent aggregation and disperse currently aggregated EBs.
Table 1. The particle size of *C. trachomatis* samples. The size of particles in a sample and their relative amount at a concentration of 1x10^4 IFU/mL in PBS. Data of three independent measurements are presented, with an error margin of one standard deviation. The chlamydial elementary bodies are aggregating, since the size of the elementary bodies is 896.4 ± 15.4 nm, which is almost three times larger than the reported size of a single elementary body.

<table>
<thead>
<tr>
<th>Size (nm)</th>
<th>Sample Composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>896.4 ±15.4</td>
<td>64.5</td>
</tr>
<tr>
<td>103.6 ±3.8</td>
<td>17.8</td>
</tr>
<tr>
<td>5364 ±25.5</td>
<td>17.7</td>
</tr>
</tbody>
</table>

Table 2. The particle size of *C. trachomatis* sample with EDTA. The size of particles in a sample and their relative amount at a concentration of 1x10^4 IFU/mL treated with 5 mM EDTA. Data of three independent measurements are presented, with an error margin of one standard deviation. The addition of EDTA significantly alleviates the aggregation of elementary bodies, as their average size is 300.9 ±2.5 nm following treatment, which is the reported size of a single elementary body.

<table>
<thead>
<tr>
<th>Size (nm)</th>
<th>Sample Composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>300.9 ±2.5</td>
<td>89.6</td>
</tr>
<tr>
<td>48.8 ± 1.8</td>
<td>7.1</td>
</tr>
<tr>
<td>5544 ±45.2</td>
<td>3.3</td>
</tr>
</tbody>
</table>

The samples that were not treated with EDTA (Table 1) contain three major particle sizes. The peak, which constitutes the largest volume of the particles accounting for 64.5% of the total particles suspended in the sample, is at 896 nm. As one EB has a diameter of 300 nm, this peak likely suggests that the EBs are aggregating. This conjecture is confirmed as the sample treated with EDTA (Table 2) displayed three distinct particle sizes. The peak, which constitutes the largest volume of the particles, accounting for 89.6% of the total particles suspended in the sample, is at 300 nm which is the size of a single EB.
These results demonstrate that the presence of excess Ca$^{2+}$ and Mg$^{2+}$ ions due to cell lysate cause the Chlamydial EBs to aggregate. These larger particle sizes are the likely underlying cause of the fouling of the LFA.

3.3.4 The LOD of Traditional Colorimetric Reporters Used in LFAs is Insufficient to Reliably Detect C. trachomatis EBs

As the aggregation issue was resolved, the testing of the LFA designed to detect C. trachomatis, with the colorimetric reporter, could proceed. A 30 µl sample of raw cell lysate was used, leading to an observed LOD of 9x10$^3$ cfu/ml (Figure 11d), which is within range of previously reported LOD for LFAs designed for Chlamydial EBs [1-7, 14]. While these results are similar to previously reported findings, and are adequate for detecting the levels of C. trachomatis found in infected tissues and secretions, several studies have shown that LFAs offer poor sensitivity even using samples taken by trained professionals [120-122]. It is therefore likely that the reported LOD of this test would be substantially higher when used in a real-world setting, and could very well be several orders of magnitude off. A change in the reporter used from colorimetric to fluorescent would allow for an increase in sensitivity and lower the LOD of the LFA.
Figure 11. A lateral flow assay (LFA) using a colorimetric reporter to detect *C. trachomatis* serovar E. The ‘+ Control’ is the area of the test strip (i.e. LFA) that will indicate whether a valid (red line present) or invalid (no line present) test occurred. The ‘Test line’ is an area of immobilized antibodies specific for the target antigen adsorbed to the nitrocellulose. The reporter-antibody-antigen complexes in the sample are captured by the antibody, and if enough complexes are present, the reporter will become sufficiently concentrated in the ‘test line’ area to cause a visible color change on the test strip (red line present). However, if no antigen is present, the reporter-antibody conjugates will not be captured, indicating a negative result (no line present) of a given sample run through the test strip. The negative control without any *C. trachomatis* elementary bodies is shown in panel (a). The remaining solutions contain *C. trachomatis* elementary bodies at concentrations of (b) $7 \times 10^3$, (c) $8 \times 10^3$, (d) $9 \times 10^3$, or (e) $1 \times 10^4$ IFU/mL. This experiment was repeated 3 times and a representative is shown here. In this experiment a concentration of $9 \times 10^3$ PFU/mL was needed to display a positive result, which was also seen in the other 2 experiments.
3.4 Fluorescent Reporters are Capable of Detecting Very Low Levels of Chlamydial EBs

Since it has been demonstrated that current colorimetric reporters lack the sensitivity to reliably detect Chlamydial EBs at levels typically found in infected tissues, a change in reporter is needed in order for LFAs to become a reliable option. Fluorescent based reporters offer the potential to greatly improve the LOD of LFAs. As the benefits of fluorescent reporters has been previously demonstrated in the detection of viral particles, a similar study was initiated for improving the LOD of LFAs for the detection of Chlamydial EBs.

3.4.1 Aggregation of Ru(bpy)_3^{2+}-doped Silica Nanoparticles

Fouling was observed at the base of both LFAs (Figure 9a & b, arrow). As both the negative control and the positive test had fouling occur, the underlying cause of fouling in the sample is likely due to the RuSNP-Ab conjugate. There are several potential underlying causes which could be responsible for the fouling issue. The first possibility is that the RuSNP-Ab conjugate could be aggregating due to insufficient charge on the surface due to the variation of the number of primary amines accessible to epoxy attack on the polyclonal Abs. The second possibility is that there are insufficient Abs compared to RuSNPs, leading to the attachment of multiple RuSNPs to a single Ab, although this is unlikely as the amount of Ab needed to fully coat 0.4 mg of nanoparticles, 50nm in diameter, is 100 μg and an excess of 150 μg was used.
Figure 12. Aggregation of Ru(bpy)$_3$$^{2+}$-doped Silica Nanoparticles (RuSNP). A LFA using a RuSNP reporter used to detect *C. trachomatis*. ‘+ Control’ is an indicator of a valid (white line present) or invalid (no line present) test. The ‘Test line’ is an area of immobilized antibodies specific for the target antigen adsorbed to the nitrocellulose. The reporter-antibody-antigen complexes in the sample are captured by the antibody, and if enough complexes are present, the reporter will become sufficiently concentrated in the ‘test line’ area to cause a visible color change on the test strip (red line present). However, if no antigen is present, the reporter-antibody conjugates will not be captured, indicating a negative result (no line present) of a given sample run through the test strip. The arrow is indicating fouling. The negative control without any *C. trachomatis* is shown in panel (a). The remaining solution contains *C. trachomatis* at the concentration of (b) 1x10$^4$ IFU/mL. This experiment was repeated two times, with a representative shown here. In this experiment fouling is occurring at the base of the strip and is due to RuSNP aggregation rather than chlamydial elementary body aggregation.

Zeta potentials were taken and the charge was 19 mV, which is below the 25 mV charge required on a particle in order to maintain stability in solution. The exact cause of this lower zeta potential is currently unknown.
3.4.2 FITC improves the limit of detection (LOD) of a lateral flow assay (LFA)

As there were difficulties in the employment of the RuSNP-Ab conjugate as a reporter, and the underlying point was to improve the LOD of the LFA, a different fluorescent reporter was used. Since FITC was previously used in the T7 bacteriophage study, FITC was chosen as a replacement for RuSNP until a solution for the aggregation could be devised.

The fluorescent signal of FITC (dotted line Figure 13) increased in a concentration-dependent manner and reached a S/B of 1.25, at the EB concentration of 1x10⁴ IFU/mL. The LOD was determined to be 1x10³ IFU/mL for the FITC labeled experiments. This value was based on the S/B being three standard deviations above the background noise, with the S/B of FITC at 1x10³ IFU/mL being 1.19. Statistical analysis was performed on the reported values and the p-value for FITC at 1x10³ IFU/mL was 0.0004 with a standard deviation of ~3%, representing a high degree of both significance and reproducibility. The data provided here demonstrates the potential for fluorescent reporters to detect very low levels of EBs, with the LOD being ~30 EBs per test.
Figure 13. FITC improves the limit of detection (LOD) of a lateral flow assay (LFA). The ratios of signal to background (S/B) in the presence of different concentrations of C. trachomatis elementary bodies are shown. The S/B is determined by dividing the signal from a test with varying concentrations of C. trachomatis elementary bodies by the signal of a negative control without C. trachomatis elementary bodies (background). Data are the mean of three independent measurements and are presented with their standard deviation. (*) The LOD for RuSNP was determined to be $1 \times 10^3$ IFU/mL. This was determined by the S/B being greater than three standard deviations above 1 (background). The signal was 1.19 with a standard deviation of $\sim$3%. The signal values were compared to the background values via the one tailed unpaired t-test, with a p-value 0.0004. FITC is a substantial improvement over colorimetric based reporters in LFAs.
CHAPTER 4

DISCUSSION

The data presented here, combined with previously published studies, demonstrate the current poor performance of traditional LFAs with relation to the diagnosis of potential viral pathogens and \textit{C. trachomatis} infections. It should be noted that the traditional LFAs performed in this study were done under substantially better conditions and with samples that were more uniform than what would occur under POC diagnostic conditions. It also should be noted that the LFAs used in this study will likely lose some sensitivity as they are further developed, since the assays lacked both sample and conjugated pads which do decrease the amount of sample that reaches the test strip.

This study provides compelling evidence that the use of fluorescent molecules, especially RuSNP, can greatly increase the sensitivity of the LFA. As previously discussed, the sensitivity of the RuSNP is 2.5 logs more sensitive than the colorimetric reporter, greatly improving the LFA and allowing for the potential use of LFAs to diagnosis viral pathogens. FITC labeled Abs also improved the sensitivity of LFAs with regards to the detection of \textit{C. trachomatis} and, should the complications of conjugating the polyclonal Abs to the RuSNP be overcome, there will likely be a similar improvement in the sensitivity of the assay.

Complications occurred with the conjugation process of the polyclonal Abs to the RuSNP. It is likely due to the polyclonal nature of the Abs used, which in turn allowed for there to be a differing number of primary amines exposed for conjugation. This
variation likely allowed for the different Abs to bind to multiple RuSNP. The variable nature of the number of RuSNP bound to each polyclonal AB is likely is the cause for the differing charges seen, leading to a zeta potential of 19 mV which is well below the 25 mV needed in order to maintain a stable suspension. In order to make the RuSNP function with polyclonal Abs, a blocking reagent with a primary amine that is heavily charged is needed in order to prevent static interaction from occurring, preventing aggregation. The other potential solution is the use of monoclonal Abs for conjugation to the RuSNP, as this will likely prevent substantial variations in the charge of the protein covered RuSNP from occurring, alleviating the low zeta potential and preventing the aggregation from occurring.

While there is more refinement required to fully exploit fluorescent reporters for use in LFAs, the data shown gives substantial credence to the potential of fluorophores as reporters in LFAs, which could greatly increase the sensitivity and accuracy of the test in POC settings.
APPENDIX

CHLAMYDIA TRACHOMATIS DEVELOPMENTAL CYCLE

*C. trachomatis* has a biphasic developmental cycle consisting of an infectious EB and a replicative, metabolically active reticulate body (RB). The EBs are spore-like infectious bodies that are metabolically inert, but stable in extracellular environments. The chlamydial nucleoid is located off-center in the cell body of the EB, and is highly compacted due to histone-like proteins HctA and HctB condensing nuclear material [133, 133]. Unlike other gram-negative bacteria, *Chlamydia* strains appear to lack a peptidoglycan (PG) layer [135], yet the EBs are able to maintain structural integrity under a diverse set of conditions. It has been postulated that inter- and intramolecular cysteine bonds exist between cysteine-rich proteins (CRPs) OmcB, OmcA, and OmpA, and that these CRPs, in conjunction with the major outer membrane protein (MOMP) and other membrane components, form a supramolecular structure that provides rigidity to the EB and allows it to resist turgor pressure. Additionally, there is a hexagonally arrayed protein layer, composed primarily of OmcB, at the inner surface of the outer-membrane complex that is thought play a role in the cellular stability of EBs [136-139].

Attachment and Entry

Infectious EBs attach to the host cell initially by electrostatic interactions [140]. The electrostatic interactions are thought to form predominantly due to sulfated macromolecules on the surface of the host cells. The knockout of genes encoding for host cell sulfation, the transport of sulfated proteins to the surface, such as glucuronosyltransferase I, galactosyltransferase I, 3′-phosphoadenosine 5′-
phosphosulfate, and other related genes has an inhibitory effect on the attachment of *C. trachomatis* to host cells [140-142]. Following the initial electrostatic interaction, an irreversible secondary attachment occurs.

Following attachment, *C. trachomatis* mediates host cytoskeleton rearrangement, which leads to a transient reorganization of the microvilli and the formation of pedestal-like structures beneath the attached EB [144, 145]. This rearrangement is induced, in part, by an effector protein, translocated actin recruiting phosphoprotein (Tarp), that is injected into the host cell via a type III secretion system (TS3) [146]. Tarp is a likely candidate for the mediation of host cytoskeletal rearrangement due to its distinct actin binding and oligomerization domains. These domains allow the clustering of multiple actin monomers to nucleate and form new actin filaments [147]. Actin filament polymerization has been shown to be an important part of chlamydial entry into host cells, as treatment of host cells with an actin polymerization inhibitor, such as cytochalasin D, inhibits the uptake of *C. trachomatis* [148]. Additionally, when host cells are microinjected with Abs specific to the actin binding domain found on Tarp prior to *C. trachomatis* infection, entry is inhibited [149].

The rearrangement of the host cytoskeleton, while likely induced by Tarp, is further mediated by Rac GTPase recruitment of WAVE2 and Abi-1 followed by the activation of the host actin related protein 2 and 3 (Arp 2/3) nucleating complex [150,151]. The disruption of the Arp2/3 complex leads to the inhibition of chlamydial entry [152]. While Tarp is capable of Arp 2/3-independent recruitment of actin, the level
of actin rearrangement required for chlamydial entry into host cells likely means that Tarp and the Arp 2/3 complex operate cooperatively to increase the rate of actin filament formation. The precise mechanism of actin rearrangement is not fully understood [153].

**Primary Differentiation**

Within the inclusion, the EB undergoes primary differentiation, shifting from an infectious EB to a metabolically active RB. The process of differentiation can be blocked by treatment with antibiotics that specifically inhibit transcription or translation, suggesting that *de novo* protein production is required for intracellular growth [154]. Primary differentiation begins with chromosomal decondensation, causing the genome to become transcriptionally active. At this point, the EB contains large quantities of mRNA and ribosomes, even though they were previously metabolically inert. This ‘carryover’ mRNA primarily encodes for late gene products predominantly used during secondary differentiation, when RBs become EBs. These late gene products, such as HtcA, if translated, would have a deleterious effect on the newly differentiating *Chlamydia* cells. *Chlamydia* cells appear to have a mechanism to differentiate newly transcribed mRNA from ‘carryover’ mRNA, allowing only for the translation of newly transcribed mRNA. It is currently unclear how *Chlamydia* species (spp.) discriminate between carryover mRNA and newly transcribed mRNA. It should be noted that the carryover mRNA is rapidly degraded in the bacterial cell and generally drops below detectable levels within 6 h post-infection (PI) [155].
Transcription begins within the differentiating RB almost immediately following internalization, with new protein expression detectable within 15 min PI [156, 157]. Genes expressed during primary differentiation serve two general purposes: establishing systems involved in nutrient acquisition (e.g. ADP/ATP translocase, nucleotide phosphate transporter, oligopeptide permease, D-alanine/glycine permease, malate dehydrogenase, nucleoside phosphohydrolase, and a methionine aminopeptidase), and modifying the inclusion to prevent its entry into the endocytic pathway leading to lysosomal fusion (e.g. inclusion-associated protein (Inc) -like genes CT228 and CT229, and the EEA1-like CT147) [155, 158, 159].

**Inclusion Modification**

Following entry into the host cell, the EB remains within the inclusion and does not fuse with the lysosome or endosome. The inclusion membrane separates EBs from the host cytosol and prevents passive diffusion of molecules greater than 520 Da [160]. While the inclusion does not fuse with the lysosomes or endosomes, it maintains the ability to selectively intercept a wide range of host vesicles [161-163]. This is due, in part, to proteins that all *Chlamydia* spp. secrete that localize to the inclusion membrane, collectively termed Incs, and the lack of late endosomal and lysosomal markers on the inclusions [164-166].

Rab GTPases regulate host vesicle fusion, and studies have identified several Rab proteins, including Rab1, Rab6, Rab10, Rab11, and Rab14, which are re-localized to chlamydial inclusions PI and are essential for bacterial survival and reproduction [167-
169]. In *C. trachomatis*, the Inc protein CT229 interacts with Rab4 and in *C. pneumoniae* the Inc Cpn0585 interacts with Rabs1, 10, and 11 [170, 171]. When these interactions are disturbed, in the case of *C. trachomatis*, with microinjection of anti-CT229 into infected cells, chlamydial development is inhibited [172]. Similarly, in the case of *C. pneumoniae* Cpn0585 was ectopically expressed in infected cells and interfered with chlamydial development [49]. The mechanisms mediating Rab6 recruitment are currently unknown, but the silencing of Rab6 decreased the formation of EBs at later points in chlamydial development. In addition to Rab proteins, soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) and Syntaxin 6 have been found to co-localize with the chlamydial inclusion. It should also be noted that SNARE-like motifs have been detected in *Chlamydia* Incs and possibly represent a mimicry mechanism which allows Chlamydia to specifically manipulate vSNAREs to the inclusion target membrane, which in turn allow it to manipulate membrane fusion [173, 174]. It has also been noted that the inhibition of Golgi fragmentation has an inhibitory effect on the growth of *Chlamydia* spp., while increased fragmentation of the Golgi promotes growth [169].

**Cell Division**

The RB arises from the internalized EB following primary differentiation. RBs are 1 μm in size. The cytoplasm appears granular with a diffuse nucleoid. RBs are non-infectious and have an inner and outer membrane, resembling other Gram-negative eubacteria. Following the conversion to RBs, they begin to undergo binary fission throughout this portion of the developmental cycle.
Unlike other eubacteria, *Chlamydia* spp. lack an identifiable *ftsZ* ortholog, which encodes a protein centrally involved in bacterial cell division and found in all other sequenced eubacteria. Interestingly, chlamydial spp. contain a complete set of genes for the synthesis of PG (*murABCDEF*), even though several studies have reported that *Chlamydia* EBs lack PG [175-178]. This apparent lack of PG is made even more peculiar as *Chlamydia* spp. contain penicillin binding proteins (Pbp) and are sensitive to penicillin and other β-lactam antibiotics [177, 178]. It has been hypothesized that the RBs synthesize small amounts of PG, which plays a role in bacterial cell division by perhaps substituting for the lack of FtsZ in the formation of nascent division septa.

*MurA* gene expression was detected early in the chlamydial cell cycle and *murB* was expressed approximately 6 h later [179]. Additionally, microarray analysis demonstrated that *murABCDEF* transcripts were not present before 16 h PI, with the highest levels being expressed 40 h PI [175]. This coincides with the time frame that *Chlamydia* undergoes binary fission. Further evidence supporting this idea has shown that *Chlamydia* has genes encoding for both Pbp2 and MreB. This is unusual, since MreB is found predominantly in rod-shaped bacteria. There is now evidence that *Chlamydia* uses MreB in place of FtsZ as the central coordinator of cell division machinery, and this guides the PG to the division septa [180].

**Secondary Differentiation**

After approximately 36 h, the RBs begin to differentiate into EBs; the signal for this process is currently unknown. Despite the accumulation of 500 to 1000 infectious EBs in the inclusion, host cell function is minimally disrupted [181]. Expression of a number of late-cycle genes occurs during secondary differentiation, including genes that
encode components of the outer membrane complex (e.g. OmcA, OmcB, and OmpA) and proteins involved in the condensation of the chromosome (e.g. HctA and B) [175,182].

At ~48 h, the release of EBs occurs through one of two mechanisms: host cell lysis, thought to be mediated by calcium dependent permeabilization of the host cell membrane, or through the extrusion of the whole inclusion, which is dependent upon host cell actin polymerization [182, 183]. The underlying mechanisms which determine which exit strategy is employed are not currently understood. It has been hypothesized that the ability of Chlamydia infections to shed away EBs while leaving behind viable infected cells is part of a cellular persistence mechanism.

Persistence

Several Chlamydia-induced diseases are associated with chronic infection and inflammation (e.g. PID and reactive arthritis). Chlamydia spp. are capable of entering a persistent state with altered growth characteristics, with enlarged pleomorphic RBs that do not differentiate into EBs or undergo binary fission, but continue to replicate their chromosomes. The conditions that induce a state of persistence in Chlamydia are well known, including penicillin treatment, amino acid starvation, IFN-γ, monocyte infection, and phage infection, and the removal of the underlying factors reverses the persistent state, allowing binary fission and secondary differentiation to resume [181-183]. While the conditions to induce persistence are well understood, the exact molecular mechanism by which Chlamydia spp. enter persistence is currently unknown. Several studies have
shown that a number of early genes were up-regulated, particularly the euo gene, which encodes a DNA-binding protein that binds to the late gene promoter region omcAB [182]. Also, several genes involved with RB to EB differentiation are down-regulated (i.e. hctAB, omcAB, MOMP, and ompA) during a state of persistence [183]. The persistent state seems to allow for Chlamydia spp. to survive the host immune response and other stresses, priming for rapid division following the alleviation of the stress.
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