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Development of a Microwave - Accelerated Metal-Enhanced Fluorescence 40 second, < 100 cfu/mL Point of Care Assay for the Detection of *Chlamydia trachomatis*

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***Abstract*—An inexpensive technology to both lyse *Chlamydia trachomatis (CT)* and detect DNA released from *CT* within 40 s is demonstrated. In a microwave cavity, energy is highly focused, using 100 nm gold films with "bow-tie" structures to lyse *Chlamydia trachomatis* within 10 s. The ultrafast detection of the released DNA from less than 100 cfu/mL *CT* is accomplished in an additional 30s by employing the microwave-accelerated metal-enhanced fluorescence (MAMEF) technique. This new "*release and detect*" platform technology is a highly attractive alternative method for the lysing of bacteria, DNA extraction and the fast quantification of bacteria and potentially other pathogenic species and cells as well. Our approach is a significant step forward for the development of a point of care test for CT.**

***Index Terms*—Chlamydia trachomatis, Lysis, Microwave-Accelerated Metal-Enhanced Fluorescence**

I INTRODUCTION

Chlamydia trachomatis (CT) is the most prevalent bacterial sexually transmitted infection (STIs) reported to the Centers for Disease Control and Prevention (CDC)*[1]* . There were 1.2 million cases of chlamydia reported to the CDC in 2008. The CDC estimates that STIs cost the health care system $1.5 billion annually *[2]*. Since these infections are most often asymptomatic, the CDC and other professional organizations recommend yearly screening for Chlamydia in all sexually active women ages 16-25 years of age*[3]*. Although there are several commercial assays available for performing nucleic acid amplifications tests (NAATs) *[4-6]*, which are now recommended by CDC as the test of choice (APHL), they are time consuming, not convenient for use and not considered point of care tests. Currently there are no commercially available POC assays for the detection of Chlamydia that have rapid, high enough sensitivity and specificity to be recommended *[7]*. In this regard, there is a great need to develop rapid detection technologies for testing Chlamydia infections using point of care (POC) assays in order to expedite immediate diagnosis and treatment in both private and public health care settings.

In recent years, a new platform technology called Microwave-Accelerated Metal-Enhanced Fluorescence (MAMEF) was introduced and demonstrated for the ultra - fast and sensitive detection of DNA hybridization assays by our laboratory*[8-11]*. The MAMEF technology couples the benefits of two technologies: 1) Metal-Enhanced Fluorescence (MEF) which increases the sensitivity of fluorescence-based bioassays[12], and 2) low power microwave heating which reduces the bioassay run time by kinetically accelerating the biological recognition events, with assay run times reduced over 1000-fold. MEF can substantially increase the sensitivity of detection. This is due to the enhancement of fluorescence emission in the near-field[13], by the presence of silver nanoparticles: the excited fluorophores partially transfer their energy to the silver nanoparticles where it is amplified and the emission from the fluorophore-silver "system" becomes greater than the emission from fluorophores alone. It is this unique combination of enhanced fluorescence emission coupled with significantly reduced bioassay run times that makes MAMEF a very powerful technology for fluorescence-based ultra- fast and sensitive bioassays, *i.e.* POC testing.

In this paper we use MAMEF for the detection of target DNA released from lysed *Chlamydia trachomatis*. The DNA was released from *Chlamydia trachomatis* by applying a low-power, low-cost, microwave based approach utilizing centimeter-sized Au disjointed "*bow-tie*" structures to focus the microwaves into a lysing volume, a technique we call "power lyse". A 10 s focused microwave burst was found to be sufficient to induce morphological changes in the CT bacteria as demonstrated by Transmission Electron Microscopy (TEM). Gel electrophoresis was also used to confirm that irradiation resulted in the release of genomic DNA from CT. Further demonstration of the detection of DNA released from 100 CFU/ mL CT, within 40 s total time

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(lysis and detection combined time) was accomplished by combining the focused microwave lysis technology with the MAMEF platform technology.

II METHODS

A. Deposition of Gold Triangles on glass substrates to lyse Chlamydia trachomatis. Glass microwave slides were covered with a mask (12.5 mm in size and 1 mm gap size) leaving a triangle bowtie region exposed. Equilateral gold triangles of 12.5 mm and 100 nm thickness were deposited onto the glass microscope slides using a BOC Edwards 306 vacuum deposition unit, with a deposition rate of 0.1nm/s. Two layers of self-adhesive silicon isolators (D=2.5 mm) were placed on top of the bow-tie region, creating a lysing chamber.

B. Lysis of *Chlamydia trachomatis* using focused microwave Irradiation. *Chlamydia trachomatis* was exposed to microwave irradiation in the lying chamber. 500 μL of different diluted aliquots of the stock bacteria (1.0×10^7 CFU.mL) were exposed to a 10 s microwave pulse. The CT solution after lysis was collected into vials for bacteria plate counting. The lysing volume and microwave power were precalibrated to 95 ^{0}C using 500 μL of buffer.

C. Bacteria plate counting. Quantitation of *C. trachomatis* was performed from stocks of chlamydia organisms grown in McCoy cells by culturing 10-fold dilutions of the chlamydia in McCoy cell in tissue culture in triplicate. Titer in includion forming units/ml (IFU/ml) was determined by the average of the 3 countable wells for number of inclusions multiplied by the dilution factor and a factor of 10 as 100ul was plated to achieve IFU/ml titer of the stock chlamydia. Aliquots of stock chlamydia organisms (1.0 x 10^7CFU/mL) were frozen for subsequent use in MAMEF experiments.

D. Gel Electrophoresis. DNA was extracted from the lysed bacteria following the scheme shown in figure 3 left. 2% Agarose gels were prepared in 1 X TAE buffer with Ethidium Bromide and run at 0.1 A on a power Pac electrophoresis device (Bio Rad Laboratories).

E. Design of anchor and fluorescent probes. The anchor and fluorophore labeled DNA probes were designed such that the anchor probe comprised 33 nucleotides and the terminal of the anchor probe was a thiol group, which readily binds to the silver surface. The fluorophore-labeled probe consisted of a region of 26 nucleotides homologous to the CT sequence. A TAMRA^{TM} NHS Ester Dye was attached to the first nucleotide, to position it close to metal when the target CT is present. This 3 pieced DNA construction has been described in detailed elsewhere[11]. The anchor probe and fluorophoe labeled probe were purchased from IDT (Coralville, IA).

F. Formation of silver island films (SiFs) on glass substrates. The procedure has been reported in detail elsewhere [14].

G. Preparation of the MAMEF assay platform for the ultra - fast detection of Chlamydia trachomatis DNA. SiFs-deposited glass slides were coated with self adhesive silicon isolators, containing 2.0 mm Diameter circular wells prior to the assay fabrication and subsequent fluorescence experiments. 100 pM of thiolated anchor probe was incubated overnight at 4°C on the surface of SiFs-deposited glass slides in EDTA Buffer, (this buffer was used in all the experiments) followed by rinsing with water to remove the unbound material. The thiolated anchor probe was subsequently covalently linked to SiFs via well-established self-assembled monolayer chemistry[15].

H. MAMEF-based Chlamydia trachomatis DNA assays. The MAMEF-based DNA capture assay was performed by the incubation of 15 μL lysed CT with varying different aliquots of stock CT solution mixed with 15 μL 1 nm TAMRA-labeled fluorescent probe on the SIFs, for 30 seconds in a microwave cavity (a 0.7 cu ft, GE Compact Microwave Model: JES735BF, max power 700 W). The power setting of the microwave cavity was set to 2 which corresponded to 140 W over the entire cavity, a fraction of which is absorbed in our assay. This power is similar to the numerous reports using low power microwaves for immunolabeling, Immunostaining, in immunocytochemistry and histological microwave processing. In all the experiments performed with low power microwaves using the SiFs surface, there was no evidence of sample drying. In the presence of the target CT DNA, the 3-piece assay is complete and enhanced fluorescence can be observed by the close proximity of the label to the silver substrate. Note: Enhanced fluorescence can only be seen in the presence of the CT target DNA.

I. Transmission Electron Microscopy. TEMs were taken with a Electron Microscope Tecnai T12. Samples were drop cast onto Formvar carbon films on copper grids (400 mesh) by placing a droplet of a 10-μL aqueous sample solution onto the grid. The grid was dried in air for 24 h.

J. Fluorescence Spectroscopy. All fluorescence assay measurements were performed by collecting the emission intensity through a long pass filter (532 nm), using a 532 nm CW diode laser for excitation and a Fiber Optic Spectrometer (HD2000) from Ocean Optics, Inc., for the detection of the fluorescence emission.

III RESULTS AND DISCUSSION

Lysis of CT Bacteria Using Gold Bow Ties and Focused Microwaves. When exposed to 2.45-GHz microwave irradiation in a conventional microwave cavity, a significant and rapid heating at the gap of the 12.5-mm gold disjoined bow-tie geometries were observed (Figure 1a). The disjoined bow-tie geometry was adopted to rapidly lyse *Chlamydia trachomatis*. In this regard, a small volume (150 μL) of bacteria (1.0×10^7 cfu/mL) was placed in the gap of the gold triangles and was irradiated for 10 s in a microwave cavity. The subsequent lysate was incubated overnight and the living bacteria counted (figure 1). We typically observed a 100 % lysing rate (Figure 1 Right), after the lysing chamber had been precalibrated not to boil over using a buffer control solution.

Using TEM, the morphologies of the CT bacteria before and after lysis are shown in Figure 2. Chlamydia are elongated ellipse-shaped ≈1000 nm in length. In the presence of the disjoined bow-tie antennas, no residual structure of CT was observed. The TEM images provided the visual evidence for the physical disruption of the bacteria and the potential release of DNA after focused microwave heating. To verify that DNA was in fact released from bacteria during

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irradiation, a quantitative evaluation of the release of DNA was undertaken using gel electrophoresis. From the gel results (Figure 3 right) we can see that the significant amount of DNA released was mostly 50 and 600 base pairs (lane 2 and lane 3). This exactly matched with the target CT DNA. These results confirmed that DNA was released from the CT in an analogous manner to how this technology was recently applied to the release of Bacillus Anthracis DNA from the spore form[11].

Microwave-Accelerated Detection of DNA Using the MAMEF Platform Technology.

The application of the MAMEF platform technology to the ultrafast and sensitive detection of target DNA sequences was previously reported[16]. The applicability of the MAMEF technology to a three piece DNA detection scheme (Figure 4) whereby a silvered glass slide is modified with an anchor probe and a target DNA sequence is detected with a fluorescence probe in less than 30 s has also been successfully demonstrated for *Bacillus anthracis*. In this scheme, the anchor probe and the fluorescent probe hybridize with different regions of the target DNA in proximity to the silver surface, where the fluorescent label is plasmon-enhanced, ultimately yielding an increased assay sensitivity. In addition, silvered surfaces preferentially focus microwave energy[17] and accelerate DNA hybridization kinetics[18]. In this paper, the MAMEF-based three-piece DNA detection scheme was adapted to detect DNA released from CT post lysing. By measuring the enhanced fluorescence spectra with varying concentrations of target DNA, we have developed a dilution curve for the fluorescence intensity and concentration of lysed CT (Figure 5). Using this approach, a detection limit of <100 cfu/mL CT has been achieved under 40 minutes total time, which includes the sample preparation time. Given that clinical samples are amenable to microwave processing, one can readily expect that we can apply the *rapid release and catch technology* to clinical samples. Work is currently underway in this regard and will be reported in due course.

IV CONCLUSIONS

In this work, the successful amalgamation of microwave accelerated and the metal-enhanced fluorescence technology for the ultrafast lysis and detection of CT was demonstrated. In this regard, disjoined bow-tie metal structures were implemented to focus 2.45-GHz microwave energy in a conventional microwave cavity to lyse and release DNA from the *CT*. The release of DNA was confirmed by TEM analysis and gel electrophoresis, respectively. The DNA extracted from as low as 100 cfu/mL CT was mixed with a fluorescent probe and captured on our MAMEF assay platform within a few seconds, where the fluorescence signal readout was readily detected, using a small detector device.

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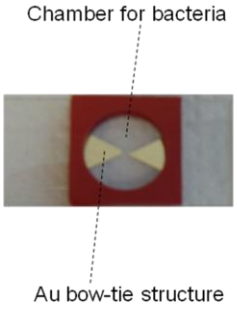
FIGURES



Before lysed (cfu)	After lysed	Lysed rate
10^6	0	100%
10^5	0	100%
10^4	0	100%
10^3	0	100%
10^2	0	100%
10	0	100%
1	0	100%
0.1	0	100%
0.01	0	100%

Figure 1. (Left) A representative substrate of the experimental geometry used to release DNA from Chlamydia, *i.e. Power lyse*. Equilateral Gold triangles of 12.5 mm size (thickness 100 nm) are deposited onto a glass microscope slide. The gap between the two triangles is 1 mm and is covered with a 2 cm Diameter silicon well. (Right) 500 uL Chlamydia in different concentrations is microwave lysed inside the silicon well for 10 secs. The lysed rate and percentage of Chlamydia remaining was calculated after undertaking plate counting.

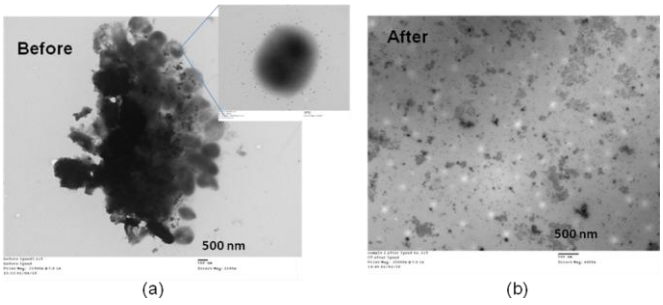


Figure 2. Transmission electron micrograph images of Chlamydia a) before b) after lysing.

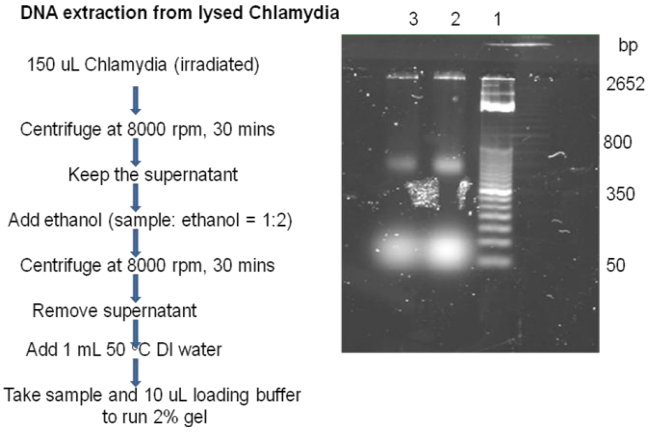


Figure 3. a) Flow diagram of DNA extraction from lysed Chlamydia. b) Ladders run on 2% agarose gel. Ladder 1. E-gel 50 bp DNA markers ladder. Lane 2. 40 ul loading volume of lysed Chlamydia bacteria. Lane 3. 20 uL loading volume of lysed Chlamydia bacteria.

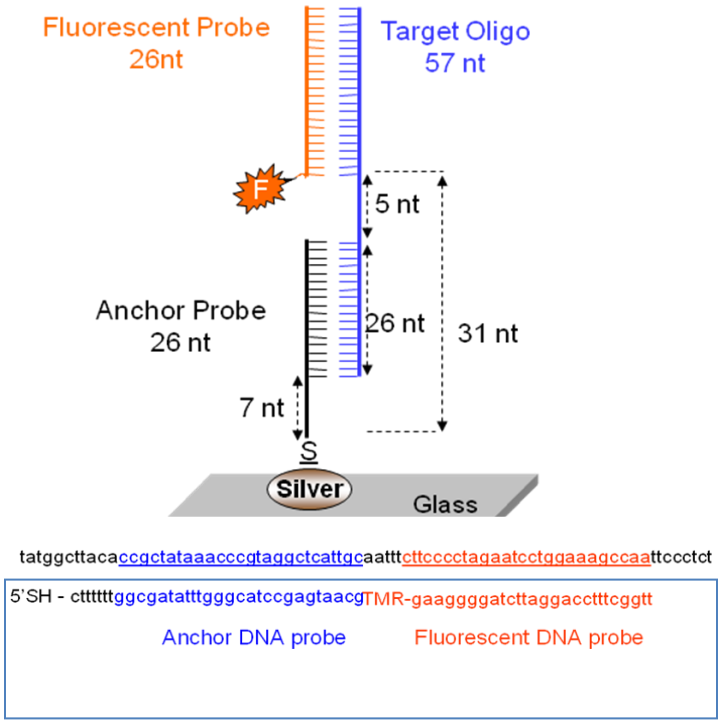


Figure 4. (Top) MAMEF assay construction for Chlamydia DNA. The anchor DNA probe, target DNA and fluorophore DNA probe, **(Bottom)**.

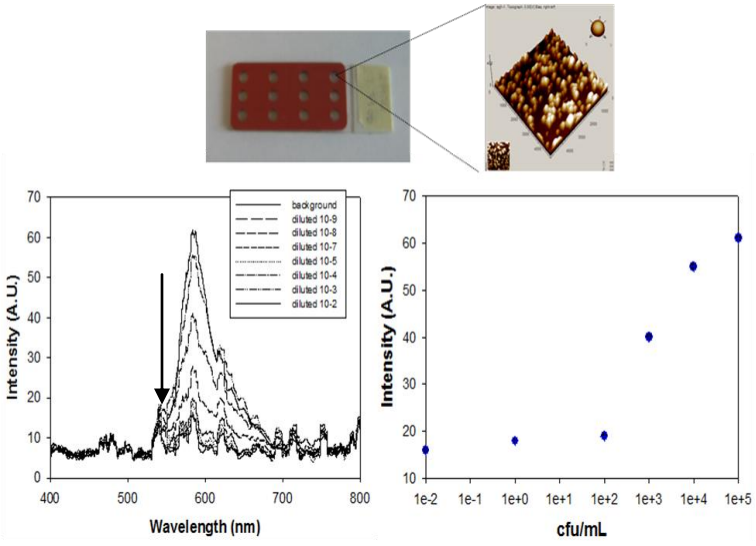


Figure 5. (Top) AFM image of SIFs as the substrate for the MAMEF assay. **(Bottom)** The fluorescence spectra of the fluorophore probe after incubation with target DNA. The intensity of the fluorophore probe is directly related to the varying

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