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RAPID COMMUNICATION

Microwave-Triggered Metal-Enhanced Chemiluminescence (MT-MEC): Application to Ultra-fast and Ultra-sensitive Clinical Assays

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Abstract In this rapid communication we describe a new approach to protein detection with chemiluminescence. By combining common practices in protein detection with chemiluminescence, microwave technology, and metal-enhanced chemiluminescence, we show that we can use low power microwaves to substantially increase enzymatic chemiluminescent reaction rates on metal substrates. As a result, we have found that we can in essence *trigger* chemiluminescence with low power microwave (Mw) pulses and ultimately, perform on-demand protein detection assays. Using microwave triggered metal-enhanced chemiluminescence (MT-MEC), we not only improve the sensitivity of immunoassays with enhanced signal-to-noise ratios, but we also show that we can accurately quantify protein concentrations by integrating the photon flux for discrete time intervals.

Keywords Immunoassays · Ultrasensitive assays · Protein detection · Low-Power microwaves · Metal-enhanced chemiluminescence · Protein quantification · Plasmons · Plasmonics · Metal-enhanced fluorescence · Radiative decay engineering · Surface enhanced fluorescence

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Abbreviations

BSA:	Bovine serum albumin
MT-MEC:	Microwave-triggered metal-enhanced chemiluminescence
MAMEF:	Microwave-accelerated metal-enhanced fluorescence
MEF:	Metal-enhanced fluorescence
Mw:	Low-power microwave heating
HRP:	Horseradish peroxidase

Introduction

Chemiluminescent reactions have become the method of choice for obtaining qualitative results in protein detection assays [1]. These analytical applications commonly include immunoassays, antioxidant assays, microbiology, protein blotting, nucleic acid analysis, cellular studies, cancer detection and disease screening [2]. One of the more commonly applied methods, immunoassays, are used for the detection and identification of a wide variety of proteins, peptides and small molecules [1–8]. These assays use antigen-antibody binding for analyte recognition and typically, fluorescence, radioactive or chemiluminescence based readout for signal transduction. Each of these detection methodologies has prospective advantages, but the sensitivity of each of these methods is fundamentally limited by poor signal-to-noise ratios at low analyte concentrations.

Fluorescent detection methods offer the advantage that the detected signal is directly proportional to the fluorescently labeled species. Thus, fluorescent detection methods are often used for protein quantification. However, these techniques are limited, as additional costly detection equipment is often required, external excitation sources are necessary to induce the photon emission, and limited sensitivity exists at low analyte concentrations.

Autoradiography is also often implemented in small molecule detection methods, such as protein detection. Although small quantities of protein (~ 1 pg) can be detected with autoradiography, sensitivity is dependent on the duration of exposure conditions (>24 h), labeling efficiencies, the identity of a radiolabeled probe, and the extent of damage caused by the radioactive label to the labeled protein. In addition, radiolabeling also suffers from inherent disadvantages such as associated health and safety risks and the high cost of material acquisition and disposal.

As an alternative, chemiluminescent assays have proved to be as sensitive as radiolabeling, but without the associated health hazards and cost of materials. Chemiluminescent reactions are often catalyzed enzymatically with horseradish peroxidase (HRP) or alkaline phosphatase. As a result, detection assays use proteins labeled with these enzymes and can detect as little as 1–5 pg of protein [3]. Consequently, chemiluminescence has become a method of choice for small molecule detection in more than 20% of clinical laboratories in the United States [6].

Although chemiluminescence detection has been successfully implemented in clinical assays, the sensitivity and specificity of these reactions require further improvements to facilitate early diagnosis of the prevalence of disease. In addition, most protein detection methodologies, most notably western blotting, are still not reliable methods for accurate quantification of low protein concentrations. The need for reliable protein quantification at low protein concentrations is still a critical issue that needs to be addressed to improve the sensitivity and the reliability of chemiluminescent small molecule assays [7–13].

In the past two decades, the use of microwave radiation has greatly increased in radar and communication systems [14], for accelerating reactions in synthetic organic chemistry applications [15–17], in drug delivery [18], assays [19–23] and in biochemistry [24–26]. We recently demonstrated the optical enhancement of chemiluminescent reactions on metal substrates [27]. In this paper here, we describe Microwave Triggered Metal-Enhanced Chemiluminescence (MT-MEC) for protein detection using low power microwaves (Mw) to accelerate chemiluminescent reactions, coupled with silver nanoparticles to plasmon-enhance local luminescent signals. Due to increased reaction rates for the triggered chemiluminescent reactions, the 'on-demand' nature of light emission provides substantial improvements in signal-to-noise ratios and a localized amplified photon flux for discrete time intervals from the 'triggered' chemiluminescent reaction. Subsequently, we show that MT-MEC not only increases the detectability of low concentrations of protein, but accurate quantification of protein concentrations can also be realized by integrating the resulting photon flux over discrete time intervals.

Experimental

Bovine-biotinamidocaproyl-labeled Albumin (biotinlyated BSA), silver nitrate (99.9%), sodium hydroxide (99.996%), ammonium hydroxide (30%), trisodium citrate, D-glucose and premium quality APS-coated glass slides (75 mm × 25 mm) were obtained from Sigma–Aldrich. CoverWell imaging chamber gaskets with adhesive (20 mm diameter, 1 mm deep) were obtained from Molecular Probes (Eugene, OR). Steptavidin-HRP prediluted solution was obtained from Chemicon® International Inc. Chemiluminscent reagents were purchased from Amersham Biosciences (ECL Plus™ Western blotting detection kit, RPN2132).

Formation of Silver Island Films (SiFs) on APS-coated glass substrates

In a typical SiF preparation a solution of silver nitrate (0.5 g in 60 ml of deionized water) in a clean 100-ml glass beaker, equipped with a Teflon-coated stir bar, is prepared and placed on a Corning stirring/hot plate. While stirring at the quickest speed, 8 drops (≈ 200 μL) of freshly prepared 5% (w/v) sodium hydroxide solution are added. This results in the formation of dark brown precipitates of silver particles. Approximately 2 ml of ammonium hydroxide is then added, drop by drop, to re-dissolve the precipitates. The clear solution is cooled to 5°C by placing the beaker in an ice bath, followed by soaking the APS-coated glass slides in the solution. While keeping the slides at 5°C, a fresh solution of D-glucose (0.72 g in 15 ml of water) is added. Subsequently, the temperature of the mixture is then warmed to 30°C. As the color of the mixture turns from yellow-green to yellow-brown, and the color of the slides become green, the slides are removed from the mixture, washed with water, and sonicated for 1 min at room temperature. SiF-deposited slides were then rinsed with deionized water several times and dried under a stream of nitrogen gas. Prior to assay fabrication and subsequent chemiluminescent experiments, imaging chamber gaskets with adhesive (20 mm diameter, 1 mm deep) were pressed against the silver coated and silica capped microscope glass slides until they were stuck together, creating a chamber.

Preparation of the model protein assay (Biotin-Avidin) on silver island films and glass substrates (control assay)

The model assay used in this paper is based on the well-known interactions of biotin and avidin. Biotin groups are introduced to the glass and silvered surfaces through biotinylated-BSA, which readily forms a monolayer on the surfaces of glass and SiFs [28–30]. Luminescent decay experiments were carried out by incubating 20 μl of 15.6 nM biotinylated-BSA solutions on the Ag and glass imaging

chambers for approximately 1 h. Concentration dependence experiments were performed with a similar protocol with the exception of the variable concentrations of the biotinylated-BSA solutions. Chambers were washed with water to remove the unbound material. Imaging chambers were then incubated with 20 μl of 1% aqueous BSA (w/v) for one hour to minimize non-specific binding of HRP-streptavidin to surfaces. Chambers were again washed with water to remove the BSA blocking solution. Stock solutions of HRP-streptavidin were diluted 1:10 to a final concentration of 100 μg/ml. Twenty microliters of the HRP-streptavidin solution was subsequently added into the biotinylated-BSA coated glass and SiFs coated imaging chambers and typically incubated at room temperature for approximately 30 min. In all the experiments performed with low power microwaves, there was no evidence of surface drying. Following incubation, imaging chambers were again washed with water to remove unbound HRP-streptavidin material prior to the chemiluminescence experiments.

Chemiluminescence reagents

The ECL™ Western Blotting Detection Kit contained two reagents that yield a bright chemiluminescent emission at 430 nm upon mixing. Solution A contained the substrate solution (peroxide formulation) and solution B contained the solution of the luminescent compound, acridan in dioxane and ethanol. HRP and hydrogen peroxide solution (solution A) catalyze the oxidation of the acridan substrate (solution B). As a result, acridinium ester intermediates are formed and further react with peroxide to generate light emission with a maximum wavelength centered around 430 nm.

Chemiluminescence from reagents on SiFs and glass surfaces

The chemiluminescence experiments were performed with and without microwave (Mw) heating inside the microwave cavity (0.7 cu ft, GE Compact Microwave Model: JES735BF, max power 700 W). During microwave heating, thirty second pulses were applied at three 100 scond intervals. The pulses were applied at 30% power, which corresponded to 210 W over the entire cavity. Solutions A and B were mixed in different proportions to optimize chemiluminescence signal for these experiments. As a result, we measured the maximum emission signal from a 20:1 mixture of solution A to B. Subsequently, all reactions were performed by combining 40 μl of solution A with 2.0 μl of solution B and immediately adding the entire solution to the imaging chamber. Data collection commenced immediately following addition of reagents, and terminated when the photon count returned to baseline. Since the rate of photon emission is directly proportional to enzyme concentration, we sum the photon flux for a fixed time interval for the points shown in Fig. 4 to the relationship between protein concentration and signal intensity, c.f. Fig. 4.

Chemiluminescence detection

Chemiluminescence spectra were collected using an Ocean Optics spectrometer, model SD 2000 (Dunedin, FL), connected to an Ocean Optics 1000 μm diameter fiber with an NA of 0.22 (Dunedin, FL). The fiber was positioned vertically on top of the slides containing the chemiluminescent reagents inside the microwave cavity. Chemiluminescent spectra and time-dependent emission intensity were collected with an integration time of 1 second for approximately 500 seconds unless otherwise noted. The integration time was kept constant between the control and silver island film sample measurements.

The *real-color* photographs were taken with an Olympus Digital camera (C-740, 3.2 Mega Pixel, 10 × Optical Zoom) without the need for optical filters.

Results and discussion

To demonstrate MT-MEC as a useful platform for protein quantification we constructed a simple surface assay as described in the experimental section. In our model assay, biotinylated-BSA is incubated on both silvered and glass substrates. HRP-streptavidin is then added to the surface,

Table 1 Recovered decay rates for glass and Ag substrates coated with 15.6 nM BSA-Biotin and Streptavidin-HRP with and without exposure to low power microwave (Mw) pulses. No Mw decays were fit to single exponential decays $I(t) = a_1 e^{-k_1 t}$ and Mw decays were fit to double exponential decays, $I(t) = a_1 a_2 e^{-k_1 t} + a_1(1 - a_2)e^{-k_2 t}$

Sample	a_1	k_1 (s^{-1})	k_2 (s^{-1})	a_2
Glass–no Mw (1 component)	1.00	1.75×10^{-4}	—	—
Glass–Mw	1.00	2.30×10^{-3}	4.48×10^{-2}	0.57
Ag–no Mw	1.00	2.64×10^{-4}	—	—
Ag–Mw	1.00	3.02×10^{-3}	7.85×10^{-2}	0.56

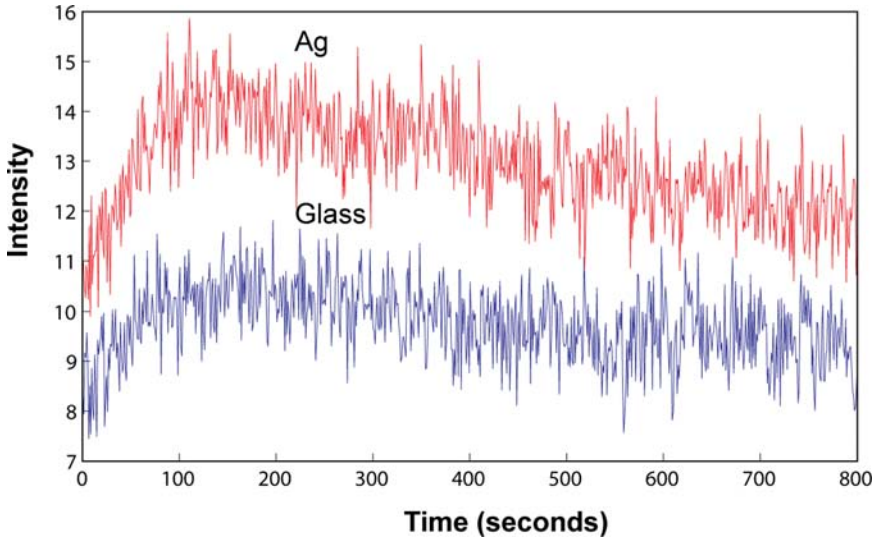

Fig. 1 Plots of Acridan chemiluminescence emission intensity as a function of time from glass and Ag substrates coated with 15.6 nM BSA-Biotin and Streptavidin-HRP

localizing the enzyme catalyst in close proximity to the silver for MT-MEC. The peroxide and Acridan (lumophore) are then added to initiate the chemiluminescence reaction. While our assay in essence determines BSA concentration, this model assay could indeed be fashioned to both localize and sense other proteins of interest.

Table 1 summarizes the results of the recovered decay rates for the chemiluminescence reactions in the presence and absence of low power microwave (Mw) heating. In the absence of low power microwave heating (Fig. 1), the chemiluminescence reaction emits a steady decaying signal, rate constant k_1. The signal decay is representative of the chemiluminescent reaction rate for the generation of light emission. In the absence of low power microwaves, the decay rates for the chemiluminescence reactions on silver and glass substrates can be fit with a single exponential decay. These results show a slight enhancement of the chemiluminescent reaction rate in the presence of silver. The results in Table 1 suggest that the chemiluminescent reaction rate on Ag is enhanced by a factor of 1.5. This finding is consistent with our recent finding of metal-enhanced chemiluminescence [27].

In the presence of low power microwave heating, we found that the decay rates were best fit to a two exponential model, c.f. Table 1 and Fig. 2. In addition to a slight increase in the slower decay component of the chemiluminescent reaction, there exists a decay rate that is approximately 200-fold greater than the slower component, i.e. k_2. This faster decaying component is a result of the accelerated reaction rates of the HRP catalyzed reaction on the surfaces of glass and Ag substrates in the presence of low power microwave heating. In addition, an additional enhancement of the microwave pulsed chemiluminescent reaction rates on silver substrates is observed. Figure 2 demonstrates the corresponding signal enhancement on glass and Ag substrates in the presence

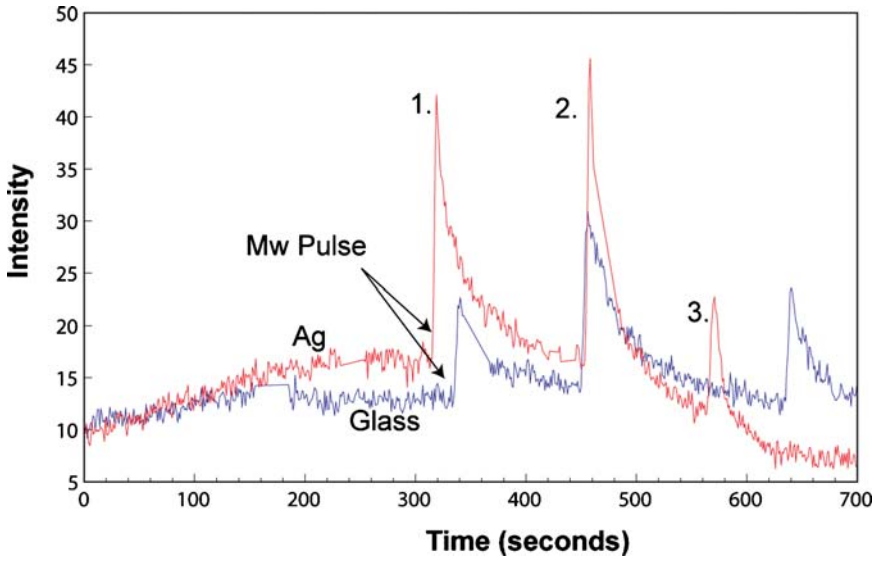

Fig. 2 Acridan chemiluminescence emission as a function of time from glass and Ag substrates coated with 15.6 nM BSA-Biotin and Streptavidin-HRP, and exposed to three (1–3) 30 s microwave (Mw) pulses

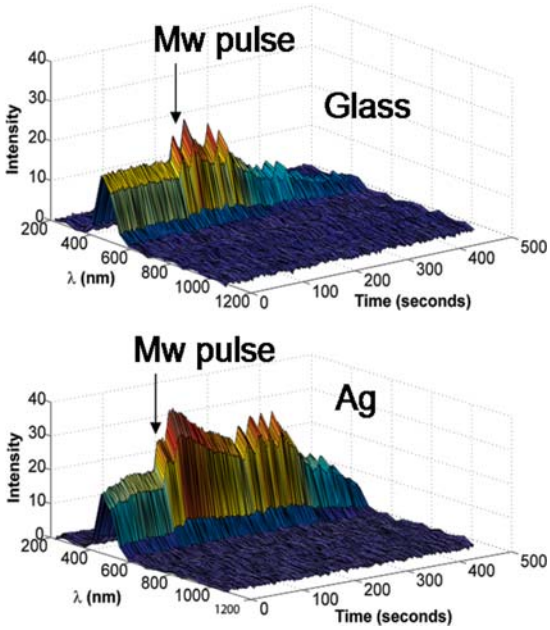

Fig. 3 3D plots of Acridan emission vs. time vs. wavelength for glass substrates (*Top*) and silvered substrates (*Bottom*), coated with 15.6 pM BSA-Biotin and Streptavidin-HRP, exposed to low power microwave pulses (Mw)

of low power microwave pulses. In Fig. 2, chemiluminescence reactions were subjected to three successive (1–3) thirty second microwave (Mw) trains of pulses. The sharp rise in signal intensity corresponds to an accelerated reaction rate and subsequent increased photon flux. These results clearly demonstrate the 'on-demand' nature of the chemiluminescent reactions in the presence of low power microwave pulses. Thus, we expect that we can induce chemiluminescence emission for discrete time intervals and subsequently, generate 'on demand' photon flux.

We chose to take advantage of the increased signal and subsequent increased signal-to-noise ratios to determine the sensitivity of the assay in the presence of low power microwaves on glass and silver substrates. In Fig. 3, we show a three-dimensional representation of the 'on demand' nature of the enhanced chemiluminescence reaction in the presence of low power microwave (Mw) pulses for glass (top) and silver (bottom) substrates incubated with a low concentration of Biotinylated-BSA solution (15.6 pM). From these observations, we determined that the implementation of low power microwaves may increase the sensitivity of protein based assays.

To test this hypothesis, we incubated glass and silver substrates with a range of BSA-biotin concentrations (the target protein). Chemiluminescence was then triggered with low power microwave pulses and the resulting photon flux was integrated over a five hundred second interval. We show in Fig. 4 not only the sensitivity of the MT-MEC assay with respect to the integrated background counts on silver and glass substrates (dashed lines), but also that signal intensity is proportional to protein concentration. Thus, the MT-MEC assay may allow for accurate protein quantification in multiple protein based assays, which presently offer only qualitative predications. We see that the 'on-demand' nature of the MT-MEC technique offers both an ultrafast and ultrasensitive approach to protein detection methods.

Conclusions

In conclusion, we have demonstrated the feasibility of using low power microwaves to 'trigger' chemiluminescence reactions. By exposing chemiluminescent reactants to low power microwaves and dramatically increasing signal-to-noise ratios, we can improve the sensitivity of protein detection assays and accurately quantify protein concentrations by integrating photon flux for discrete time intervals. With the implementation of improved detection schemes, alternative substrate design and optimized assay conditions, we believe that we can improve the sensitivity of MT-MEC beyond currently available protein detection methodologies.

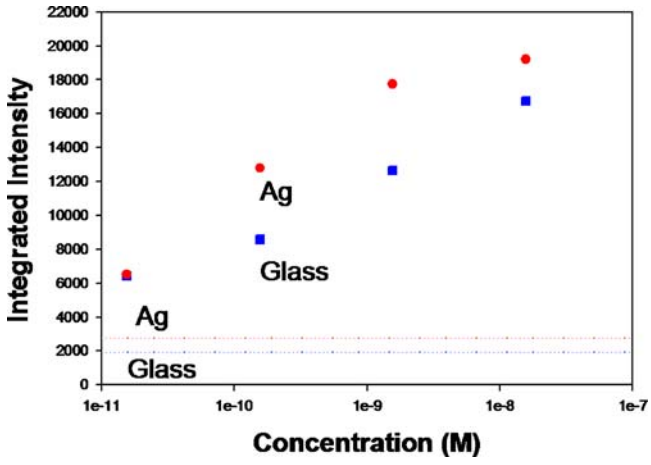

Fig. 4 Integrated photon flux for different concentrations of BSA-biotin from both glass and silvered glass surfaces (Ag). Baselines correspond to integrated photon counts for Ag and glass substrates incubated with HRP-streptavidin and no BSA-biotin

For example, alternative substrates, such as silver nanorods and silver fractal patterns, show 50-fold and 3000-fold enhancements of fluorescent signals respectively [31–34]. Furthermore, we recently demonstrated that microwave accelerated metal-enhanced fluorescence (MAMEF) improves the specificity and sensitivity of surface protein assays on silver substrates [35–37]. Combining the advantages of MAMEF and MT-MEC, we believe that we can further improve the sensitivity and rapidity of protein detection with chemiluminescence. Using low power microwaves to induce the combined effect of a kinetically accelerated binding, increased binding specificity, and accelerated 'triggered' chemiluminescent reactions, we anticipate that Microwave Triggered Metal-Enhanced Chemiluminescence (MT-MEC) can be implemented to not only accurately quantify protein concentrations, but also alleviate the current bottlenecks of immunoassay sensitivity and rapidity.

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