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Microwave-Accelerated Metal-Enhanced Fluorescence (MAMEF): Application to Ultra Fast and Sensitive Clinical Assays

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In this rapid communication we describe an exciting platform technology that promises to fundamentally address two underlying constraints of modern assays and immunoassays, namely sensitivity and rapidity. By combining the use of Metal-enhanced Fluorescence (MEF) with low power microwave heating (Mw), we can significantly increase the sensitivity of surface assays as well as >95% kinetically complete the assay within a few seconds. This technology is subsequently likely to find significant importance in certain clinical assays, such as in the clinical assessment of myoglobin, where both the assay rapidity and sensitivity are paramount for the assessment and treatment of acute myocardial infarction.

KEY WORDS: rapid assays; ultra bright assays; low-power microwaves; metal-enhanced fluorescence; radiative decay engineering; silver nanostructures; surface-enhanced fluorescence.

INTRODUCTION

Immunoassays are widely used for the detection and determination of a wide variety of proteins, peptides and small molecules [1–4]. While there exists a large and diverse family of immunoassays today, the basic principles are mostly the same [1–4]. These typically use antigen-antibody binding for analyte recognition and mostly fluorescence based readout for signal transduction. The antigen-antibody recognition step is most often kinetically very slow, requiring long incubation times, very few assays subsequently being complete under 10 min [1–4]. In addition, the sensitivity of fluorescence based immunoassays is mostly governed by the quantum yield of the tagging fluorophore and the efficiency and sensitivity of the detection system [1–4]. These two physical constraints underpin both the rapidity and sensitivity of current immunoassays [1–4].

To address assay sensitivity, we have employed the use of metal-enhanced fluorescence (MEF), a new technology which has been described by our laboratories in detail over the last 5 or so years [5–16]. Excited fluorescent species in close proximity to silver nanostructures, can induce dipoles in metal structures, which under certain conditions [5–16], radiate the photophysical properties of the fluorophore. Remarkably, the efficiency of fluorophore coupling to surface plasmons combined with their high efficiency to radiate, produces fluorophore-metal "systems" which display high fluorescence quantum yields combined with reduced lifetimes [5–16]. It is this unique combination of an increased emission intensity, coupled

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Abbreviations: AFM, Atomic Force Microscopy; BSA, Bovine Serum Albumin; MAMEF, Microwave-Accelerated Metal-Enhanced Fluorescence; MEF, Metal-Enhanced Fluorescence; Mw, Low-Power Microwave exposure; RDE, Radiative Decay Engineering; SiFs: Silver Island Films.

with a reduced lifetime (increased photostability) that has recently lead to the use of MEF in many biotechnology applications; such as in the increased detectability and photostability of fluorophores [5–7] improved DNA detection [17], the release of self-quenched fluorescence of over labeled proteins [18] and the application of metallic surfaces to amplified wavelength-ratiometric sensing [19], to name but just a very few.

To address assay rapidity we have employed the use of low power microwaves (2450 MHz) to heat the samples. Interestingly, metallic particles in the microwave cavity appear to be preferentially heated as compared to solvents, which advantageously localizes both the MEF effect and heating around the silver nanostructures. A detailed description of this effect, temperature calibrations as well as discussions pertaining to protein denaturation are to be published elsewhere [20]. For metals, the attenuation of microwave radiation, arises from the creation of currents resulting from charge carriers being displaced by the electric field [21]. These conductance electrons are extremely mobile and unlike water molecules can be completely polarized in 10^{-18} s. In our microwave cavity, the time required for the applied electric field to be reversed is far longer than this, in fact many orders of magnitude. If the metal particles are large, or form continuous strips, then large potential differences can result, which can produce dramatic discharges if they are large enough to break down the electric resistance of the medium separating the large metal particles. Interestingly, and most appropriate for our new assay platform described here, small metal particles do not generate sufficiently large potential differences for this "arcing" phenomenon to occur [21]. However, the charge carriers, which are displaced by the electric field, are subject to resistance in the medium in which they travel due to collisions with the lattice phonons [21]. This leads to Ohmic heating of the metal nanoparticles in addition to the heating of any surface solution-phase polar molecules.

Finally, most immunoassays employ high quantum yield fluorophores to facilitate analyte detectability [1–4,22]. Interestingly, immunoassays designed to utilize the MEF phenomenon are well-suited for use with low quantum yield fluorophores, Fig. 1. This occurs due to

1. The maximum metal-fluorophore system fluorescence emission enhancement and therefore analyte detectability, is roughly given by $1/Q_0$, where Q_0 is the quantum yield of the fluorophore alone in the absence of metal [5,6]. Hence low quantum yield fluorophores are generally more suited.
2. Low quantum yield fluorophores will invariably produce greater S/N ratios. This is because the MEF phenomenon is a through space phenomenon, occurring up to about 10 nm from the surface [5, 6]. Hence, low quantum yield material distal from the metal surface and outside the enhancement region, contributes little to unwanted background fluorescence.

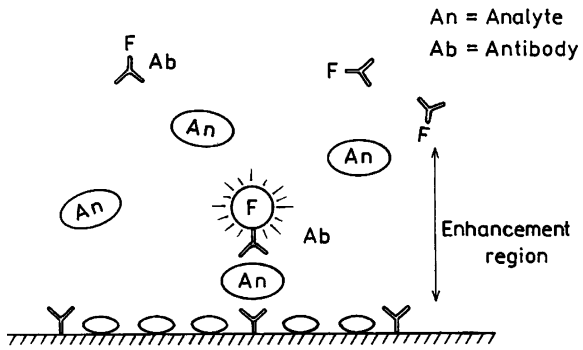

Fig. 1. Model metal-enhanced fluorescence immunoassay. A silvered surface features immobilized antibodies for a particular analyte. Upon formation of the sandwich immunoassay, the low quantum yield fluorophore is brought into close proximity to the surface (within the 10 nm enhancement region), the system becoming highly luminescent and facilitating analyte detectability. Adopted form Ref. [7].

EXPERIMENTAL

Materials

Silver nitrate (99.9%), sodium hydroxide (99.996%), ammonium hydroxide (30%), trisodium citrate, *D*-glucose and premium quality APS-coated glass slides (75 × 25 mm) and bovine serum albumin (BSA) were obtained from Sigma-Aldrich. Myoglobin (recombinant) and monoclonal anti-myoglobin antibodies (capture anti-Myo antibodies clone 2 mb-295, reporter anti-Myo antibodies clone 9mb-183r) were obtained from Spectral Diagnostics, Canada. All chemicals were used as received.

Reporter anti-Myo antibodies were labeled with Alexa Fluor-647 using a labeling kit from Molecular Probes; the kit provided dyes with reactive succinimidyl ester moieties, which react effectively with the primary amines of proteins. While Alexa Fluor-647 has a moderately high quantum yield, we chose this fluorophore due to its availability in the reactive form and the subsequent simplicity to demonstrate the MAMEF concept.

Methods

Silver Island Films were formed as described previously [20]. In a typical SiFs preparation, a solution of

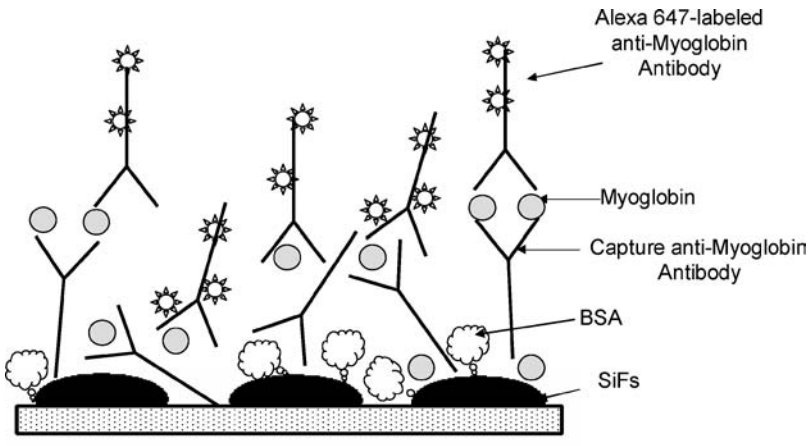

Fig. 2. Metal-enhanced fluorescence myoglobin immunoassay.

sodium hydroxide and ammonium hydroxide are added to a continuously stirred solution of silver nitrate at room temperature. Subsequently, the mixture is cooled down in an ice bath, Silane-prep^{TM} glass slides (Sigma) are inserted and a solution of D-glucose is added. As the temperature is increased, the color of the mixture turns yellow–brown and the SiFs-deposited slides are removed from the mixture, washed with water, and sonicated for 1 min at room temperature. The effects of microwaves on SiFs were investigated by optical absorption spectroscopy and atomic force microscopy.

Myoglobin (Myo) immunoassays were performed in a sandwich format as described previously, but with a few modifications [23], and as shown in Fig. 2. In this regard, slides were non-covalently coated with capture anti-Myo antibody at room temperature. The glass/SiFs surfaces were blocked with BSA in order to minimize the nonspecific interaction of the antibodies and myoglobin with the surfaces. These surfaces were then incubated with Myoglobin antigen (100 ng/mL) at room temperature, and then used for end-point measurements. The end-point measurements were performed by incubating the antigen-coated surfaces in a solution of Alexa 647-labeled anti-Myoglobin Antibody for 30 min at room temperature, or by microwaving the antigen-coated surfaces with Alexa 647-labeled anti-Myoglobin Antibody for 20 s. Fluorescence measurements were performed by collecting the emission intensity at 45° to the excitation through a long pass filter, using a Fiber Optic Spectrometer (HD2000) from Ocean Optics, Inc.

Further details of the microwave cavity, its temperature and power calibration, as well as discussion pertaining to the denaturation of proteins by low power microwaves can be found elsewhere [20].

RESULTS AND DISCUSSION

Figure 3 shows the plasmon absorption spectra and AFM images of Silver Island Films (SiFs), both before and after low power microwave heating for 30 s. The cavity power was approximately 140 W, which is the same as utilized in the myoglobin assay discussed later and in earlier studies by our group on a model protein system [20], and also is of a similar power commonly used for immunostaining [24, 25]. As can be seen from Fig. 3 right, the microwaves and heating had no effect on the surface plasmon absorption of the SiFs, indicating no structural or surface silver shape changes, where the surface plasmon absorption is well-known to be characteristic of the shape of the nanoparticles [26,27], which is due to the mean free path oscillation of surface charges [26,27]. Further, no "sparking" was evident from the silvered surfaces, a known consequence of surface charge build-up and dissipation [21] for larger sized particles or continuous surfaces [21].

We additionally measured the structural morphology of the silvered surfaces both before and after low power microwave heating using Atomic Force Microscopy, Fig. 3 Left. While it is was somewhat difficult to probe the exact same area after microwave heating, very little, if no change in surface morphology was observed between the locations. While not shown in Fig. 3, we additionally exposed the silvered surfaces, both wet and dry, to several hundreds watts of microwave cavity power over many minutes. In all of these investigations no evidence for surface structural changes was found by microwave heating, clearly demonstrating the compatibility of the nanostructured surfaces to microwave exposure and therefore heating. In addition,

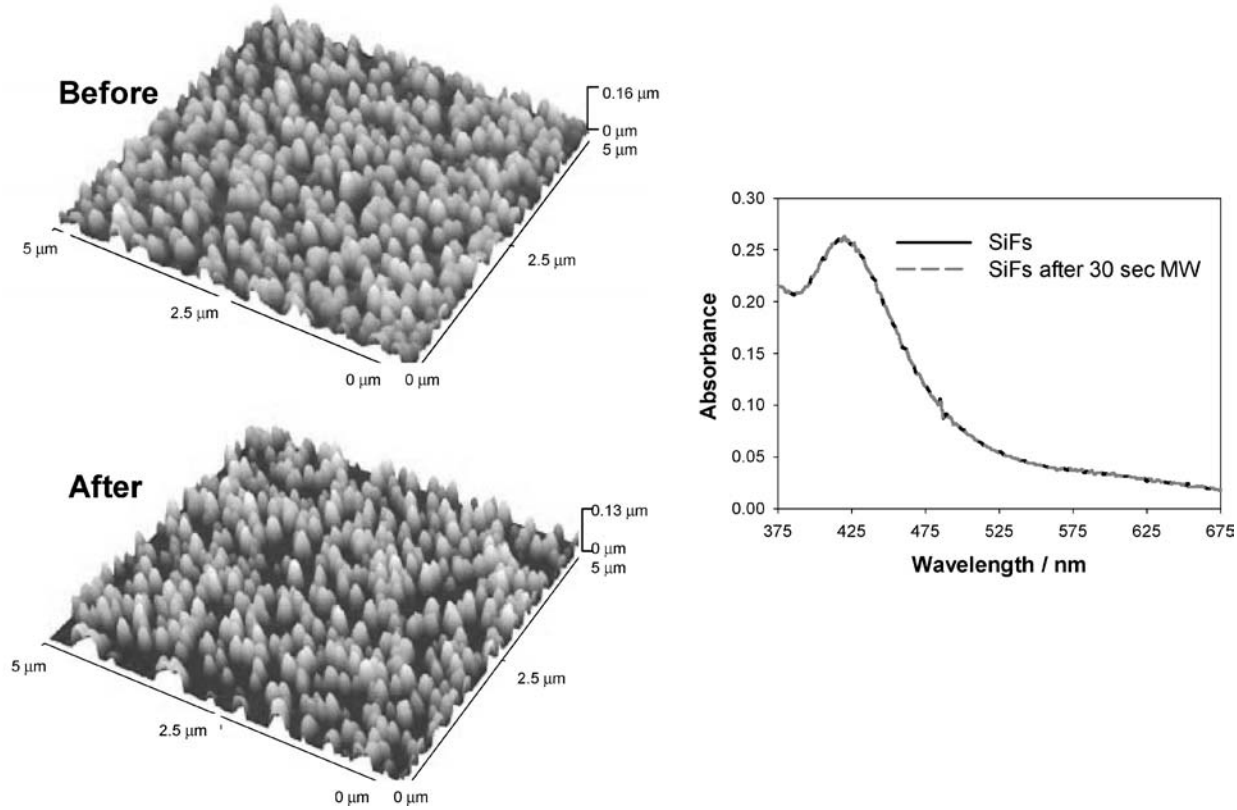

Fig. 3. AFM images both before and after microwave heating (top left and bottom respectively) and the corresponding plasmon absorption spectrum (right).

we also undertook control experiments to investigate both the absorption and emission intensity of Alexa-647 (fluorophore stability) upon exposure to microwaves. Similarly to the silvered surfaces, the Alexa-647 was unperturbed.

In a clinical setting there are several assays that could significantly benefit from both rapidity and sensitivity. One particular assay is for the determination of myoglobin and its role in the clinical assessment of a myocardial infarction [23,28–32]. Subsequently, we constructed a myoglobin assay in a similar manner to the model assay shown in Fig. 1, see Fig. 2. In addition, we also prepared a control assay, identical to the silvered myoglobin assay except that the control assay had no silver and was constructed on bare glass. This was constructed to rationalize the benefits of using the MAMEF technique.

Figure 4 left shows the Alexa-647 emission intensity, both on silver and glass after 30 min incubation and with no microwave heating. A myoglobin concentration of 100 ng/mL was used to sandwich the immunoassay, which is the clinical cut-off concentration for the assessment of a myocardial infarction [23,28–32]. The emission spectrum, which was collected through a long-pass filter, shows an approximate 7.5-fold greater intensity from the silver as compared to the glass control. As mentioned in the introduction, this effect is due to an apparent radiative decay rate modification of the fluorophore as it is brought into close proximity to the silver nanostructures upon formation of the sandwich immunoassay [23], and is consistent with the numerous publications from our laboratories on the MEF phenomenon [5–7]. In Fig. 4 left, the sample was incubated for 30 min at room temperature, which was predetermined to be sufficient enough time to allow the assay to go to >95% completion. The spectra on both glass and silver were found to be identical after normalization (data not shown).

Figure 4 (right) shows the combined effect of both low power microwave heating and the optical amplification due to the silver for an identical immunoassay. Remarkably, the myoglobin immunoassay yields a

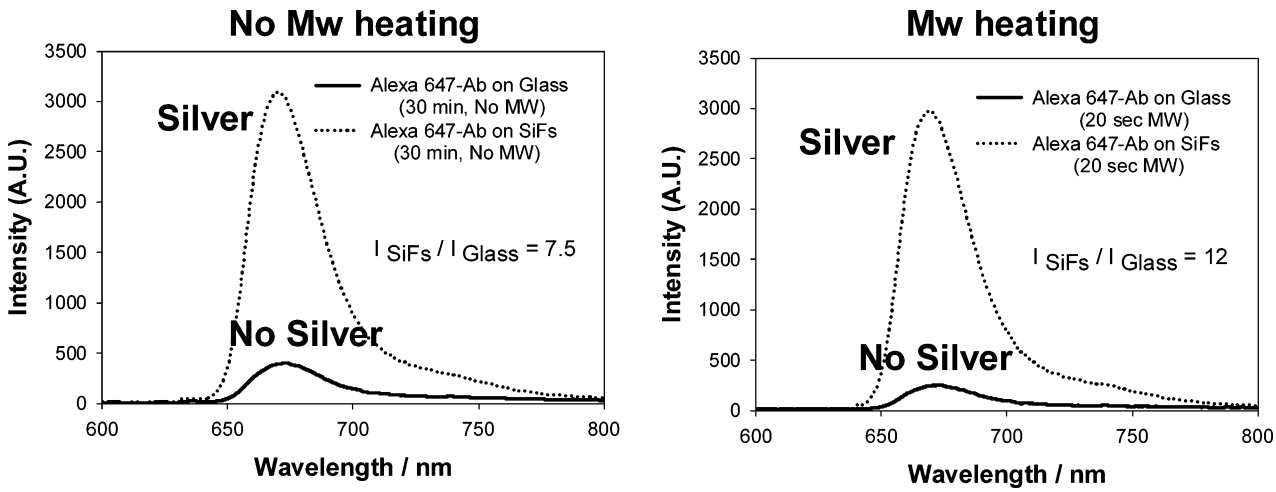

Fig. 4. Fluorescence intensities of the myoglobin immunoassay in the presence and absence of silver with no microwave heating (left) and after low power microwave heating (right).

similar final fluorescence intensity after 20 s microwave heating (Mw), ≈3000 arbitrary units, as compared to a 30 min incubation, but with no microwave heating, Fig. 4 left. In addition, the silver still maintains its ability to optically amplify the Alexa-647 fluorescence emission after microwave heating.

A close inspection of both Fig. 4 (left and right) reveals that the final fluorescence intensities on glass are different, which manifests itself in different fluorescence enhancement factors for both the assays, i.e., the enhancement is 7.5-fold with no microwave heating and 12-fold after low power microwave heating. Our detailed temperature studies of the assays have revealed that the bulk temperature jump in the system was only 8°C after microwave exposure (50 μL of sample), which does not account for the ≈90-fold increase in assay rapidity. Subsequently, we believe that this effect is due to the preferential localized heating around the silver nanostructures [21], rapidly accelerating mass transport to the surface and therefore the kinetics of the assay. In this regard, Mingos and coworkers [21] have reported that metal-powders and particles can couple with microwave fields at 2.45 GHz, and heat up to temperatures in excess of 1000°C in very short periods of time, without causing visible electric discharges [21], supporting our hypothesis of localized heating and accounting for the ultra-fast assay kinetics.

Finally, while not shown or described in this rapid communication, we have also addressed issues relating to the denaturation of proteins by low power microwave heating and the effects of microwaves on nonspecific absorption. These findings can be found elsewhere [20].

CONCLUSIONS

In this paper we have demonstrated how a clinically important assay can be both optically amplified as well as rapidly accelerated. The new approach undertaken, Microwave-Accelerated Metal-Enhanced Fluorescence (MAMEF), could potentially be applied to many other fluorescence based assays currently in use today. For the myoglobin assay described here, the use of MAMEF provided for a 12-fold increase in fluorescence detectability (emission intensity) which can easily be translated into assay sensitivity, and a 90-fold increase in rapidity, the assay being kinetically complete (100%) within 30 s of microwave heating (95% measured at 20 s).

In clinical settings, a myoglobin immunoassay can take over 1 h to get an answer. This is due to the need to separate blood and the time required to run the serum assay to completion. MAMEF provides a platform technology which could both amplify and kinetically increase assays to completion within a few seconds, potentially safeguarding life and alleviating the need to separate blood. In addition, low power microwave devices could be constructed for heart attack risk assessment, alleviating the need for unnecessary hospital visits, or even facilitate the early prescription of drugs with the onset of chest pains. Further, highly sensitive and rapid devices are also likely to be of immense importance to first responders of bioterrorism

threats, who are also faced with rapidity and sensitivity constraints.

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