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Research paper

Microwave-Accelerated Metal-Enhanced Fluorescence (MAMEF) with silver colloids in 96-well plates: Application to ultra fast and sensitive immunoassays, High Throughput Screening and drug discovery

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Abstract

Fluorescence detection is the basis of most assays used in drug discovery and High Throughput Screening (HTS) today. In all of these assays, assay rapidity and sensitivity is a primary concern, the sensitivity determined by both the quantum yield of the fluorophores and efficiency of the detection system, while rapidity is determined by the physical and biophysical parameters of temperature, concentration, assay bioaffinity, etc.

In this paper we describe a platform technology that promises to fundamentally address these two physical constraints of sensitivity and rapidity. By combining the use of Metal-Enhanced Fluorescence (MEF), a near-field effect that can significantly enhance fluorescence signatures, with low power microwave heating, we can significantly increase the sensitivity of surface assays as well as >95% kinetically complete the assay within a few seconds. In addition, the metallic nanostructures used to facilitate MEF appear to be preferentially heated as compared to the surface assay fluid, advantageously localizing the MEF and heating around the nanostructures. To demonstrate proof of principle, a 96-well plate has been functionalized with silver nanostructures, and a model protein avidin–biotin assay studied. In our findings, a greater than 5-fold fluorescence enhancement coupled with a ≈ 90-fold increase in assay kinetics was observed, but with no assay washing steps needed due to the silver-enhanced evanescent field mode of excitation. These findings promise to strongly facilitate high throughput fluorescence-based processes, such as in biology, drug discovery and general compound screening.
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Keywords: Rapid assays; Ultra bright assays; Low-power microwaves; Metal-Enhanced Fluorescence; Radiative Decay Engineering; Silver nanostructures; High Throughput Screening and biology; Surface-enhanced fluorescence

Abbreviations: AFM, Atomic Force Microscopy; BSA, Bovine Serum Albumin; HTS, High Throughput Screening; MAMEF, Microwave-Accelerated Metal-Enhanced Fluorescence; MEF, Metal-Enhanced Fluorescence; Mw, Low power microwave heating; RDE, Radiative Decay Engineering; TIRF, Total Internal Reflection Fluorescence.

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1. Introduction

The major thrusts and pioneering works in High Throughput Screening (HTS) can be traced to the late 1970s (Shayne, 2005). While the first microtiter plate was described in 1954 (Takatsy, 1955), the use of the 96-well plate became popular with the increased use of enzyme-linked immunosorbent assays (ELISA) in the late 1970s (Shayne, 2005; Bange et al., 2005; Hemmilam, 1992; Van Dyke and Van Dyke, 1990; Ozinkas, 1994; Gosling, 1990; Davidson and Hilchenbach, 1990; Schweitzer and Kingsmore, 2002). Since that time, it is widely thought that three main technologies have driven the development of HTS practices. These include: the reaction vessels, methods to reproducibly deliver small volumes of reagents, and methods of measuring the endpoint of the assays, including absorbance, fluorescence and luminescence based technologies (Shayne, 2005; Hemmilam, 1992).

While fluorescence based detection is the basis of most assays used in both HTS and general biotechnology today (Lakowicz, 1999; Aslan et al., 2005a), it is the quantum yield of the tagging fluorophore and the efficiency and sensitivity of the detection system that underpins assay sensitivity (Hemmilam, 1992). In addition to assay sensitivity, it is the kinetically slow antigen–antibody recognition step that often governs assay rapidity, very few assays being >95% complete in less than 10 min (Bange et al., 2005; Ozinkas, 1994; Van Dyke and Van Dyke, 1990). Subsequently assay rapidity and assay sensitivity are bottlenecks in the HTS process today (Shayne, 2005; Bange et al., 2005; Ozinkas, 1994; Van Dyke and Van Dyke, 1990).

In this regard we have combined the use of Metal-Enhanced Fluorescence (MEF), a relatively new technology which can dramatically increase fluorescence signatures and the photostability of fluorophores (Aslan et al., 2005b; Geddes et al., 2004b, 2005), with the use of low power microwaves, which can kinetically accelerate (heat) assays. The unique combination of both technologies to HTS well-plates, serves to demonstrate in this paper that the current bottlenecks of assay (potentially immunoassay) sensitivity and rapidity can be alleviated.

In almost all current fluorescent based assays, fluorophores are in the “free space condition” (Lakowicz, 1999; Geddes and Lakowicz, 2002; Geddes et al., 2003a) in which they emit energy into a homogeneous transparent environment such as aqueous solutions and solvents. However, in the presence of silver nanostructures, fluorophores display unique spectral properties, not observed in the free space condition (Aslan et al., 2005a,b,c,d,e,f; Aslan and Geddes, 2006; Geddes et al., 2003a,b,c,d,e; Geddes et al., 2004c; Lakowicz, 2001; Lakowicz et al., 2001, 2002, 2003). These include, dramatically increased system quantum efficiencies, reduced lifetimes (increased photostabilities) and under appropriate conditions, directional fluorescence emission (Geddes et al., 2003e; Lakowicz, 2004). Over the last 5 or so years, our laboratories have demonstrated many applications of Metal-Enhanced Fluorescence, including improved DNA detection (Malicka et al., 2003) and the application of metallic surfaces to amplified wavelength-ratiometric sensing (Aslan et al., 2005b), to name but just a very few (Geddes et al., 2003a,b,c,d,e; Lakowicz, 2001; Lakowicz et al., 2001, 2002, 2003). More recently, we have shown that the Metal-Enhanced Fluorescence process is due to the ability of excited metal surface plasmons to radiate the fluorophores’ photophysical characteristics with high efficiency, subsequently named the Radiating Plasmon Model (Aslan et al., 2005f). While the first observation of MEF was in the 1970s (Drexhage, 1974), more descriptive explanations followed in the early 1980s (Weitz et al., 1982; Wokaun et al., 1983). However, in all these and later phenomenological reports (Lobmaier et al., 2001), the underlying enhancement mechanism was not discussed or even utilized for diagnostic applications.

In the past two decades, the use of microwave radiation has greatly increased in radar and communication systems (Sridar, 1998), for accelerating reactions in synthetic organic chemistry applications (Caddick, 1995; Sridar, 1997; Varma, 2002), in drug delivery (Lin et al., 1998), assays (Akins and Tuan, 1995; Rhodes et al., 2001; Van Triest et al., 2000) and in biochemistry (Roy and Gupta, 2003; Bismuto et al., 2003; Porcelli et al., 1997). The development of medical microwave based devices for clinical diagnosis and therapy has also prompted widespread interest and stimulated much research on the mechanisms of interaction of low power microwaves with living organisms (Adam, 2003; Whittaker and Mingos, 1995; Kappe, 2002). While the heating of water and other solvents by microwaves is well understood and established, there are relatively fewer reports with regard to metals, and even fewer with regard to metallic nanostructures (Whittaker and Mingos, 1993, 1995). For metals, the attenuation of microwave radiation arises from the creation of currents resulting from charge carriers being displaced by the electric field (Whittaker and Mingos, 1993). These conductance electrons are extremely mobile and unlike water molecules can be completely polarized in 10^{-18} s. 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 HTS assay platform described here, small metal particles do not generate sufficiently large potential differences for this "arcing" or "sparking" phenomenon to occur at the bottom of the wells (Whittaker and Mingos, 1993). 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 (Whittaker and Mingos, 1993). This leads to Ohmic heating of the metal nanoparticles in addition to the heating of any surface polar molecules, i.e. surface assay water. Intuitively, in our assay, this leads to localized heating around the silver nanostructures at the bottom of the wells, in addition to the buffer, rapidly accelerating assay kinetics. Further, the close proximity of assay fluorophores, additionally leads to fluorophore (system) quantum yield modifications, i.e. MEF, and the subsequent increase in fluorescence emission (Geddes et al., 2004a, 2005). Hence metallic nanoparticles, fluorophores and microwaves can be combined to yield kinetically accelerated and optically amplified protein based assays and potentially immunoassays in HTS 96-well platforms.

2. Experimental

2.1. Materials

Silver nitrate (99.9%), trisodium citrate, Bovine-biotinamidocaproyl-labeled Albumin (biotinlyated BSA), FITC-labeled avidin and SigmaScreen™ Poly-D-Lysine coated High Throughput Screening (HTS) plates (96 wells) were obtained from Sigma-Aldrich. All chemicals were used as received.

2.2. Methods

2.2.1. Synthesis of silver colloids and the coating of HTS wells with silver colloid films

The synthesis of silver colloids was performed using the following procedure: 2 ml of 1.16 mM trisodium citrate solution was added drop wise to a heated (90 °C) 98 ml aqueous solution of 0.65 mM of silver nitrate while stirring. The mixture was kept heated for 10 min, and then it was cooled to room temperature.

The coating of the HTS plates was achieved by incubating 0.5 ml of silver colloid solution inside the HTS wells (48 wells) overnight. The HTS wells were coated with silver colloids due to the binding of silver to the amine groups of the surface poly-lysine (Malicka et al., 2003), as demonstrated previously by our laboratories. The other half of the wells (48 wells) in the same HTS plates were left intentionally blank for the control experiments. The silver colloid deposited HTS wells were rinsed with deionized water several times prior to the fluorescence experiments.

2.2.2. Metal-Enhanced Fluorescence (MEF) and Microwave-Accelerated Metal-Enhanced Fluorescence (MAMEF) from silver colloids-modified HTS wells

In previous reports of Metal-Enhanced Fluorescence (MEF), our laboratories have coated silvered surfaces with fluorophore labeled protein (Geddes et al., 2004a, 2005). This experimental format has been adopted for two main reasons, the first, being that the protein coverage with Human Serum Albumin (HSA) is known to bind to silvered surfaces and indeed forms a monolayer (Green, 1975; Wilchek and Bayer, 1990, 1998) and secondly, the dimensions of the protein being such that the protein allows for a mean ≈ 4 nm separation of the silver and the fluorophore, MEF being a through space phenomenon, as demonstrated by the late T. Cotton and indeed our laboratories. (Geddes et al., 2004a, 2005; Sokolov et al., 1998). In contrast, Surface Enhanced Raman Scattering (SERS) is known to be a consequence of mostly contact between the species of interest and the silvered surface (Sokolov et al., 1998).

The model assay used in this paper is based on the well-known interactions of biotin and avidin. Biotin groups are introduced to the surface through biotinylated-BSA, which, similar to HSA, readily forms a monolayer on the surface of glass and silver colloid films (Green, 1975; Wilchek and Bayer, 1990, 1998). Binding the biotinylated-BSA to the silver colloid-modified and unmodified part of the HTS wells was accomplished by incubating 10 μM biotinylated-BSA solution in the wells for 1 h, followed by rinsing with water to remove the unbound material. For the model assay, then 100 μl of 1 μM FITC-labeled avidin was subsequently added to the biotinylated-BSA coated wells, 30 min for the control experiments at room temperature (20 °C), and 30 s in the microwave cavity (0.7 cu ft, GE Compact Microwave Model: JES735BF, max power 700 W), followed by rinsing with water to remove the unbound material. The power setting was set to 2 which corresponded to 140 W over the entire cavity. This power is similar to the numerous reports using low power microwaves for immunolabeling (Chicoine and Webster, 1998), Immunostaining (Micheva et al., 2001; Petrali and Mills, 1998), in immunocytochemistry (Madden,

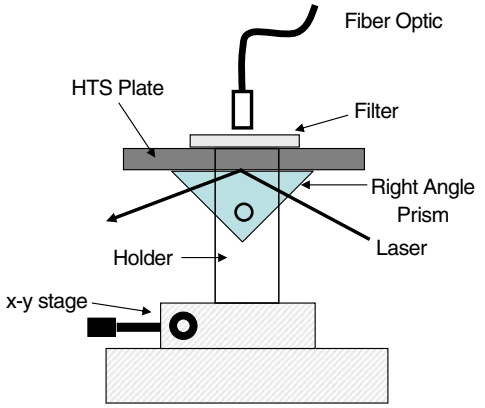

Fig. 1. TIRF—Total Internal Reflection Fluorescence experimental set-up mounted on an XY stage, for analysis of both the silvered and unsilvered HTS wells.

1998; Rangell and Keller, 2000; Schichnes et al., 1999) and histological microwave processing (Ressner et al., 1997; Schray et al., 2002). In all the experiments performed with low power microwaves using HTS plates, there was no evidence of drying of the aqueous media.

Several control experiments were also performed on the silver colloid-modified and unmodified HTS wells to investigate the extent of non-specific binding of proteins to the HTS wells: 1) Incubation of FITC-avidin without Biotinylated-BSA and without microwave heating for 30 min, 2) Incubation of FITC-avidin without Biotinylated-BSA and without microwave heating for 30 s, 3) Incubation of FITC-avidin with microwave heating and without Biotinylated-BSA for 30 s.

2.2.3. Absorption and fluorescence measurements

All absorption measurements were performed using a HP 8453 UV–Vis spectrophotometer. Fluorescence measurements on HTS plates were performed by placing the HTS plates on a Total Internal Reflection (TIR) stage equipped with a fiber-optic mount on a 15-cm-long arm (normal to sample), Fig. 1. The output of the fiber was connected to an Ocean Optics HD2000 spectrofluorometer to measure the fluorescence emission spectra. The excitation was from the second harmonic (473 nm) of the diode-pumped Nd:YVO4 laser (output power ≈30 mW) at an angle of 45°. This configuration allowed easy changes of the incident angle and the evanescent excitation spot position. The emission was observed through a 488 nm razor-edge filter (Semrock).

3. Results

To demonstrate proof of principle that our MAMEF approach can be applied to high-throughput formats, we have used commercially available 96-well plates, with silver deposited on the bottom of the plates, Fig. 2. Silver colloids can be readily deposited on the bottom of the clear plastic plates, the number density determined by the incubation conditions, the colloid size determined by the experimental conditions of preparation, all monitored by measuring the surface plasmon absorption, Fig. 3.

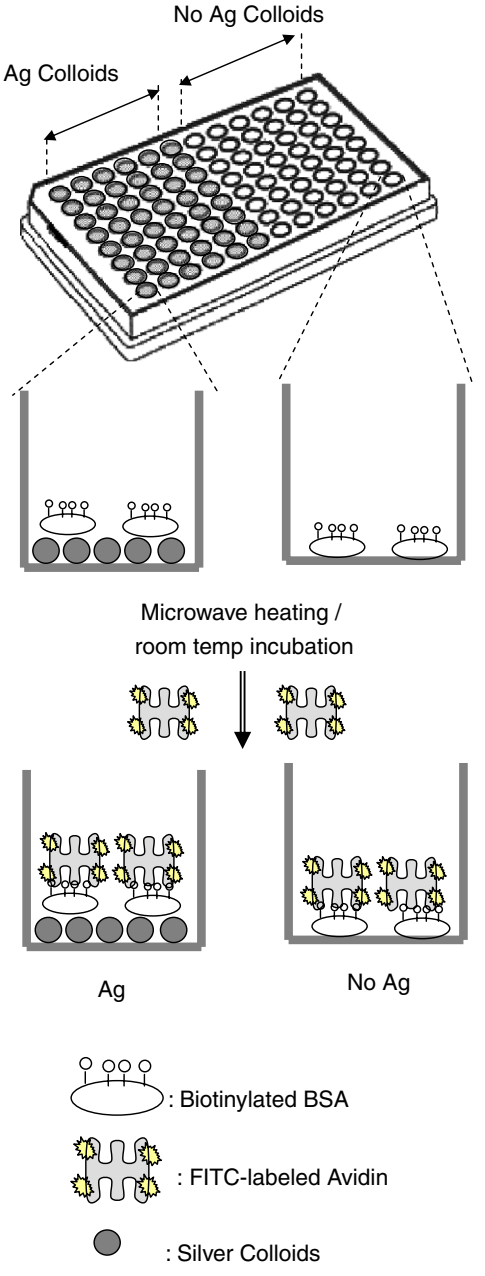

Fig. 2. Sample geometry in HTS wells.

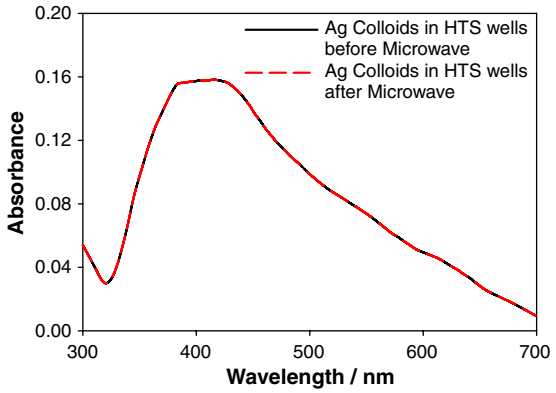

Fig. 3. Absorption spectra of silver-colloid coated plastic-bottomed HTS wells, before and after low power microwave heating.

The model protein system chosen was the Biotin–Avidin system, where the simple kinetics and bioaffinity of both for each other is well-known (Green, 1975; Wilchek and Bayer, 1990, 1998). Earlier studies by our laboratories had shown that biotinylated-BSA readily forms an equal monolayer on both silver and plastic substrates (Aslan et al., 2005e). Subsequently, biotinylated BSA was coated on all 96-well plates, half of which had been silvered, the other half poly-lysine coated bare plastic, acting as control samples by which to compare the benefits of using the Metal-Enhanced Fluorescence (MEF) phenomenon (Aslan et al., 2005e). The enhancement ratio I_{SiFs}/I_{HTS} (the benefit of using MEF) is the fluorescence intensity observed on the silver colloids divided by the intensity on the non-silvered substrate. In addition, this model protein system, Fig. 2 positions the fluorophore (which is fluorescein labeled avidin), >4 nm from the surfaces. This is ideal for MEF, which we have shown in numerous publications to be a through space phenomenon, occurring over the range 4–10 nm (Geddes et al., 2004a, 2005), as compared to Surface-Enhanced Raman Scattering (SERS), a similar effect primarily reported to be due to surface contact interactions (Sokolov et al., 1998).

For excitation of the assay shown in Fig. 2, we chose Total Internal Reflection Fluorescence (TIRF) (Matveeva et al., 2004). TIRF is an approach that readily allows the selective excitation of fluorophores within ≈200 nm proximity to surfaces (Matveeva et al., 2004), an approach ill used for surface assays (Matveeva et al., 2004). In the presence of silver islands however, Fig. 3, the evanescent field generated using the TIRF format, has been shown to much stronger than as compared to uncoated glass (Matveeva et al., 2004), penetrating further into solution. Subsequently, evanescent wave excitation confines the excitation volume at the assay surface, eliminating the need for washing the solution from above the assay. Interestingly, because the MEF phenomenon only occurs out to about 10 nm from the silver nanoparticles, then unwanted background fluorescence from assay material distal from the surface is not observed, increasing the S/N in the HTS wells for sensing. In contrast, most fluorescence HTS based assays both excite and collect back-fluorescence from above the open HTS wells, usually in a faster scanning mode or using fibers for both excitation and fluorescence collection (Shayne, 2005). In these geometries, all the fluorophores in the entire solution are excited, mandating an assay washing step to remove the unbound assay material, which inherently increases both the screening time and cost, as well as reduces the S/N ratio of the system. To the best of our knowledge, this is the first report of enhanced evanescent field geometry (by silver) for sensing in plate wells.

Fig. 3 shows the plasmon absorption spectrum of silver colloids deposited at the bottom of HTS wells, both before and after low power microwave heating for 30 s. The cavity power was ≈140 W, which is the same power as utilized in the assays discussed later, and is of a similar power used by others for immunostaining (Micheva et al., 2001; Petrali and Mills, 1998). From Fig. 3, we can see that the microwaves had no effect on the surface plasmon absorption of the silver colloids, strongly indicating no surface silver shape or size changes, where the surface plasmon absorption is well-known to be characteristic of the shape and size of noble metal nanoparticles (Geddes et al., 2004a, 2005). In this regard, we are confident of our interpretation here, as we have recently reported that silver colloids thermally annealed up to 200 °C in a vacuum oven show no evidence of shape change, with no changes evident in their surface plasmon absorption or even their atomic force microscopy images (Aslan et al., 2005f). For the HTS well samples, we were unable to undertake AFM images of the silver colloids on the base of the wells both before and after microwave heating, due to the physical size constraints of the wells.

As briefly mentioned in the Introduction, large particles or even continuous metallic surfaces produce “sparking” when heated in microwave cavities. This effect is due to the charge build up, and subsequent “arching” as charge builds between the particles (Whittaker and Mingos, 1993, 1995). For the nanostructures deposited in the HTS wells described here, no sparking was evident at all, suggesting that the charge build up on the nanometer sized colloids is too small to induce dielectric breakdown of the medium separating

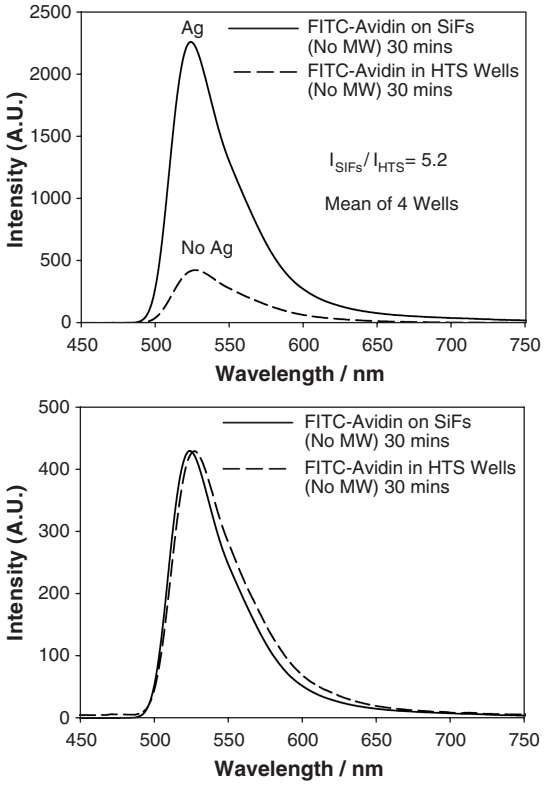

Fig. 4. Fluorescence emission intensity of fluorescein from both silvered and non-silvered HTS wells (Top) after 30 min room temperature incubation. The spectra are the average of 4 wells. (Bottom) Normalized spectra from both silvered and non-silvered wells.

them. A similar finding has also been observed by the authors for even larger sized silver island films deposited on glass microscope slides (Aslan et al., 2005f).

Fig. 4 top shows the fluorescein emission intensity from both silvered and non-silvered HTS wells after room temperature incubation of the assay. The assay was incubated for 30 min. The emission, which was collected through a 488 nm razor edge filter (Semrock), shows an approximate 5-fold greater intensity from the silver as compared to the non-silvered wells. These values were the mean of 4 wells each, the data quickly collected from each well by moving the plate well on the XY stage, Fig. 1. As mentioned in the Introduction, this increase is not due to reflected photons from the silvered surface, i.e. scattering, but is in fact a consequence of a new near-field fluorescence phenomenon reported by the authors (Geddes et al., 2004a), whereby fluorophores in close proximity (<10 nm) to a silver nanostructures can be made highly fluorescent. This was originally thought to be due the ability of the metal to modify the intrinsic radiative decay rate of fluorophores, but is now a process thought to be underpinned by the ability of the fluorophore to transfer energy to surface plasmons, which themselves radiative with high efficiency (Aslan et al., 2005f). Subsequently, the net fluorophore–metal system is highly fluorescent (Aslan et al., 2005f) and highly photostable, the photophysical characteristics of the metal-coupled emission, very similar to that of the fluorophore alone (Aslan et al., 2005f). Fig. 4 bottom shows almost identical spectra after normalization from both the silvered and non-silvered wells, indicating the only differences in emission being the relative intensities.

Fig. 5 shows the fluorescence intensity of fluorescein from both the silvered and non-silvered HTS wells after 30 s microwave heating. Similar to Fig. 4, a ≈4–5-fold fluorescence enhancement can be seen on silver as compared to the non-silver wells. Interestingly, similar emission intensities were observed for 30 min incubation as compared to 30 s microwave heating, an ≈90-fold kinetic increase. The slight differences in the final fluorescence intensities (1700 vs. 2250 arbitrary units)

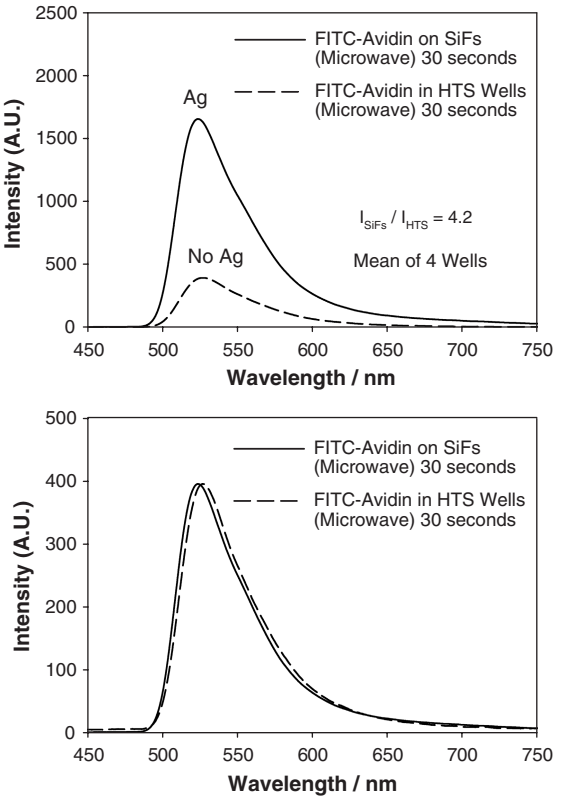

Fig. 5. Fluorescence emission intensity of fluorescein from both silvered and non-silvered HTS wells (Top) after 30 s microwave heating. The spectra are the average of 4 wells. (Bottom) Normalized spectra from both silvered and non-silvered wells after microwave heating.

are due to the fact that 45 s of heating was actually required to take the assay to >95% kinetically complete and not 30 s as shown in Fig. 5 top (data not shown). Interestingly, the emission intensity on the unsilvered plates was similar for both 30 min incubation and 30 s low power microwave heating. Clearly, by considering both Figs. 4 and 5 top, we can see the benefits of using low power microwave heating to accelerate assays in HTS wells, as well as the combined benefit of amplified fluorescence to facilitate detection by the presence of the silver colloids. Surprisingly, the ≈90-fold kinetic increase can not be explained by the ≈8 °C temperature jump, as determined using the temperature dependent probe, Thymol blue (experimental protocol described elsewhere) (Aslan and Geddes, 2005). Subsequently, it is thought that localized and indeed preferential heating on an around the silver nanoparticles occurs. In this regard, Mingos and co-workers (Whittaker and Mingos, 1993) 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 (Whittaker and Mingos, 1993). Subsequently metal powders have been used to accelerate the synthesis of a wide range of metal chalogenides and as reducing agents in the formation of low-oxidation-state metal cluster compounds (Whittaker and Mingos, 1993).

The MAMEF comparison in the 96-well plates is also evident visually, Fig. 6, the photographs showing the benefits of both microwave heating and the use of silvered substrates. Top left and right visually compare the emission with and without silver, while bottom left and right show the effects of low power microwave heating, with and without silver colloids. Remarkably, by comparing both bottom-left with the photograph top right, we can see the benefits of using the MAMEF technique. All photographs were taken through a 488 nm razor edge filter with 473 nm evanescent wave excitation.

As with many assays it is the bioactivity, concentration of the species of interest and nature of the surfaces that governs the extent of non-specific reactions or non-specific surface assay absorption (Bange et al., 2005; Hemmilam, 1992). Subsequently, we questioned if the use of low power microwaves in HTS well formats indeed increased the rate of non-specific absorption in our model assay. In these experiments, the well bottoms were not pre-coated with biotinylated-BSA as is the case in the model assays shown in Figs. 4–6. From Fig. 7 top we can see that after 30 min incubation in the wells with FITC-Avidin, followed by a washing step, fluorescein emission was evident, more so on the silvered as

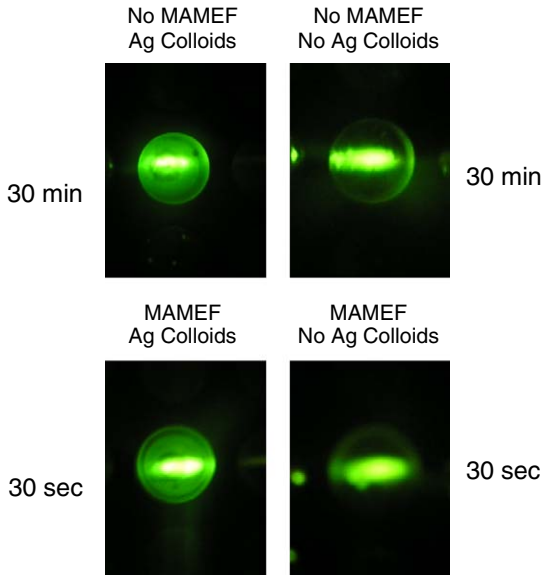

Fig. 6. Photographs of actual HTS wells, with and without silver, before and after microwave heating. The photographs were taken through a long-pass filter with 473 nm TIR evanescent wave excitation.

compared to the unsilvered well bottom, although the extent of absorption was much smaller than the actual assay shown in Fig. 4. Interestingly, a significantly large portion of the non-specific absorption occurred in the first 30 s of room temperature incubation, Fig. 7 middle. After low power microwave heating for 30 s, Fig. 7 bottom, similar fluorescence intensity was observed as compared to just 30 s room temperature incubation, cf. Fig. 7 middle and bottom. This suggests that low power microwaves do not further accelerate non-specific absorption, beyond that normally present in this assay system. While a <20% extent of non-specific absorption would be unacceptable in many assays settings, our results are focused on demonstrating the MAMEF platform technology and not the individual assay. For actual assays, non-specific absorption would need to be investigated and determined thoroughly before application. Finally, in a recent paper, we have shown that low power microwaves do not increase the extent of non-specific absorption for the same protein system, but on silvered and unsilvered glass microscope slides. In these findings (Aslan and Geddes, 2005), the total extent of non-specific absorption was very low indeed, suggesting that the <20% total absorption shown here is due to protein interactions with the poly-lysine HTS well coating.

Fluorophore photostability is a primary concern in many applications of fluorescence, particularly platform

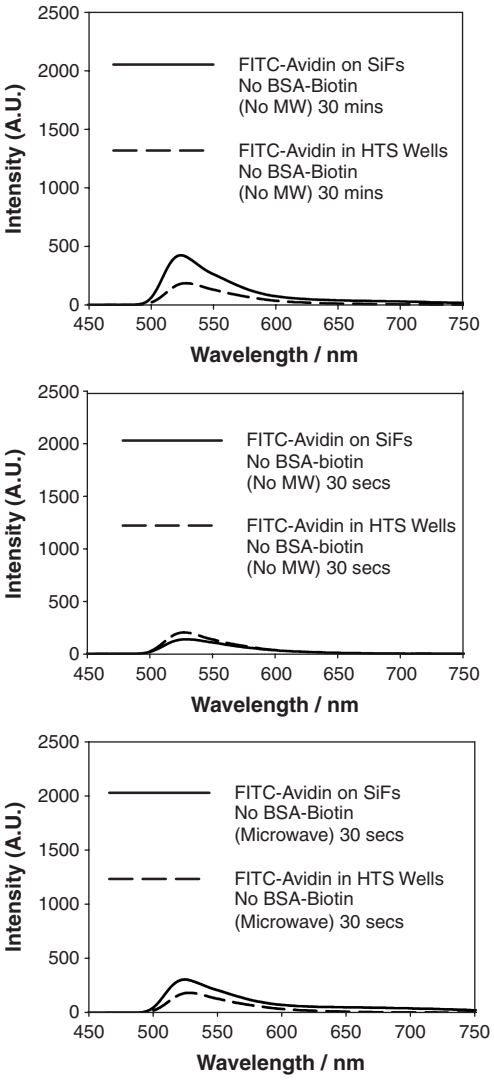

Fig. 7. Control experiments showing the rate of non-specific absorption of fluorescein–avidin to bare and silvered surfaces after 30 min incubation (Top), 30 s incubation (Middle), and after 30 s low power microwave heating (Bottom). The well bottoms were not coated with Biotinylated-BSA.

type assays and in single molecule studies (Lakowicz, 1999; Axelrod et al., 1992). The maximum number of photons emitted (photon flux) is roughly limited by its excited state lifetime (Lakowicz, 1999). Due to the geometry of the wells, it was not possible to measure the fluorescein lifetime using either frequency or time domain fluorescence spectroscopy (Lakowicz, 1999). However, our numerous reports published to date pertaining to fluorophores in close proximity to silver nanoparticles (Geddes et al., 2004a, 2005) suggest that the fluorescein lifetime will be significantly shorter than the free space lifetime (Lakowicz, 1999), affording for an enhanced photostability due to the fluorophore spending less time in an excite state, and therefore being less prone to photoxidation, the major excited state photo destructive pathway (Lakowicz, 1999).

In addition to traditional fluorescence photostability, we also investigated the effects of low power microwave heating on the emission intensity of the fluorescein assay, where the assay was initially incubated for 30 min at room temperature. After this time, the assay was microwave heated in 5 s cumulative increments up to 1 min, the fluorescence intensity measured at 530 nm after 473 nm excitation. After 1 min of total heating and re-excitation, no change in the emission signal intensity was evident, indicating that low power microwaves do not perturb fluorescein fluorescence. Interestingly, under the conditions employed with this assay, it took greater than 5 min microwave heating to completely dry multiple plate well assays, up until which point, both the fluorescein emission spectra and peak intensity remained mostly constant.

4. Discussion

In this paper we have demonstrated a cheap and simplistic approach to overcoming some of the physical constraints imposed by current assay platforms in HTS, namely assay rapidity and sensitivity (Bange et al., 2005; Hemmilam, 1992). Our new Microwave-Accelerated Metal-Enhanced Fluorescence (MAMEF) approach has several notable advantages including:

- The fluorescence amplification provided by the silver nanostructures has been shown to be applicable to many fluorophores and therefore wavelengths, from the UV to near IR Geddes et al., 2003a,b,c,d,e; Lakowicz, 2001; Lakowicz et al., 2001, 2002, 2003). Hence fluorophores currently employed in assays would still be suitable. However, the use of low quantum yield fluorophores would lead to much larger fluorescence enhancements (i.e. $1/Q_0$) and could significantly reduce unwanted background emission from fluorophores distal from the silvered assay, recalling that MEF is a close-range (<10 nm) through-space interaction (Geddes et al., 2004a, 2005).
- The Metal-Enhanced Fluorescence phenomenon has been shown by us to provide for increased emission intensities (Geddes et al., 2004a, 2005), up to several thousand-fold (Geddes et al., 2003c). This substantially increases detection limits (i.e. lower concentrations detectable), which is a major criterion in HTS assay screening today (Shayne, 2005).

- A whole variety of silvered surfaces for the bottom of HTS 96-well plates can be routinely prepared, which do not require the benefits of a nanofabrication lab and sophisticated instrumentation such as electron beam lithography. These include the preparation of silver island films (Lakowicz et al., 2002), silver colloids (Geddes et al., 2003b), silver nanotriangles (Aslan et al., 2005c), silver nanorods (Aslan et al., 2005d) and silver fractals (Parfenov et al., 2003), to name a few. In addition, we have recently reported the simple modification of plastic substrates for silver deposition. These procedures (Aslan et al., 2005e), could readily and cheaply be employed, alleviating the need to use poly-lysine coated HTS 96-well plates.
- The reduced lifetime of fluorophores in close proximity to silver nanostructures provides for a substantially increased fluorophore photostability (Geddes et al., 2004a). In addition, shorter lifetimes allow for higher fluorophore cycling rates (Lakowicz, 1999), also providing for increased fluorophore and therefore assay detectability.
- The low power microwaves employed here do not perturb the silvered surfaces and do not produce "arcing" which is commonly observed for larger metallic objects in microwave cavities (Whittaker and Mingos, 1993).
- Low power microwaves provide for effective rapid heating of the assays, producing identical final fluorescence intensities as compared to longer room temperature incubation. This is likely to significantly reduce assay times and cost in HTS.
- The silver-enhanced evanescent field mode of excitation localizes the excitation volume in close proximity to the silver nanostructures. This eliminates the need for assay washing steps. In this regard the assay shown in Fig. 4, was shown to have almost identical spectral characteristics and intensities, with or without a washing step.
- Finally, it is informative to comment on the use of microwaves in biochemical and biological systems. In the mid 1980s much controversy surrounded the use of microwaves due to the so-called "non-thermal" effects (Adam, 2003; Whittaker and Mingos, 1993; Kappe, 2002) on biological systems, where some authors believed that the rates of reaction in many microwave assisted reactions could not be explained by heating alone (Adam, 2003; Whittaker and Mingos, 1993; Kappe, 2002), and have since questioned enzyme, DNA and protein function and conformation after microwave exposure (Adam, 2003; Whittaker and Mingos, 1993; Kappe, 2002). On the other hand, some workers assert that there are no "non-general thermal effects", and the so-called non-thermal effect is due to the superheating of solvents above their boiling points (Kappe, 2002). While this debate is likely to continue for time, as it is difficult to experimentally prove a non-thermal effect, it is clearly evident that the use of low power microwaves is rapidly growing, with many companies now selling microwave based laboratory equipment and the publication of many articles a year embracing the technology. In this regard, it is likely that we could see the future development of microwave assisted HTS plate readers, combining silver particles for thermal localization and enhanced fluorescence signatures.

5. Conclusions

In this paper we have demonstrated a model assay sensing platform for potential use in HTS 96-well plate formats. This new approach provides for both ultra fast and brighter assays as well as immunoassays in HTS platforms, which is likely to find popular use.

The new MAMEF approach combines the use of silver nanostructures to optically amplify fluorescence signatures, a new near-field phenomenon, with the use of low power microwaves to kinetically accelerate the assays. The microwaves do not perturb the silver nanostructures, but simply increase the mass transport of protein to the plate well-bottoms. Interestingly, the $\approx$90-fold increase in assay kinetics can not be explained by the $\approx$8 °C bulk temperature jump, suggesting that localized heating occurs around the silver nanostructures.

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References

Adam, D., 2003. Microwave chemistry: out of the kitchen. Nature 421, 571.

Akins, R.E., Tuan, R.S., 1995. Ultrafast protein determinations using microwave enhancement. Molecular Biotechnology 4, 17.

Aslan, K., Geddes, C.D., 2006. Microwave-Accelerated Metal-Enhanced Fluorescence (MAMEF): Application to Fast and Sensitive Clinical Assays. J. of Fluoresc. 16 (1), 3.

Aslan, K., Geddes, C.D., 2005. Microwave-Accelerated Metal-Enhanced Fluorescence: A New Platform Technology for Ultra Fast and Ultra Bright Assays. Anal. Chem. 77, 8057.

Aslan, K., Gryczynski, I., Malicka, J., Matveeva, E., Lakowicz, J.R., Geddes, C.D., 2005a. Metal-enhanced fluorescence: an emerging tool in biotechnology. Curr. Opin. Biotechnol. 16 (1), 55.

Aslan, K., Lakowicz, J.R., Szmacinski, H., Geddes, C.D., 2005b. Enhanced ratiometric pH sensing using SNAFL-2 on silver island films: metal-enhanced fluorescence sensing. J. Fluoresc. 15, 37.

Aslan, K., Lakowicz, J.R., Geddes, C.D., 2005c. Rapid deposition of triangular silver nanoplates on planar surfaces: application to metal-enhanced fluorescence. J. Phys. Chem., B 109, 6247.

Aslan, K., Leonenko, Z., Lakowicz, J.R., Geddes, C.D., 2005d. Fast and slow deposition of silver nanorods on planar surfaces: application to metal-enhanced fluorescence. J. Phys. Chem., B 109, 3157.

Aslan, K., Badugu, R., Lakowicz, J.R., Geddes, C.D., 2005e. Metal-enhanced fluorescence from plastic substrates. J. Fluoresc. 15, 99.

Aslan, K., Lakowicz, J.R., Geddes, C.D., 2005f. Annealed silver-island films for applications in metal-enhanced fluorescence: interpretation in terms of radiating plasmons. J. Fluoresc. 15, 643.

Axelrod, D., Hellen, E.H., Fulbright, R.M., 1992. In: Lakowicz, J.R. (Ed.), Topics in Fluorescence Spectroscopy. Biochemical Applications, vol. 3. Plenum Press, New York, p. 289.

Bange, A., Halsall, H.B., Heineman, W.R., 2005. Microfluidic immunosensor systems. Biosens. Bioelectron. 20, 2488.

Bismuto, E., Mancinelli, F., d'Ambrosio, G., Massa, R., 2003. Are the conformational dynamics and the ligand binding properties of myoglobin affected by exposure to microwave radiation? Eur. Biophys. J. 32, 628.

Caddick, S., 1995. Microwave-assisted organic-reactions. Tetrahedron 51, 10403.

Chicoine, L., Webster, P., 1998. Effect of microwave irradiation on antibody labeling efficiency when applied to ultrathin cryosections through fixed biological material. Microbiol. Res. Technol. 42, 24.

Davidson, R.S., Hilchenbach, M.M., 1990. The use of fluorescent-probes in immunochemistry. Photochem. Photobiol. 52, 431.

Drexhage, K.H., 1974. In: Wolfe, E. (Ed.), Progress in Optics. North-Holland, Amsterdam, p. 161.

Geddes, C.D., Lakowicz, J.R., 2002. Metal-enhanced fluorescence. J. Fluoresc. 12, 121.

Geddes, C.D., Gryczynski, I., Malicka, J., Gryczynski, Z., Lakowicz, J.R., 2003a. Metal-enhanced fluorescence: potential applications in HTS. Comb. Chem. High Throughput Screen. 6, 109.

Geddes, C.D., Cao, H., Gryczynski, I., Gryczynski, Z., Fang, J., Lakowicz, J.R., 2003b. Metal-enhanced fluorescence due to silver colloids on a planar surface: potential applications of Indocyanine green to in vivo imaging. J. Phys. Chem., A 107, 3443.

Geddes, C.D., Parfenov, A., Lakowicz, J.R., 2003c. Photodeposition of silver can result in metal-enhanced fluorescence. Appl. Spectrosc. 57, 526.

Geddes, C.D., Parfenov, A., Roll, D., Fang, J., Lakowicz, J.R., 2003d. Electrochemical and laser deposition of silver for use in metal-enhanced fluorescence. Langmuir 19, 6236.

Geddes, C.D., Gryczynski, I., Malicka, J., Gryczynski, Z., Lakowicz, J.R., 2003e. Fractal silver structures for metal-enhanced fluorescence: applications for ultra-bright surface assays and lab-on-a-chip-based nanotechnologies. J. Fluoresc. 14, 119.

Geddes, C.D., Aslan, K., Gryczynski, I., Malicka, J., Lakowicz, J.R., 2004a. In: Geddes, C.D., Lakowicz, J.R. (Eds.), Review Chapter for Annual Reviews in Fluorescence. Kluwer Academic/Plenum Publishers, New York, USA, p. 365.

Geddes, C.D., Parfenov, A., Roll, D., Gryczynski, I., Malicka, J., Lakowicz, J.R., 2004b. Roughened silver electrodes for use in metal-enhanced fluorescence. Spectrochim. Acta, Part A 60, 1977.

Geddes, C.D., Gryczynski, I., Malicka, J., Gryczynski, Z., Lakowicz, J.R., 2004c. Photonics Spectra 38, 92.

Geddes, C.D., Aslan, K., Gryczynski, I., Malicka, J., Lakowicz, J.R., 2005. In: Geddes, C.D., Lakowicz, J.R. (Eds.), Topics in Fluorescence in Fluorescence Spectroscopy. Kluwer Academic/Plenum Publishers, New York, USA, p. 401.

Gosling, J.P., 1990. A decade of development in immunoassay methodology. Clin. Chem. 36, 1408.

Green, N.M., 1975. Adv. Protein Chem. 29, 85.

Hemmilam, L.A., 1992. Applications of Fluorescence in Immunoassays. John Wiley and Sons, New York.

Kappe, C.O., 2002. High-speed combinatorial synthesis utilizing microwave irradiation. Curr. Opin. Chem. Biol. 6, 314.

Lakowicz, J.R., 1999. Principles of Fluorescence Spectroscopy. Kluwer, New York.

Lakowicz, J.R., 2001. Decay engineering: biophysical and biomedical applications. Anal. Biochem. 298, 1.

Lakowicz, J.R., 2004. Radiative decay engineering 3: surface plasmon-coupled directional emission. Anal. Biochem. 324, 153.

Lakowicz, J.R., Shen, Y., Gryczynski, Z., D'Auria, S., Gryczynski, I., 2001. Intrinsic fluorescence from DNA can be enhanced by metallic particles. Biochem. Biophys. Res. Commun. 286, 875.

Lakowicz, J.R., Shen, Y., D'Auria, S., Malicka, J., Fang, J., Grcyzynski, Z., Gryczynski, I., 2002. Radiative decay engineering 2. Effects of silver island films on fluorescence intensity, lifetimes, and resonance energy transfer. Anal. Biochem. 301, 261.

Lakowicz, J.R., Malicka, J., D'Auria, S., Gryczynski, I., 2003. Release of the self-quenching of fluorescence near silver metallic surfaces. Anal. Biochem. 320, 13.

Lin, J.C., Yuan, P.M.K., Jung, D.T., 1998. Enhancement of anticancer drug delivery to the brain by microwave induced hyperthermia. Bioelectrochem. Bioenerg. 47, 259.

Lobmaier, C., Hawa, G., Gotzinger, M., Wirth, M., Pittner, F., Gabor, F., 2001. Direct monitoring of molecular recognition processes using fluorescence enhancement at colloid-coated microplates. J. Mol. Recognit. 14, 215.

Madden, V.J., 1998. Micro Microanal. 4, 854.

Malicka, J., Gryczynski, I., Lakowicz, J.R., 2003. DNA hybridization assays using metal-enhanced fluorescence. Biochem. Biophys. Res. Commun. 306, 213.

Matveeva, E., Gryczynski, Z., Malicka, J., Gryczynski, I., Lakowicz, J.R., 2004. Metal-enhanced fluorescence immunoassays using total internal reflection and silver island-coated surfaces. Anal. Biochem. 334, 303.

Micheva, K.D., Holz, R.W., Smith, S.J., 2001. Regulation of presynaptic phosphatidylinositol 4,5-biphosphate by neuronal activity. J. Cell Biol. 154, 355.

Ozinkas, A.J., 1994. In: Lakowicz, J.R. (Ed.), Principles of Fluorescence Immunoassay. Topics in Fluorescence Spectroscopy, vol. 4. Plenum Press, New York.

Parfenov, A., Gryczynski, I., Malicka, J., Geddes, C.D., Lakowicz, J.R., 2003. Enhanced fluorescence from fluorophores on fractal silver surfaces. J. Phys. Chem., B 107, 8829.

Petrali, J.P., Mills, K.R., 1998. Micro Microanal. 114.

Porcelli, M., Cacciapuoti, G., Fusco, S., Massa, R., d'Ambrosio, G., Bertoldo, C., DeRosa, M., Zappia, V., 1997. Non-thermal effects of microwaves on proteins: thermophilic enzymes as model system. FEBS Lett. 402, 102.

Rangell, L.K., Keller, G.A., 2000. Application of microwave technology to the processing and immunolabeling of plastic-embedded and cryosections. J. Histochem. Cytochem. 28, 1153.

Ressner, U.A., Crumrine, O.A., Nau, P., Elias, P.M., 1997. Microwave incubation improves lipolytic enzyme preservation for ultrastructural cytochemistry. Histochem. J. 29, 387.

Rhodes, A., Jasani, B., Balaton, A.J., Barnes, D.M., Anderson, E., Bobrow, L.G., Miller, K.D., 2001. Study of interlaboratory reliability and reproducibility of estrogen and progesterone receptor assays in Europe—Documentation of poor reliability and identification of insufficient microwave antigen retrieval time as a major contributory element of unreliable assays. Am. J. Clin. Pathol. 115, 44.

Roy, I., Gupta, M.N., 2003. Applications of microwaves in biological sciences. Curr. Sci. 85, 1685.

Schichnes, D., Nemson, J., Sohlberg, L., Ruzin, S.E., 1999. Microwave protocols for paraffin microtechnique and in situ localization in plants. Micro Microanal. 4, 491.

Schray, C.L., Metz, A.L., Gough, A.W., 2002. Histologic 35, 7.

Schweitzer, B., Kingsmore, S.F., 2002. Measuring proteins on microarrays. Curr. Opin. Biotechnol. 13, 14.

Shayne, Gad (Ed.), 2005. Drug Discovery Handbook. Wiley and Sons, New Jersey.

Sokolov, K., Chumanov, G., Cotton, T.M., 1998. Enhancement of molecular fluorescence near the surface of colloidal metal films. Anal. Chem. 70, 3898.

Sridar, V., 1997. Rate acceleration of Fischer-indole cyclization by microwave irradiation. Indian J. Chem., Sect. B: Org. Chem. Incl. Med. Chem. 36, 86.

Sridar, V., 1998. Microwave radiation as a catalyst for chemical reactions. Curr. Sci. 74, 446.

Takatsy, G., 1955. Acta Microbiol. Hung. 3, 191.

Van Dyke, K., Van Dyke, R. (Eds.), 1990. Luminescence Immunoassay and Molecular Applications. CRC Press, Boca Raton, FL.

Van Triest, B., Loftus, B.M., Pinedo, H.M., Backus, H.H.J., Schoenmakers, P., Telleman, F., 2000. Thymidylate synthase expression in patients with colorectal carcinoma using a polyclonal thymidylate synthase antibody in comparison to the TS 106 monoclonal antibody. J. Histochem. Cytochem. 48, 755.

Varma, R.S., 2002. Advances in Green Chemistry, Chemical Synthesis using Microwave Irradiation. Astrazeneca Research Foundation, India, Banglore.

Weitz, D.A., Garoff, S., Hanson, C.D., Gramila, T.J., Gersten, J.I., 1982. Fluorescent lifetimes of molecules on silver-island films. Opt. Lett. 7, 89.

Whittaker, A.G., Mingos, D.M.P., 1993. Microwave-assisted solid-state reactions involving metal powders and gases. J. Chem. Soc. Dalton Trans. 16, 2541.

Whittaker, A.G., Mingos, D.M.P., 1995. Microwave-assisted solid-state reactions involving metal powders. J. Chem. Soc. Dalton Trans. 12, 2073.

Wilchek, M., Bayer, E.A., 1990. Methods of Enzymology, vol. 184. Academic Press, San Diego.

Wilchek, M., Bayer, E.A., 1998. The avidin–biotin complex in bioanalytical applications. Anal. Biochem. 171, 1.

Wokaun, A., Lutz, H.-P., King, A.P., Wild, U.P., Ernst, R.R., 1983. Energy transfer in surface enhanced luminescence. J. Chem. Phys. 79, 509.