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Preface

This is the tenth volume in the very popular fluorescence series, Reviews in Fluorescence, by Springer (http://www.springer.com/series/6946). To date, nine volumes have been both published and well received by the scientific community, the very first volume 14 years ago in 2004. Since that time we have seen the continued growth of fluorescence techniques, as well as recognition for two fluorescence-based Nobel Prizes. In addition, The *Journal of Fluorescence*, http://link.springer.com/journal/10895, continues to be the major repository for fluorescence-based peer-reviewed publications, also a Springer journal, celebrating 27 years of publishing excellence this year.

In this 2017 volume, we are pleased again with the broad and timely fluorescence content from contributors around the world. We subsequently thank the authors for their very timely and exciting contributions again this year. We hope you all will find this volume as useful as the past volumes.

In closing, I would like to thank both Sara Germans and Meran Owen at Springer for their help in compiling this volume and with the broader series.

http://theinstituteoffluorescence.com/

http://www.chrisgeddes.com/

Baltimore, MD, USA — Chris D. Geddes

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Chapter 1
Microwave-Accelerated Metal-Enhanced Fluorescence (MAMEF): A Rapid, < 10 Copy Number Detection Platform

Tonya M. Santaus and Chris D. Geddes

Keywords Microwave-accelerated metal-enhanced fluorescence · DNA detection · Protein detection · Rapid detection · Low-copy number detection

1.1 Introduction

There is a current need for rapid, sensitive, and easy-to-use detection platforms, for a variety of proteins and DNA/RNA [1–4]. Technologies such as polymerase chain reaction (PCR), nucleic acid amplification tests (NAAT), and culturing methods have the potential to provide results anywhere between 30 min and a couple of days. Pathogen detection and identification on these platforms can take a substantial amount of time, due to sample preparation requiring lengthy growth rates and numerous processing reagents. In many academic and research laboratories today, PCR, NAAT, and culturing are still performed and are considered the gold standard for bacterial detection and identification. Our new method encompasses microwaves, metal-enhanced fluorescence, and the added benefit of protein specificity or nucleic acid hybridization.

Microwaves are electromagnetic waves with frequencies between 0.3 and 300 GHz. For this application, a domestic microwave is utilized with a frequency of 2.45 GHz. The electromagnetic energy of the microwave interacts with materials at the molecular level, where a transfer of energy is converted into heat through solvent molecular motion. This results in volumetric heating. A thermal gradient is generated via three heat mechanisms: conduction, convection, and radiation. This gradient allows for rapid protein binding and/or DNA hybridization to occur. Selective heating is considered the crux of microwave-accelerated metal-enhanced fluorescence (MAMEF) and has been reviewed in Aslan, K. Plasmonics (2008) 3:89–101.

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1.2 Development of the Microwave-Accelerated Metal-Enhanced Fluorescent Assay

At the Institute of Fluorescence in Baltimore, Maryland, researchers have developed a novel detection platform known as microwave-accelerated metal-enhanced fluorescence (MAMEF), which has the potential to influence both diagnostic and research settings. With the use of a standard microwave, a thermal gradient is generated allowing for rapid protein binding and/or DNA hybridization to occur. This platform is generic, in that it can be designed for a wide range of proteins or bacterial and viral target DNA.

1.2.1 The Principles Behind MAMEF

In the life sciences, it is pivotal to detect small quantities of proteins and/or DNA in biological fluids or tissues. Overcoming the challenge of low limits of detection can be achieved by finding approaches that can detect small amounts of the respective cellular component. Until recently, polymerase chain reaction (PCR) and culturing methods were employed exclusively for the growth, amplification, and detection of small quantities of DNA coding for certain protein or genomic DNA regions [4–8]. In the last 10 years, metal-enhanced fluorescence (MEF) has become a promising approach to detect very low copy number DNA. Metal-enhanced fluorescence occurs when a fluorophore is within close proximity to a metal surface (Fig. 1.1). The plasmon/dye coupling substantially increases the chromophore brightness as compared to a free dye in bulk solution. The MEF effect is employed in DNA MAMEF by anchoring a probe DNA (anchor probe) to surface-bound nanoparticles. Upon hybridization with labeled DNA, the fluorophore comes within proximity (5–10 nm) of the metal nanoparticles. Under these conditions, the system produces a strong fluorescent signal due to the near-field bimolecular recognition event.

To facilitate MEF, surface-immobilized nanoparticles are used. While silver is the ideal plasmon-supporting substrate, surfaces made from gold, copper, zinc, and aluminum can be employed. The silver island films (SiFs) used for MAMEF were characterized before and after microwave irradiation to verify that the films were not changing with microwave irradiation with a glass microscope slide as a control. Plasmon absorption spectra were taken before and after low-power microwave heating for 30 s. Atomic force microscopy (AFM) was also used to analyze the silver island film surface morphology. As seen in Fig. 1.2, microwave heating had no effect on the surface plasmon absorption (Fig. 1.2b) of the SiFs. This indicates no abnormalities in the surface structure or surface shape of the silver nanoparticles. There was no variation in the metal surface morphology of the nanoparticles as confirmed through AFM (Fig. 1.2c). These tests demonstrate the compatibility of the metallic nanoparticle structures with microwave heating. The combination of metal-

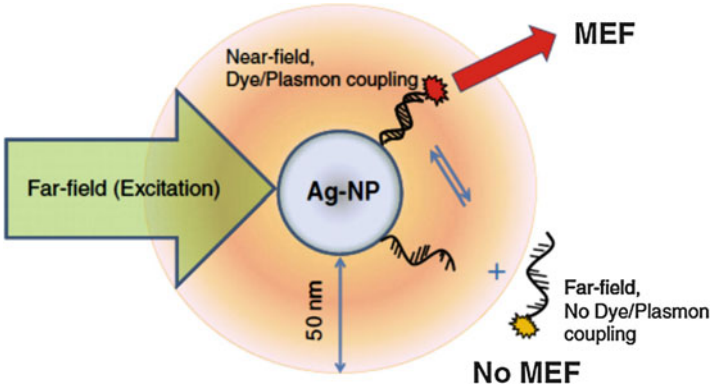

Fig. 1.1 Metal-enhanced fluorescence (MEF) is the principle technology behind the MAMEF platform. The fluorescence occurs in the near field when the fluorophore is coupled to the metal plasmons in both the ground and excited states

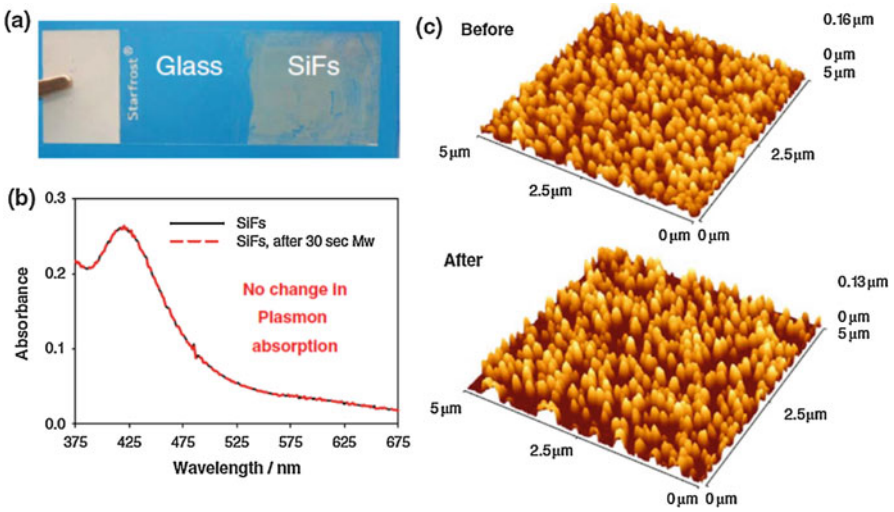

Fig. 1.2 (**a**) Photograph of silver island films (SiFs) coated on a glass slide. (**b**) Absorbance of SiFs before and after 30 s of microwave irradiation. (**c**) AFM images of SiFs before and after microwave irradiation. No change in plasmon absorption or SiF structure is seen post microwave irradiation

enhanced fluorescence with microwave acceleration is termed microwave-accelerated metal-enhanced fluorescence (MAMEF) [8].

"Indirect" and "direct" approaches have been used to study the thermal gradient generated between the aqueous media and the metallic nanoparticles, in particular the heating of assay components. For the indirect method, microwave heating was determined by monitoring the ratiometric absorbance of a temperature-sensitive dye such as thymol blue. Thymol blue was heated in a microwave and transferred to a

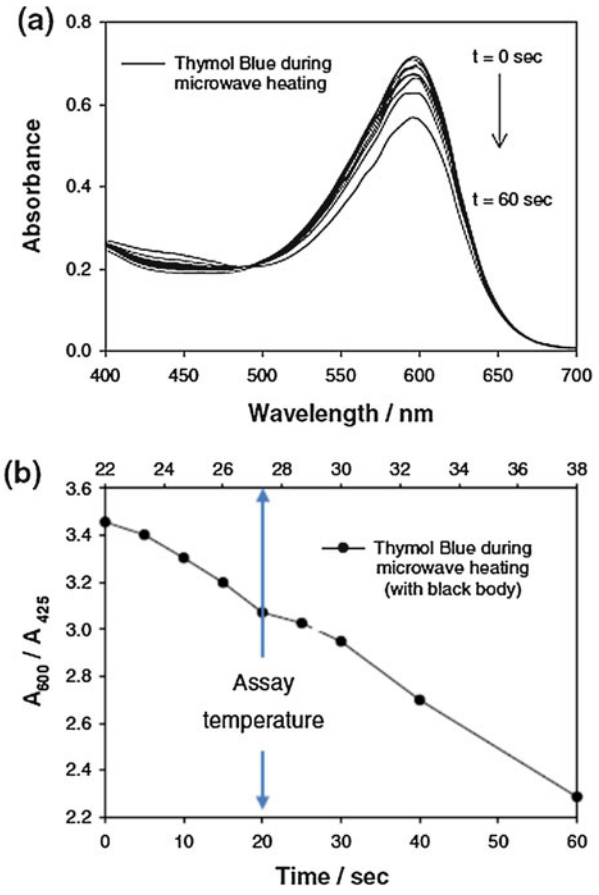

Fig. 1.3 Real-time monitoring of temperature changes during microwave irradiation. (a) 30 μL thymol blue absorption spectra as a function of temperature measured during microwave heating. (**b**) Temperature vs. time ratiometric respective absorbance

spectrophotometer where the absorption spectra were taken. Figure 1.3a, b depicts the temperature-dependent absorption spectra of thymol blue and the calibration curve for microwave heating up to 60 s for the volume of fluid heated. The direct method utilizes a thermal camera that captures the infrared (IR) radiation of temperature changes on the assay surface during microwave irradiation. In order to detect the IR radiation, the SiFs were deposited on to sapphire plates. It was shown that heat is transferred from the warm water to the colder water near the silver nanoparticles resulting in a rapid diffusion of biomolecules toward the silver surface [9]. Figure 1.4a depicts the general thermal gradient generated in MAMEF. A comparison study was undertaken with biomolecules to demonstrate the gradient generated with and without the use of the SiF substrate (Fig. 1.4b). A temperature difference of ~6 °C is seen with the use of SiFs; without the metal substrate, there is no change in temperature across the substrate and solution [10].

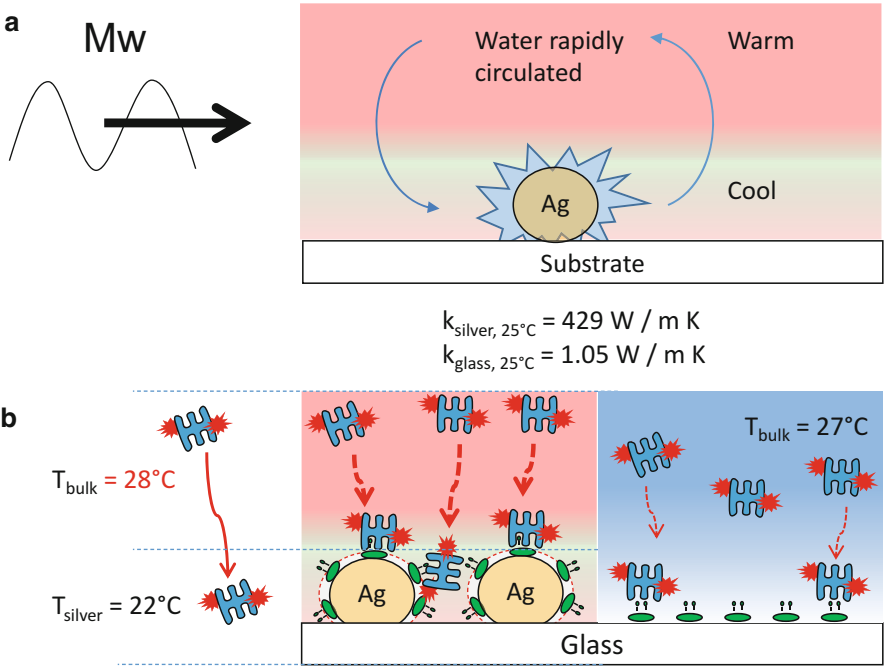

Fig. 1.4 (**a**) Thermal gradient generated during microwave irradiation. (**b**) Cartoon depicting the microwave heating of solutions both with (left) and without (right) silver nanoparticles. In the presence of silver nanoparticles, a rapid thermal gradient is created between the cooler silver nanoparticles and microwave-heated aqueous solution, which facilitates antibody-antigen or other biomolecule associations such as DNA/RNA hybridization

1.2.2 Early Development of Protein-Based MAMEF Assays

One of the first model MAMEF protein assays was performed in 2005. Biotinylated bovine serum albumin (BSA) was used as the protein to bind streptavidin-labeled fluorescein isothiocyanate (FITC). Experimentation was undertaken with and without the use of a silver colloid film to elucidate the fluorescent intensity changes between the glass and the fluorophore and the silver and the fluorophore (i.e., MEF). As noted above, when a metal substrate is deposited onto the glass and a biomolecule solution is microwaved, a thermal gradient is generated facilitating rapid binding events to occur. Since fluorescein is within close proximity to the silver substrate, (MEF) can then also occur. The setup of the experimental assay is depicted in Fig. 1.5a. Streptavidin-labeled fluorescein isothiocyanate was incubated on a silver colloid-coated BSA film and on a glass BSA-coated film for 30 min. The same setup was also performed, but the system was microwave irradiated for 20 s. The same fluorescent intensity is seen when the FITC-Avidin is incubated at room temperature on silver colloid BSA-coated film or microwave irradiated for 20 s (Fig. 1.5b). This

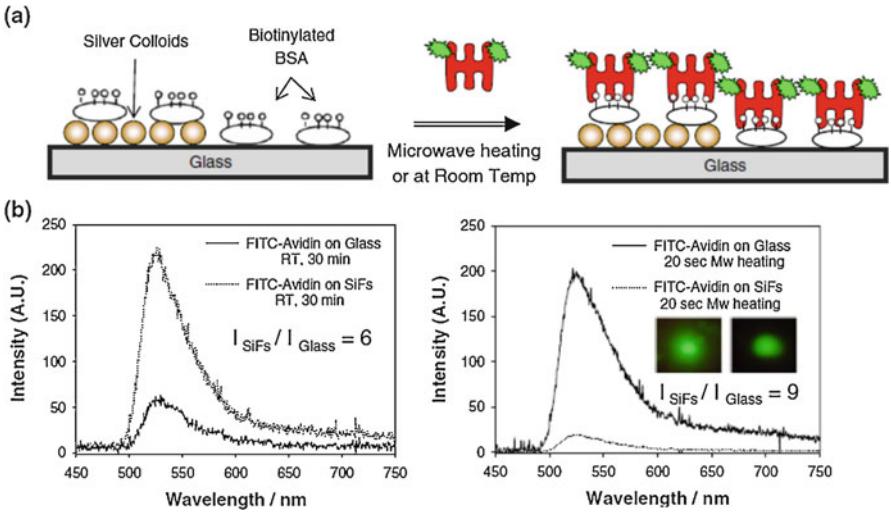

Fig. 1.5 A model MAMEF protein-based detection assay. (**a**) A protein-fluorophore system used to demonstrate MAMEF on both silver and glass. (**b**) Fluorescein isothiocyanate emission spectra at room temperature incubation for 30 min (left) and with 20 s microwave heating (right). The signal from the silvered assay is significantly brighter than from the simple glass substrate

validates that using microwave irradiation can significantly shorten the molecular binding event time, yet while still having the same degree of signal intensity as generated by room temperature incubation [9]. When the model assay is compared on both glass and the silvered surface, there is a significant increase in the fluorescence from the silver, i.e., MEF. It is this optical amplification of the fluorescent signal, coupled with the very fast assay time due to microwave heating (microwave acceleration), which underpins the MAMEF technique.

Shortly thereafter at the Institute of Fluorescence, a protein MAMEF assay was developed for use of high-throughput screening (HTS). Many fluorescence-based HTS applications are limited by the quantum yield of the labeling fluorophore, the antigen-antibody recognition step, and the low amount of material used. Based on the MAMEF model assay in 2005, the Institute of Fluorescence subsequently demonstrated the application of the MAMEF technology to run in a HTS-based format [9]. Commercially available polylysine-coated HTS wells were modified with silver nanoparticles to facilitate the metal-enhanced fluorescent effect. The model FITC-Avidin assay was used on the HTS wells, and the fluorescence emission from both the non-silvered and silvered wells was collected after incubation at room temperature for 30 min or after microwave heating for 30 s (Fig. 1.6). There was a five- and fourfold emission intensity increase from silvered wells compared to non-silvered wells, as well as for assays run at room temperature and after microwave heating, respectively. This study demonstrated the applicability of the MAMEF technology to be used in HTS formatted applications [9, 11]. Today,

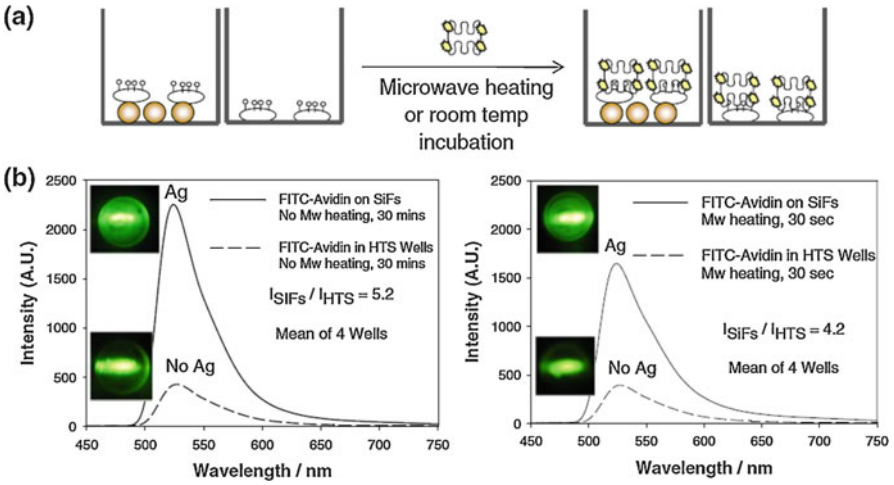

Fig. 1.6 (**a**) High-throughput screening 96-well plates coated with and without silver nanoparticles. (**b**) The fluorescent intensity mean of four wells incubated at room temperature for 30 min with and without the use of SIFs (left) and the fluorescent intensity before and after 30 s of microwave irradiation

silvered 96-well plates can be readily purchased from Ursa BioScience under the trade name Quanta-plate™ (www.urasebioscience.com).

1.2.2.1 Anthrax Toxin MAMEF Protein Assay

Following the development of the MAMEF protein model assay and the high-throughput plate screening model, a real-world assay was developed for anthrax toxin. Anthrax toxin (PA) assay was chosen as an essential real-world protein assay due to the clinical importance of detecting anthrax rapidly, noting that patients are usually deceased approximately 48 h post infection. Based on previous studies optimizing the surface properties for metal-enhanced fluorescence (MEF), a MEF-based anthrax toxin assay was developed (Fig. 1.7). The general procedure for the assay included microwave irradiation of a primary antigen solution on the silver nanoparticle plate for 30 s. The wells were then washed and blocking was performed three times with SuperBlock solution. A second wash was performed with a 30 min incubation of secondary antibody labeled with fluorescein in the silvered wells. A third wash was performed with buffer to remove any free antibodies, and fluorescence detection was performed. Fluorescent signals were compared from SiF-containing plates and plates containing no silver. As seen in previous studies, the fluorescent signal from the silver-containing plates was significantly larger than the plates containing no silver. Sensitivity of the assay was completed using different concentrations of primary antigen ranging from 10 ng/mL to 0.01 pg/mL with a control containing no primary antigen. The MEF-PA assay demonstrated

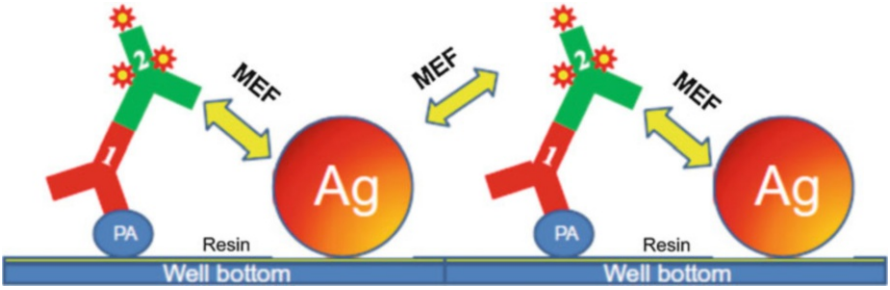

Fig. 1.7 Scheme of the MEF-anthrax toxin assay. The multiprotein complex (PA, primary antibody, and secondary antibody labeled with fluorescein) is attached to HBX plate bottom resin with a coating of silver nanoparticles

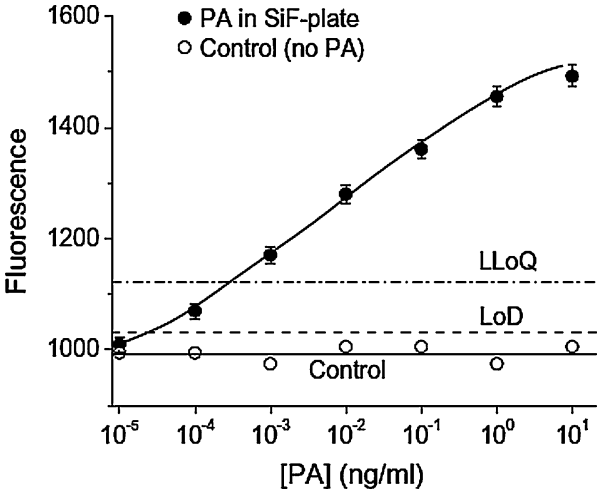

Fig. 1.8 Anthrax toxin sensitivity curves measured in SiF plates. LoD = mean fluorescence +3× standard deviation (dashed line) (LLoQ = 10× standard deviation (dashed-dotted line)

high sensitivity to the primary antigen (Fig. 1.8). Fluorescence readings from all the plates could be reliably registered down to a concentration of 0.1 pg/mL. The limit of detection and the lower limit of quantitation were 0.1 pg/mL and 1 pg/mL, respectively [12]. The resulting assay from this study still remains about 1000 times more sensitive and 5000 times quicker than the US Navy standard ELISA immunoassays [12].

1.2.2.2 Myoglobin Detection: Potential Application for Myocardial Infarction Diagnosis

Cardiovascular disease is a leading cause of mortality in many countries. Most assessments for myocardial infarction are based on cardiac markers like troponin T, troponin I, and myoglobin, although myoglobin is not a specific marker in itself

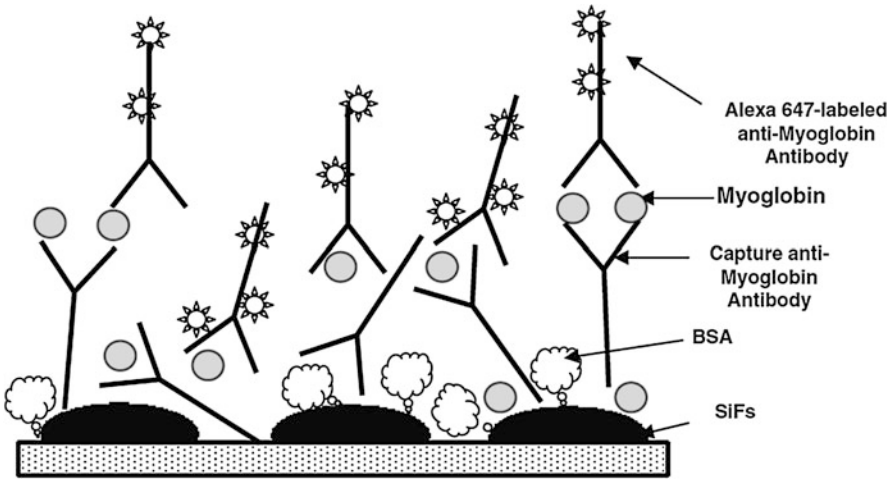

Fig. 1.9 General scheme of the myoglobin MAMEF immunoassay

[13–17]. Myoglobin was chosen for this study because of the clinical cutoff level for myoglobin detection in the clinic (100 ng/mL). The general assay scheme is shown in Fig. 1.9. Slides were coated with a capture anti-myoglobin antibody at room temperature. The glass/SiF surfaces were blocked by BSA to minimize the interaction of antibodies and myoglobin with the surface (i.e., nonspecific interactions with the surface). The clinical cutoff concentration of myoglobin was added and incubated. Endpoint fluorescent measurements were performed after incubating the coated surface in a solution of Alexa 647-labeled anti-myoglobin antibody for 30 min at room temperature or by microwave irradiation for 20 s. Fluorescence was then taken using a 650 nm diode laser and a fiber-optic spectrometer. An increase in fluorescent intensity is seen from the silvered slides when the system is incubated at room temperature or via microwave irradiation (Fig. 1.10) [17]. The myoglobin assay demonstrates the potential of MAMEF for use for clinical diagnosis. Most importantly, this assay demonstrates the significant potential of the MAMEF technique for detecting clinically important analytes, noting in particular that the assay in Fig. 1.10 is 90 times faster than a standard room temperature incubation.

1.3 Development of the DNA-Based MAMEF Platform

DNA hybridization assays are currently used in detection techniques such as gene chips, diagnostic settings, and fluorescence hybridization studies [9, 18, 19]. In nearly all of these methods, a fluorophore with a high quantum yield is employed to increase the overall sensitivity and detectability of the assay. However, complications such as high background emissions and low fluorophore photostability are primary concerns. To maximize the efficiency and the sensitivity of DNA

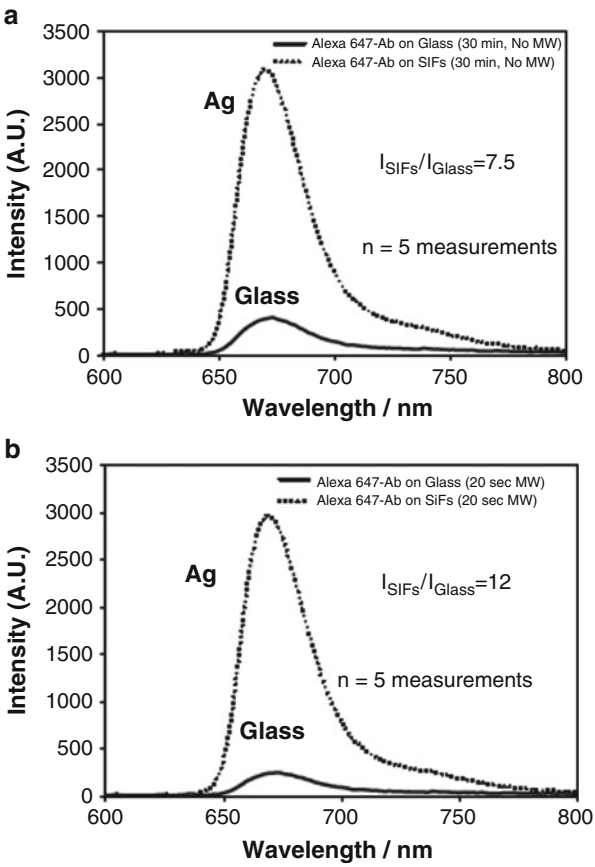

Fig. 1.10 Emission spectra of Alexa 647 on coated and non-coated glass slides after 30 min incubation (**a**) and after microwave irradiation for 20 s (**b**). A significant fluorescent intensity increase is seen with the use of the silver-coated slides, i.e., MEF. Microwave acceleration was about 90 times faster as compared to room temperature incubation

hybridization, MAMEF assays can be employed. This is because MEF-based assays utilize low quantum yield fluorophores, which are preferentially plasmon-amplified near-the-metal and contribute little to the overall background fluorescent noise, i.e., the distal fluorophores do not fluoresce.

The first proof-of-concept design was a two-piece DNA hybridization construct. Two complementary oligonucleotides, one labeled with fluorescein, were hybridized on silver nanoparticles and microwave irradiated for 20 s (Fig. 1.11a). The anchor probe, i.e., the capture oligonucleotide, was attached to silver nanoparticles via a sulfhydryl-metal bond after overnight incubation. The assay, incubated with complementary DNA, was carried out at room temperature for 3.5 h or with a low-power microwave heating for 20 s. The fluorescein emission spectra from the room temperature incubation yielded approximately a 2.5 field larger intensity than the corresponding control assay. Identical fluorescein emission intensity was also observed after the low-power microwave heating, demonstrating a remarkable 600-fold decrease in the assay run time (Fig. 1.11b). This two-piece DNA

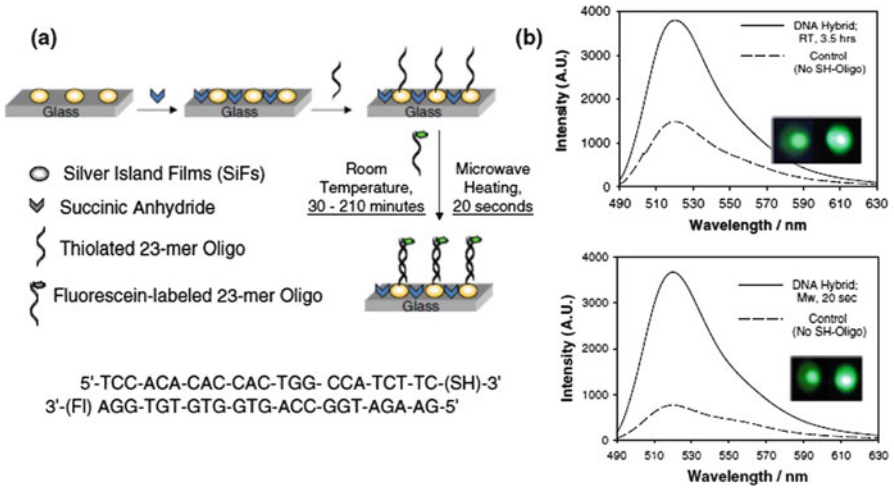

Fig. 1.11 (**a**) Two-piece DNA assay. (**b**) Fluorescence of the assay incubated at room temperature for 3.5 h (top) and microwave irradiated for 20 s (bottom)

hybridization assay demonstrated a reduced DNA hybridization time and a high sensitivity for DNA detection and quantification.

Nonspecific binding is a major factor that influences the sensitivity of many hybridization assays. The silver nanoparticles enhance the fluorescence emission, increase the photostability of the fluorophore, and serve as the platform for anchor oligonucleotides. Considering the silver nanoparticles act as the substrate for the assay, they need to be protected against nonspecific binding of complementary target oligonucleotides. Therefore, self-assembled monolayers of alkanethiol nonreactive groups can be used to occupy the spaces between the anchor probes on the metal nanoparticles. Interestingly, the microwave-accelerated assays typically result in a reduced nonspecific absorption of the assay biomaterial, which is a significant opportunity for bioassay development. It is thought that the reduction in the nonspecific binding kinetics is due to the change in the binding rate (k_A) during microwave heating versus room temperature incubation. Another important factor to the assay is the effect of microwave heating on the oligonucleotides. The effects of low-power microwave heating were employed by analyzing the heating, melting, and rehybridization of the assay (Fig. 1.12). Fluorescence emission spectra of the two-piece fluorescein-labeled assay were taken after microwave heating, post DNA melting, removal of the fluorescein-labeled oligonucleotide, and rehybridization with fresh-labeled oligonucleotide. After rehybridization occurred with microwave heating, a similar fluorescent intensity was observed, indicating that the anchor probe was remarkably unaffected during microwave heating. These results indicate that the anchor oligonucleotides are reusable, which is a notable factor in the preparation of low-cost hybridization assays.

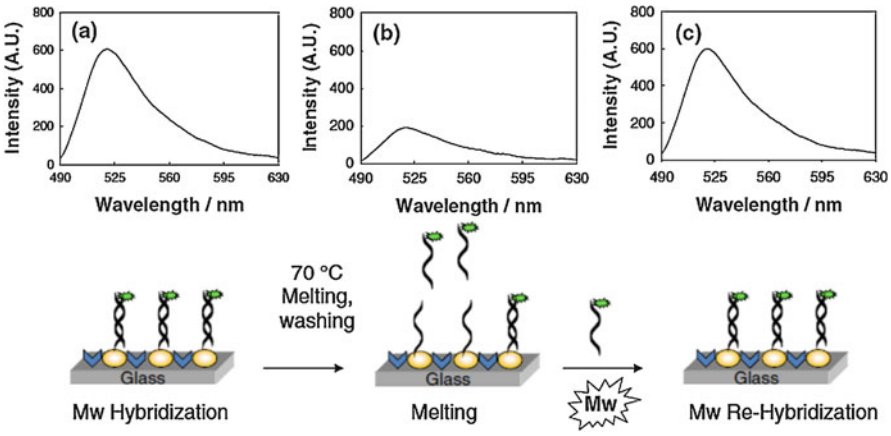

Fig. 1.12 Reversibility of the DNA hybridization assays on glass substrates. Emission spectra of DNA oligos labeled with fluorescein (**a**) after hybridization, (**b**) after melting, (**c**) after rehybridization and 250 nm DNA addition. The results show that MAMEF does not denature the DNA, both in solution and on the surface

1.3.1 The Three-Piece DNA Hybridization Assay: Ideal for Real-World DNA Detection

Following the success of the two-piece assay, a three-piece assay was subsequently developed for use with real-world samples. The assay consists of an anchor probe connected to the metal nanoparticle surface via thiol bonds, a fluorescent probe, and the target DNA of interest (Fig. 1.13) [8]. Anchor and fluorescent probes that are complimentary to the specific genome of interest can similarly be designed. This approach produces highly specific recognition/hybridization, which is similar in principle to DNA primers for polymerase chain reaction (PCR) based DNA detection [8].

1.3.1.1 Benefits of a Fluorophore-Metal System

Underpinning the metal-enhanced fluorescence effect, there is a fluorophore-metal radiative decay rate modification. This is characterized mathematically by an increased quantum yield (increased fluorescence intensity) coupled with a decreased lifetime. The fluorophore-metal system is coupled in both the ground and excited state. This is represented in Eq. 1.1 where the system quantum yield (Q_m) in metal-enhanced fluorescence is a function of the radiative decay rate (Γ), the metal-modified radiative decay rate (Γ_m), and non-radiative rates (k_{nr}).

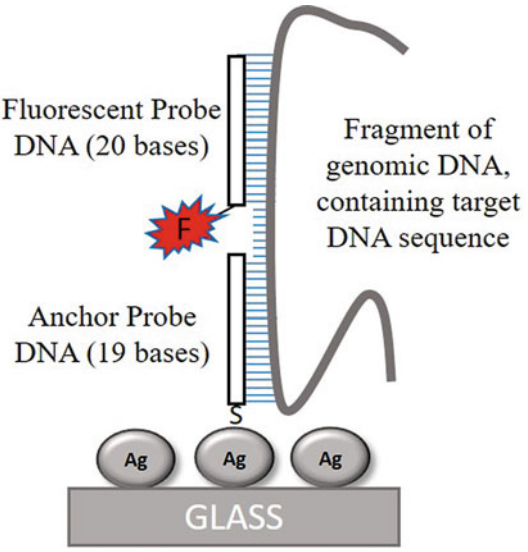

Fig. 1.13 Three-piece DNA MAMEF assay construct consisting of an anchor probe, fluorescent probe, and target DNA. Only when the target DNA is present will the three-piece DNA assay form and the fluorophore be close enough to the surface to facilitate metal-enhanced fluorescence

$$Q_{\mathrm{m}} = (\Gamma + \Gamma_{\mathrm{m}})/(\Gamma + \Gamma_{\mathrm{m}} + k_{\mathrm{nr}}) \tag{1.1}$$

Following the equation for quantum yield, the equation for the system lifetime can then be modified to include the metal (Eq. 1.2) where τ_{m} is the metal-modified lifetime.

$$\tau_{\mathrm{m}} = 1/(\Gamma + \Gamma_{\mathrm{m}} + k_{\mathrm{nr}}) \tag{1.2}$$

These generalized equations for both Q_{m} and τ_{m} (plasmon/metal-modified quantum yield and lifetime, respectively) do not account for the modified non-radiative rates but do suggest some important photophysical possibilities. From Eq. 1.1, we can see as Γ_{m} increases, the quantum yield of the system also increases. However, from Eq. 1.2, as Γ_{m} increases, the lifetime subsequently decreases. Fluorophores near-to-metal in the above equations indicate an increase in brightness accompanied by a simultaneous drop in the radiative lifetime. This is in contrast to classical fluorescence, where the quantum yield and lifetime always change in unison. To observe this, fluorescein decay rates were measured from an assay on both glass and silvered glass after a 30 min incubation or 30 s low-power microwave heating, respectively (Fig. 1.14) [9, 20]. The assay was very similar to that shown in Fig. 1.5. The intensity decay curves for fluorescein on silver after room temperature incubation or 30 s microwave heating were almost identical. However, significantly reduced lifetimes as compared to the glass control were observed. The decay curves serve to confirm modification in the fluorophore-metal system radiative decay rate [20]. In terms of immunoassays, this reduced lifetime is significant as assay

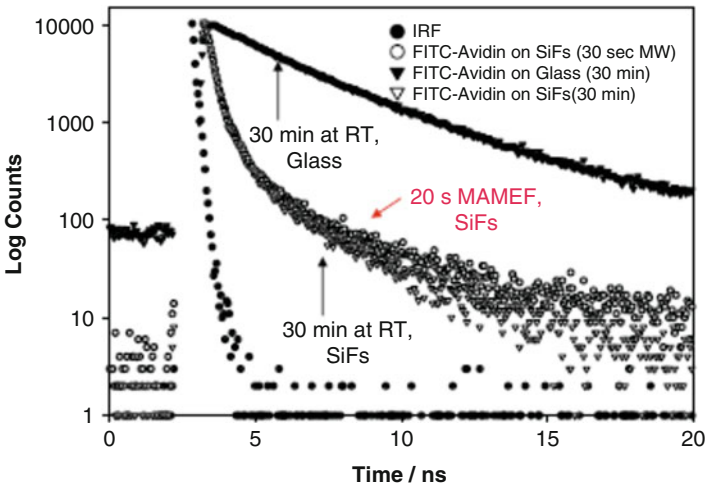

Fig. 1.14 Lifetime studies for a fluorophore used in MAMEF assays. Intensity decays for fluorescein isothiocyanate (FITC)-Avidin on both and glass and silver nanoparticles before and after microwave irradiation. The intensity decays on the SiFs are almost identical. IRF = instrument response function. A slightly reduced lifetime is observed for fluorophores near-to-metal

detectability is underpinned by the photostability of the fluorophore used in the assay. In MAMEF assays, the fluorophore typically has a reduced lifetime near-to-metal, which enhances the assay photostability, allowing the signal to be integrated for longer without degradation.

1.3.2 MAMEF Assays for the Detection of Salmonella, Chlamydia, *and Gonorrhea*

The MAMEF assay is a versatile tool for the detection of various bacteria. One of the first MAMEF assays was used for the detection of *Salmonella* in 2011. *Salmonella* is a gram-negative bacterium, which contains several serovars that can cause human disease. Non-typhoidal *Salmonella* (NTS) usually produces gastroenteritis which constitutes vomiting, fever, and diarrhea. Non-typhoidal *Salmonella* can cause severe and fatal disease in industrialized and developing countries [21–29]. *Salmonella typhimurium* and *Salmonella enteritidis* are the most common NTS serovars isolated from blood and other sterile sites found in patients in Europe and sub-Saharan Africa [30–32]. Currently, there is a pressing need for sensitive and specific rapid diagnostic test to detect NTS bacteremia.

To address the need for a rapid diagnostic test, a MAMEF assay was developed for the detection of *Salmonella* [29]. The target DNA was the *oriC* locus of the *Salmonella* genome. The lysis of the bacteria was performed with the Lyse-It®

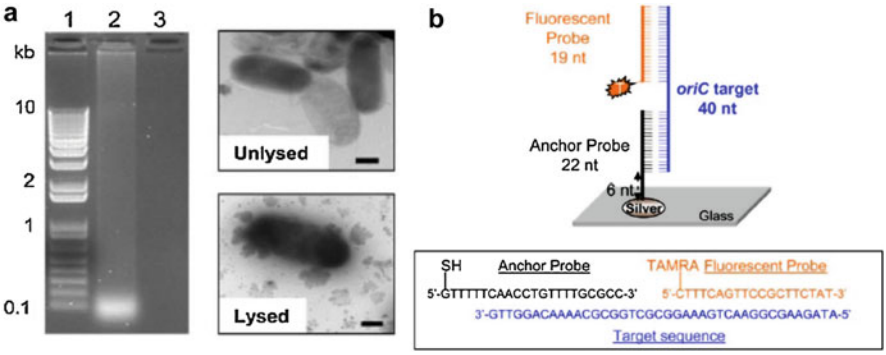

Fig. 1.15 Microwave lysis (Lyse-It®) and DNA fragmentation of *Salmonella* followed by MAMEF detection. (**a**) Gel electrophoresis displaying DNA fragments below 100 base pairs, TEM images of unlysed cells (top), and microwave irradiated lysed cells (bottom). (**b**) The DNA target and fluorescent probe sequences used in the MAMEF assay hybridized to the *ori*C target

technology (www.lyse-it.com). Gold lysing triangles with a bowtie configuration were used with high-power microwave irradiation for 13 s. The lysis was analyzed through gel electrophoresis and transmission electron microscopy (TEM) (Fig. 1.15a). Gel electrophoresis showed that DNA was extracted and lysed using the Lyse-It® technology. TEM confirmed that *Salmonella* cells were being broken open to release inner cellular components. Following cellular lysis, MAMEF was performed for the detection of the *oriC* DNA target. Previously, PCR primers were used to detect the target, and thus those primers were the basis of the design for the anchor and fluorescent probes of the MAMEF assay [27, 29, 33]. The anchor probe was 22 nucleotides long, while the fluorescent probe was 19 nucleotides long, hybridizing with a 40 nucleotide *oriC* target (Fig. 1.15b). Following the optimization of lysis and detection, two methods were tested to detect DNA using the MAMEF platform. Overnight 10^3 CFU/mL cultures of suspended CVD 1920 were diluted to biologically relevant concentrations. Two milliliters of the concentrations were microwave irradiated, and 1 mL of the lysed bacteria was tested on MAMEF. The intensity of the fluorescent signal was concentration dependent (Fig. 1.16a). To validate the concentration dependence of the target DNA in the MAMEF assay, concentrations of *oriC* ranging from 0.5 to 500 nM were tested on the platform (Fig. 1.17). The higher the concentration of target DNA, the greater the fluorescent intensity increase. Whole blood studies were also performed. Preliminary experiments with spiked blood were performed, and fluorescence was not observed greatly from the baseline. However, the *oriC* target DNA is readily detected when the sample was diluted with an equal volume of phosphate buffer saline (PBS) (Fig. 1.16b) [29, 33]. With the success of the *Salmonella* assay with synthetic oligonucleotides and detection of the gene target, *oriC*, more assays have designed for rapid and sensitive detection of sexually transmitted infections, in particular for *Chlamydia* and Gonorrhea.

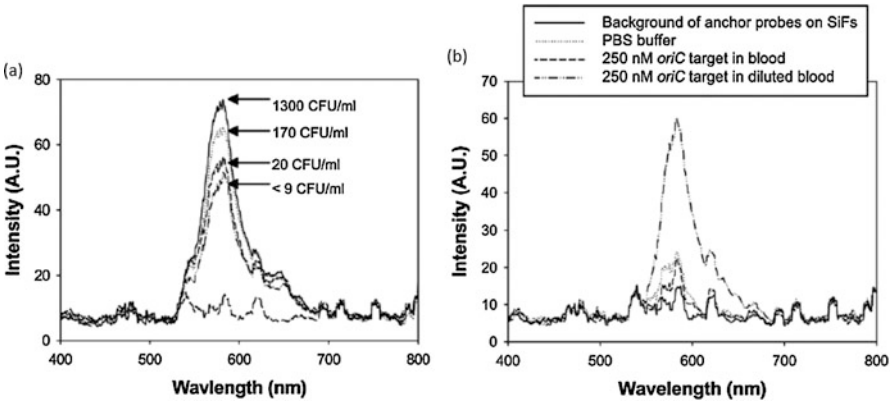

Fig. 1.16 Detection of DNA released from lysed *Salmonella.* (**a**) Tenfold serial dilutions of independent 10^3 CFU/mL suspension. (**b**) MAMEF-based detection of synthetic *oriC* suspended in whole blood

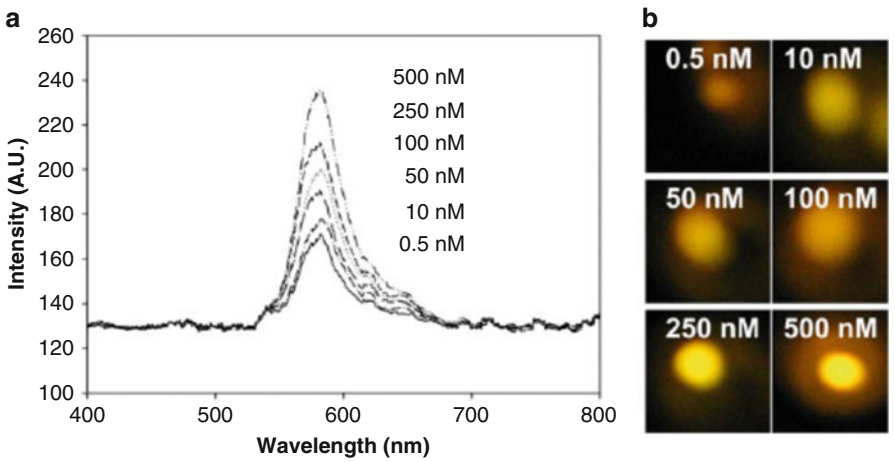

Fig. 1.17 (**a**) Concentration dependence of target *oriC* from *Salmonella* on the MAMEF assay. (**b**) Direct dependence of fluorescent intensity to the concentration of target DNA. The more the target DNA, the brighter the fluorescence and the greater the intensity difference from the baseline

1.3.2.1 Blinded *Chlamydia* Clinical Trial

Chlamydia trachomatis is the bacterium responsible for the infection, *Chlamydia*, which is the most commonly reported bacterial sexually transmitted infection (STI). According to the Center for Disease Control, in 2011, there were 1,412,718 chlamydial infections in the United States and the District of Columbia [10]. To address this significant clinical need, a collaboration between the Johns Hopkins University and the Institute of Fluorescence at the University of Maryland, Baltimore County,

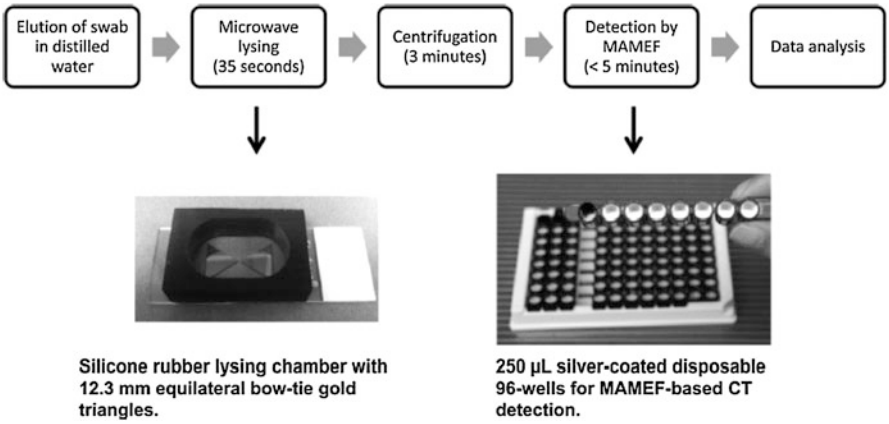

Fig. 1.18 Flowchart of the steps for the MAMEF clinical *Chlamydia* assay

began a blinded clinical validation study. The MAMEF assay was compared against common methods such as nucleic acid amplification tests (NAAT). The first assay targeted the *C. trachomatis* 16S rRNA gene, while the second assay targeted the *C. trachomatis* cryptic plasmid. The general procedure followed the elution of a vaginal sample swab in distilled water followed by microwave lysing with the Lyse-It® technology, centrifugation, and detection on the MAMEF platform (Fig. 1.18). The detection was undertaken on silver-coated microtiter plates (Ursa BioScience). All samples were tested in duplicate using both the 16S rRNA and the cryptic plasmid. A negative control sample of pooled *C. trachomatis*-negative specimens was tested in parallel. The silver-coated wells were subject to an initial washing step to remove any unbound fluorescent probe, and a secondary wash was carried out for all samples with elevated signals. The number of positive and negative samples from the NAAT and MAMEF tests is shown in Fig. 1.19. There were 257 vaginal swab samples available for analysis. As shown in the figure, 45 samples were identified to be positive for *C. trachomatis*, and 212 samples were identified as negative through NAAT testing. From the MAMEF 16S rRNA and cryptic plasmid assays, 33/45 and 197/212 samples were identified correctly. The two assays were also tested with commonly found bacteria in vaginal swabs. An average fluorescent intensity plot was created to determine the limit of detection (LOD) for both MAMEF assays (Fig. 1.20). The cryptic plasmid has a limit of detection of ten inclusion-forming units per milliliter (IFU/mL), while the 16s rRNA assay had an LOD of 100 IFU/mL. In this study, the overall specificity and sensitivity were greater than 90%. The total time for the assays to run in parallel was less than 9 min, which included the sample preparation time from a clinical vaginal swab. The *C. trachomatis* detection through MAMEF is low-cost, rapid, specific, and has the potential to be adopted as a point-of-care test [10].

MAMEF assay result	No. of samples with a NAAT (ProbeTec or Gen-Probe) result of: Positive	Negative	Total no. of samples
Combination of 16s rRNA and cryptic plasmid-based MAMEF assays			
Positive	33	15	48
Negative	12	197	209
Total	45	212	257
16s rRNA-based MAMEF assay			
Positive	34	15	49
Negative	11	197	208
Total	45	212	257
Cryptic plasmid-based MAMEF assay			
Positive	37	15	52
Negative	8	197	205
Total	45	212	257

Fig. 1.19 *Blinded* MAMEF assay results versus NAAT results

Microbial organisms	MAMEF targets: 16S rRNA[1]	Cryptic plasmid[2]
C. Trachomatis serovar K	+	+
C. trachomatis serovar E	+	+
C. trachomatis serovar L	+	+
C. pneumoniae T4	+	-
C. pneumoniae AR39	+	-
C. psittaci	-	-
Neisseria gonorrhoeae	-	-
Neisseria meningitidis	-	-
Trichomonas vaginalis	-	-
Herpes simplex virus 1 & 2	-	-
Haemophilus ducreyi	-	-

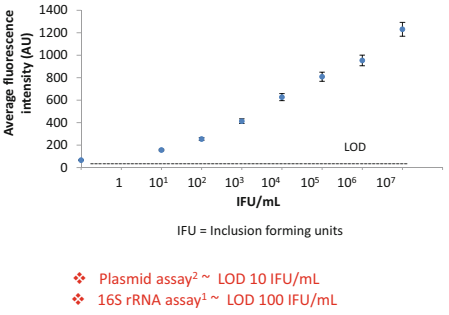

Fig. 1.20 (Left) Specificity of the MAMEF assays against microbial organisms commonly found in vaginal samples. (Right) Determination of the limit of detection for the 16S rRNA and cryptic plasmid assays

1.4 Conclusions and Future Directions

Over the last few years, the microwave-accelerated metal-enhanced fluorescence (MAMEF) technology has been shown to be a powerful, rapid, highly sensitive platform for the detection of either DNA, RNA, or proteins. When used in conjunction with Lyse-It®, a sample preparation technology, DNA/RNA and protein of interest can be detected from clinical samples within a few minutes. Microwave-

accelerated metal-enhanced fluorescence is currently undergoing several future clinical validations, all of which will be reported in due course.

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Chapter 2
Hydroporphyrins in Fluorescence *In Vivo* Imaging

Marcin Ptaszek

Keywords Near-IR fluorophores · Chlorins · Bacteriochlorins · Fluorescence bioimaging · Multicolor fluorescence imaging

2.1 Hydroporphyrins – Structure, Synthesis, and Basic Photochemical Properties

Hydroporphyrins (Fig. 2.1) are tetrapyrrolic macrocycles with a partially saturated pyrrolic subunits [9]. In porphyrins four pyrrole moeties are connected through sp^2 carbon atoms to form fully conjugated, aromatic macrocycle (Fig. 2.1). Aromaticity is granted through the conjugated 18π-electronic system [10]. The partial saturation at carbon-carbon double bond between *β-β* positions) leads to the formation of hydroporphyrins, of which optical and photochemical properties are significantly different from those for poprhyrins [11, 12].

There are three types of hydroporphyrins [11, 12], which retain the fully conjugated, 18π-electron aromatic system: chlorins, bacteriochlorins, and isobacteriochlorins (Fig. 2.1). Chlorins possess two sp^3 carbon atoms, which constitute one extra carbon-carbon single bond compared to porphyrins, and one partially saturated pyrroline ring. There are isomeric hydropoprhyrins, bacteriochlorin and isobacteriochlorin, which incorporate four sp^3 carbon atoms in the macrocyclic core, which give rise to two pyrroline rings, on the opposite sides of the macrocycle (bacteriochlorin) or adjacent to each other (isobacteriochlorin). The vast majority of hydroporphyrins utilized for bio-imaging applications, are based on chlorin or bacteriochlorin systems, and therefore only these two classes of macrocycles will be discussed here.

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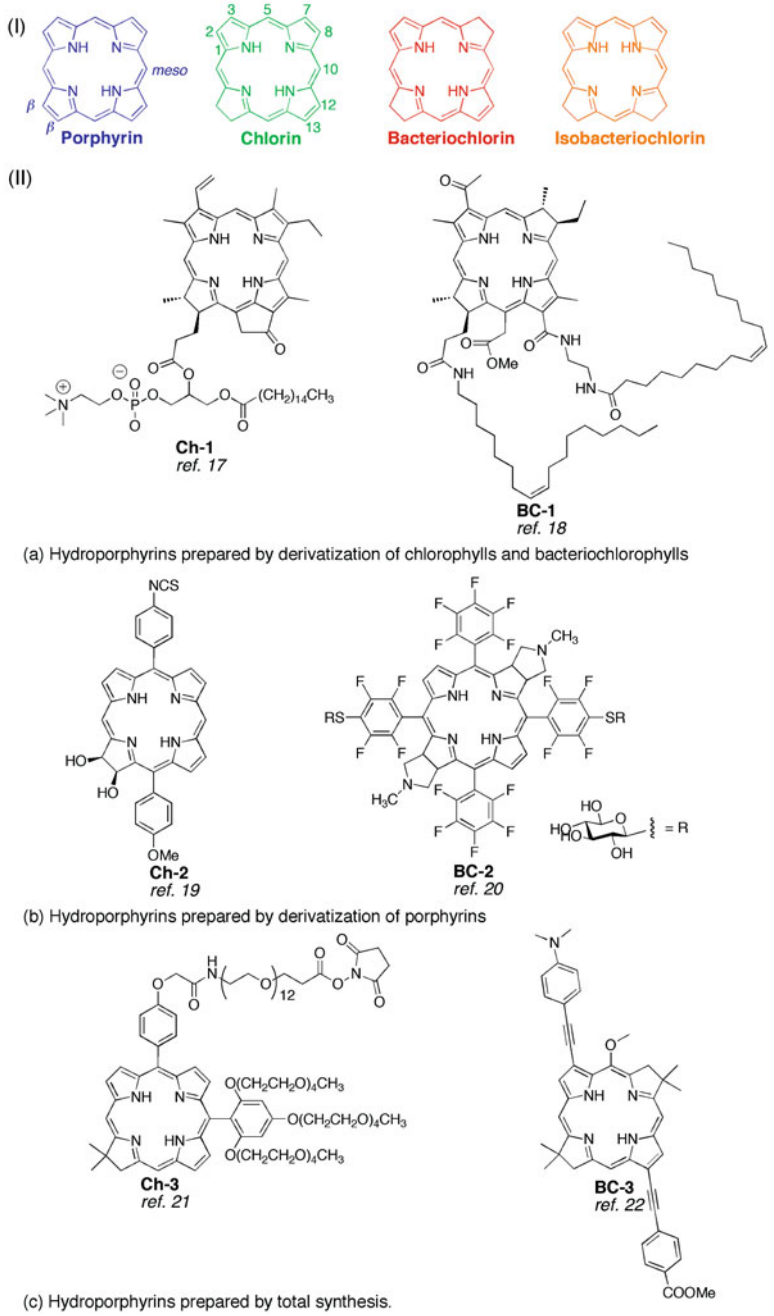

Fig. 2.1 (**I**) General structures of porphyrin and hydroporphyrins. (**II**) Examples of hydroporphyrins utilized in bioimaging prepared through (**a**) derivatization of natural (bacterio) chlorins, (**b**) modifications of porphyrins, and (**c**) total (or *de novo*) synthesis