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ORIGINAL PAPER

Nanosphere Templated Metallic Grating Assisted Enhanced Fluorescence

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Abstract In this paper, enhanced fluorescence from a silver film coated nanosphere templated grating is presented. Initially, numerical simulation was performed to determine the plasmon resonance wavelength by varying the thickness of the silver film on top of a monolayer of 400 nm nanospheres. The simulation results are verified experimentally and tested for enhancing fluorescence from fluorescein isothiocyanate whose excitation wavelength closely matches with the plasmon resonance wavelength of the substrate with 100 nm silver film over nanosphere. The 12 times enhancement in the intensity is attributed to the local field enhancement in addition to the excitation of surface plasmon polaritons along the surface.

Keywords Metal enhanced fluorescence · Surface plasmons · Nanosphere monolayer · Finite-difference time domain method

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Introduction

Enhancement of molecular fluorescence is of great interest due to the widespread popularity of molecular-fluorescence-based measurements and devices in fields such as chemistry, molecular biology, materials science, photonics, and medicine. In most of these applications the fluorophores emit fluorescence isotropically into free space and are observed in the far field optical space. Ever since Drexhage [1] reported the effect of metallic surfaces on fluorescence, there have been numerous studies utilizing metals for fluorescence enhancement. In his work, Drexhage found that the fluorescence emission was dictated by an interference condition between direct and reflected fields, giving rise to oscillations in the excited-state lifetime and striking features in the radiation pattern from the emitting layer. This phenomenon was successfully modeled to describe the emitting ions as forced, damped, oscillating electric dipoles [2]. Metallic nanoparticles can influence the fluorescence emission of nearby molecules in several other ways: (a) by enhancing the optical intensity incident on the molecule through near field enhancement, (b) by modifying the radiative decay rate of the molecule, and (c) by increasing the coupling efficiency of the fluorescence emission to the far field through nanoparticle scattering. For enhanced electric field, the excitation is directly proportional to the square of the intensity of the E-field which leads to a close match between the resonance wavelength of the metal and the absorption wavelength of the fluorophore.

Recently, there has been a great interest in exploring metal enhanced fluorescence (MEF) and its applications in biotechnology [2–15]. High enhancements were thought to be associated only with fluorescent dyes with low quantum yields [8, 9] and hardly any enhancement for those above 50% quantum yields. However, some reports have shown

promising results for higher quantum yield dyes [10]. Recent results using fluorescein isothiocyanate (FITC) which has a quantum yield of around 0.9 have indicated that an enhancement well over one to two orders is possible under highly optimized conditions [11]. Substrates with good plasmonic activity are preferred for better enhancement and hence it plays a vital role in enhancing the fluorescence. Most of the recently reported experimental works related to MEF were performed using silver colloids [11], fractal silver surfaces [12], patterned nano-structures using nanosphere lithography [13], electron beam lithography (EBL) [14] and by core-shell nanoparticles [15]. However, these methods of fabricating the substrate for MEF suffer from difficulty in controlling the process parameters to achieve, high reproducibility and also involves time consuming and expensive approaches.

Several reports have discussed the formation of 2D monolayers of a particular size of nano/micro spheres [16] and also fabricating nanotriangles [17] by removing the spheres after coating metal and utilizing this for surface enhanced Raman spectroscopy (SERS). Fluorescence enhancement using 1D grating-coupled surface plasmons have been demonstrated previously using time consuming and expensive fabrication techniques like electron beam lithography and 2-beam interference lithography [18–20]. In these experiments it was shown that enhancement of fluorescence emission was due to excitation of surface plasmons propagating along the grating surface.

In this paper, we have demonstrated a simple method of fabricating a controlled, reproducible large active area of metal coated 2D nanosphere arrays for enhancing fluorescence from FITC, which is one of the most common fluorescent labeling reagents. A standardized method to form a monolayer of 400 nm polystyrene (PS) nanospheres which can readily be used for applications in MEF has been proposed. Effective coupling of incident photons into the surface is achieved through high periodicity of the fabricated array of nanospheres which also acts like a 2D reflective surface grating.

Numerical Simulations

Three-dimensional finite difference time domain (FDTD) method has been used for numerical analysis. The simulated structure comprises of a hexagonal structure of the 2D monolayer of 400 nm nanosphere coated with silver, which was modeled as shown in Fig. 1. The FDTD simulation was performed on the substrate to determine the plasmon resonance wavelength and map the local field distribution on the substrate by varying the silver film thickness on the nanosphere monolayer. The film thickness was varied from 40 nm to 120 nm in steps of 20 nm.

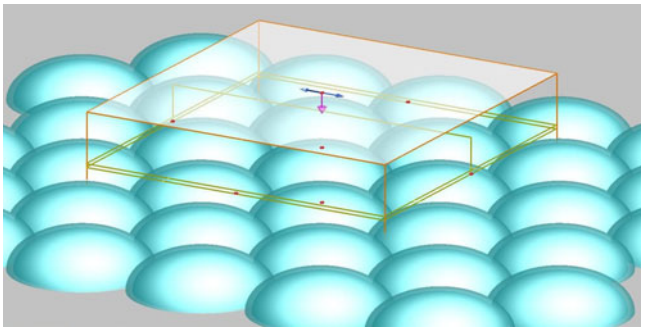

Fig. 1 A three dimensional simulation model using FDTD replicating the closely packed hexagonal structure of nanospheres with silver film, the simulation area and the source

The perfectly matched layer (PML) boundary condition has been used along the X, Y and Z-axis. In the simulation, the refractive index of the polystyrene is taken as 1.56 and frequency-dependent dielectric constant of silver is used as reported [21]. To mimic a broad band source, the light source for this simulation was taken as a plane wave source propagating in free space which was incident normally covering the wavelength range from 400 nm to 700 nm. The simulations were done by assuming a background relative dielectric constant of 1.0. Frequency-domain monitors were used that perform discrete Fourier transforms of the time-domain fields while the simulation is running. In this manner, continuous wave information is obtained at any pre-specified wavelengths for the various electric and magnetic field components.

Experimental Method

Nanosphere Monolayer

400 nm polystyrene (PS) nanosphere suspensions (Kisker-Biotech) were used without further preparation. 100 μl of the 2.5 wt.% PS bead suspension solution was mixed with 6 μl of 15 wt.% sodium dodecyl sulphate (SDS). 10 μl of this mixture was pipetted onto a cleaned glass slide cut into 10 mm x 10 mm and spin coated at 2,000 rpm for 30 s. SDS (Sigma-Aldrich) acts like a surfactant, which chemically modifies the nanosphere. As the solvent evaporates, capillary forces draw the nanospheres together resulting in a hexagonally close-packed pattern on the substrate. Due to the effect of interference on layers of PS, it was possible to examine the formation of monolayer with the naked eye. The substrates were dried in a dessicator overnight. 100 nm of silver was sputtered onto the nanosphere monolayer using a table top sputtering machine (JFC-1600, JEOL).

Spacer Layer

Poly vinyl alcohol (PVA) which acts as a good dielectric was purchased from Sigma-Aldrich. The PVA powder was used to make 0.1% solution and spin coated at 3,000 rpm for 30 s onto the substrate to form the spacer layer. PVA also helps in preventing the silver from getting oxidized.

Fluorophore Layer

100 μM solution of FITC was prepared and was spin coated on top of the spacer layer.

The schematic summarizing the whole process of substrate preparation for MEF is as shown in Fig. 2.

Characterization

The UV–Vis reflection spectrum of the silver coated substrate was measured in reflection mode using a Y-shaped fibre optic reflection probe. One of the arms of the probe was connected to the tungsten halogen light source (LS-1 Ocean Optics) to incident light normally onto the substrate. The reflected light was collected and delivered to the fibre optic spectrometer (USB 4000 Ocean Optics) by the other arm of the probe.

Scanning electron microscope (SEM) images were taken on a 3500N Hitachi (Hitachi Science Systems Ltd.) instrument. The applied voltage on the cathode was 15 kV. Before taking the images from samples with dielectric surfaces, a thin layer of gold (5 nm) was deposited using a table top gold sputtering machine.

Atomic force microscope (AFM) images were collected using Dimension 3000 (Digital Instruments). The AFM with 5 nm tip was operated in tapping mode under ambient conditions.

Fluorescence from the substrates was observed using an automated upright fluorescence microscope (Nikon eclipse 90i). The light for excitation was provided by the inbuilt white light source filtered at the required wavelength, in our case it was filtered at the FITC excitation wavelength. The images were acquired and the intensity measurements were performed using NIS-Elements software (Nikon Imaging).

Results and Discussion

The reflection spectrum obtained from the FDTD simulation of the substrate coated with 40 nm, 60 nm, 80 nm, 100 nm and 120 nm of silver film is represented in Fig. 3a. The graph shows the variation in reflection intensity with incident wavelength varied from 400 nm to 800 nm. The reflection spectrum minimum, red shift's as the film thickness is decreased. This effect shows that the optical resonance is dependent on the relative film thickness and its underlying nanosphere. At 100 nm silver thickness the minimum reflection was obtained at 480 nm. This represents the plasmon resonance wavelength for a monolayer of 400 nm nanospheres coated with 100 nm silver film. Figure 3b shows the local field distribution on the surface of the substrate at the plasmon resonance wavelength obtained from the substrate coated with 100 nm silver film. It is evident from the figure that there exists enhanced local field distribution around the nanosphere. In order to achieve maximum enhancement from this substrate, we have to use a fluorescent dye which has an excitation wavelength close to 480 nm as predicted from simulation results.

The substrates were fabricated by forming a monolayer of PS on the glass substrate using 400 nm PS. A 2D monolayer formation was achieved by optimizing the PS suspensions, SDS, spin speed and spin duration. Large surface areas with only few defects such as 'line defects' and 'vacancies' were easily achieved as shown in Fig. 4a. A closer inspection of the monolayer reveals that the spheres are very closely packed with prefect hexagonal structure

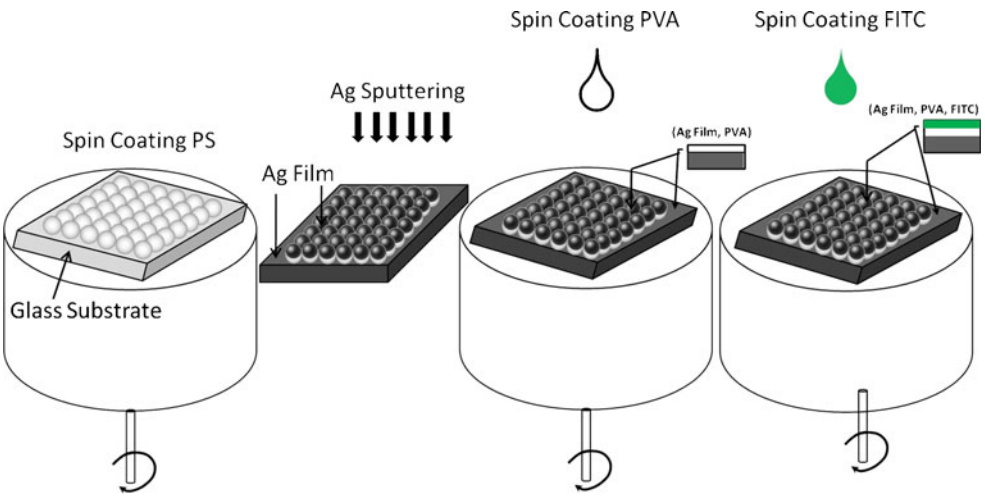

Fig. 2 This schematic illustrates the step by step process of fabricating the substrate for MEF. The PS nanospheres are first spin coated to form a monolayer followed by deposition of silver film. To prevent quenching of fluorescence, a 40 nm PVA spacer layer is spin coated prior to coating FITC

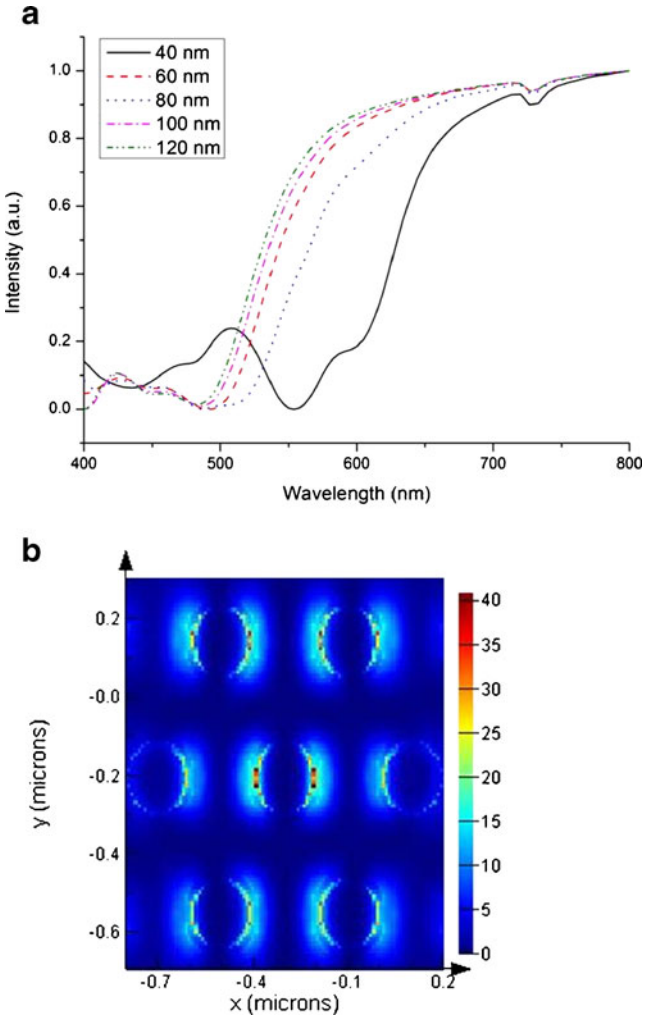

Fig. 3 **a** Reflection spectrum obtained from the substrate for film thickness varied from 40 nm to 120 nm. The dip in the spectrum corresponds to the plasmon resonance wavelength. **b** Local field distribution corresponding to one hexagonal structure on the nanosphere substrate clearly shows the enhanced field

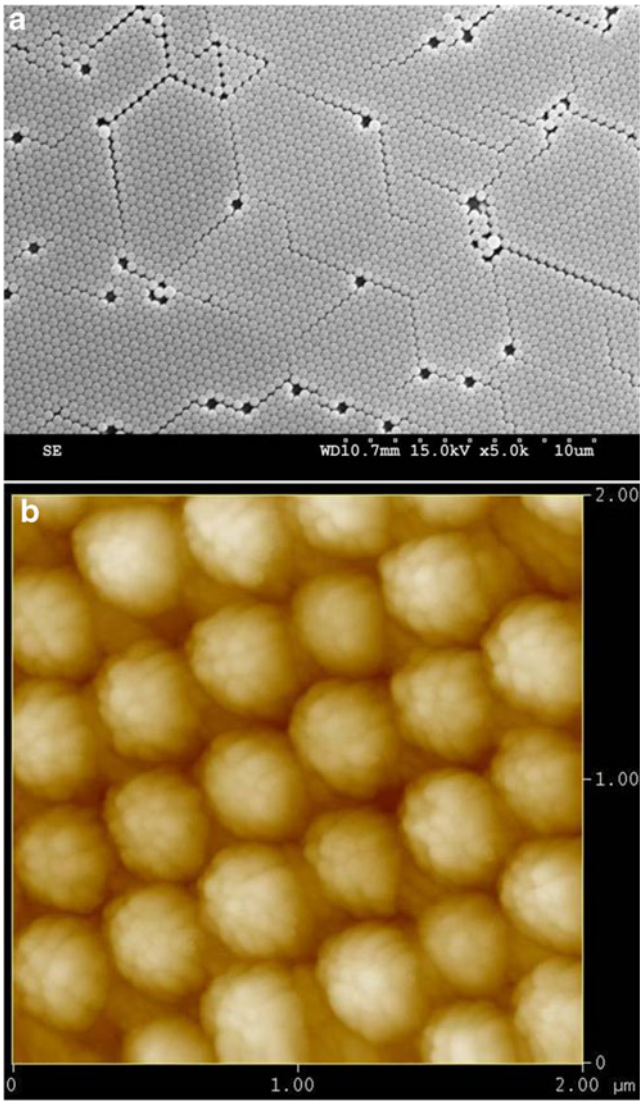

Fig. 4 **a** SEM image showing the close packing and the defects in monolayer of 400 nm nanospheres. **b** AFM image showing the surface roughness on the nanospheres after coating with 100 nm silver

forming 2D monolayer which can be considered as a 2D surface grating. The 2D surface gratings obtained from the closely packed nanospheres are one of the ways to provide the additional momentum for coupling incident photons into surface plasmon excitations. This substrate enables excitation of surface plasmons over large areas via top side illumination using an upright fluorescence microscope. Based on the simulation results, 100 nm of silver was sputtered onto the monolayer surface. The general variation in overall height profile characteristics and surface roughness of the silver substrate was determined using the AFM. An average roughness of 35.9 nm was obtained for 2 μm^2 area. Figure 4b clearly shows the surface roughness on each sphere after coating with 100 nm of silver.

The plasmon resonance for non-transparent samples can be associated with the minimum reflectivity [22]. The optical characterization in the reflection mode of the substrate was carried out to determine its plasmon resonance wavelength. The plasmon resonance wavelength for nanospheres coated with 100 nm silver was found to be at around 480 nm which matches with the simulation results. Figure 5 shows the simulated and experimental reflectance spectra of the substrate under normal incidence. FITC has been used as the fluorescent dye as it has an excitation wavelength at 488 nm which closely matches with the plasmon resonance wavelength of the substrate. It is also widely used in medical imaging and sensing applications.

Fluorophores within a few nanometers from a metal surface severely quenches the fluorescence [23]. In particular, noble metal particles with characteristic absorption in the UV–Vis, coupling with the absorption spectrum of the fluorophore leads to severe quenching. To prevent this,

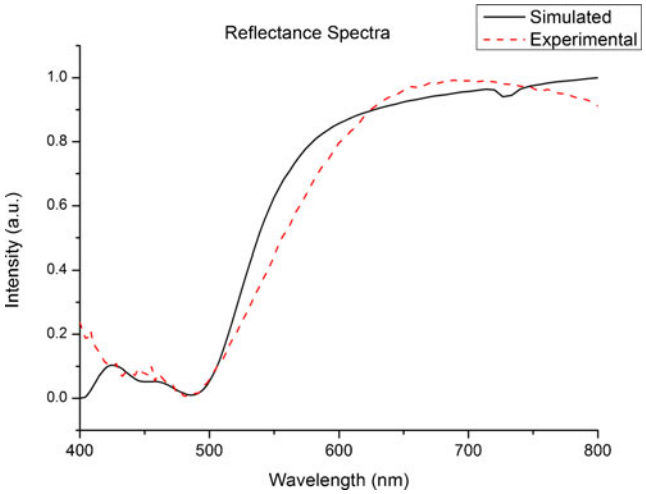

Fig. 5 Simulated and experimental reflectivity spectrum of the nanosphere surface coated with 100 nm silver with a plasmon resonance wavelength at around 480 nm

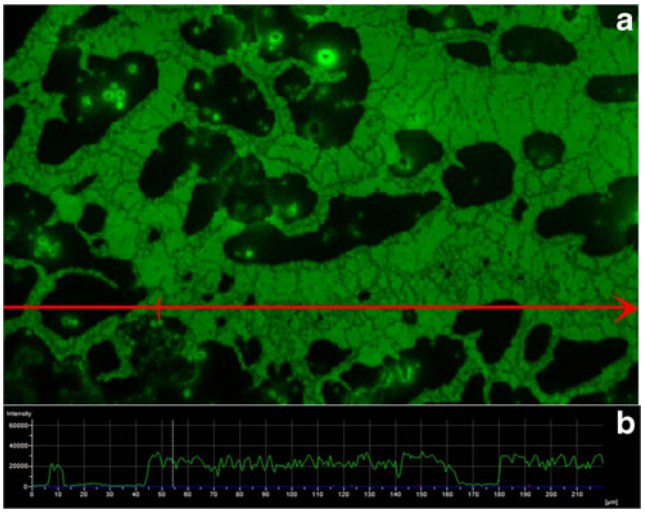

Fig. 6 **a** Fluorescence image with 10× magnification and 500 ms exposure time. **b** The fluorescence intensity profile showing an increase in intensity on the nanosphere templated 2D metallic grating surface as compared to the bare film

PVA was spin coated to form a 40 nm film which acts as a spacer layer. The optimization of spacer layer for exciting the fluorophores in the near field is still a challenge for achieving maximum enhancement [24]. After spin coating the fluorescent layer (FITC), the substrates were observed using an automated upright fluorescence microscope.

In analyzing the fluorescence images, we adopt the common definition for calculating the enhancement [25]

$$R = \frac{I_n - I_B}{I_f - I_B}$$

Here, I_n is the raw fluorescence intensity measured on top of nanosphere array, I_f is the fluorescence intensity measured from the silver film, which is void of the nanosphere array, and I_B is the background fluorescence of silver-coated nanosphere. The intensity ratio R, describes the fluorescence enhancement due to the silver coated 2D array of nanospheres.

The fluorescence image in Fig. 6a captured with 10× magnification and 500 ms exposure time clearly shows the enhancement of fluorescence from the regions of silver coated monolayers of PS. The intensity profile along the red line indicates the rise in intensity due to the presence of nanosphere coated with silver as shown in Fig. 6b. Compared to silver film, a twelve fold increase in intensity from FITC was achieved.

The close packing and the periodicity of the nanospheres greatly contributes to the enhancement mainly in two ways: (a) the uniform array of silver coated nanospheres produce narrower resonances; this allows better control of the absorption spectrum and an optimization of spectral overlap between the particle plasmon resonances and the fluorescent molecules, (b) the interstices formed by the intersection of nanospheres create hotspots where the plasmonic activity is high which also contributes significantly to the fluorescence enhancement. The intensity profile in Fig. 6b shows some sharp dips, which are due to the defects or imperfections on the substrate. These defects are usually more than the diameter of the nanosphere. Surface area of 5–10 μm^2 with sub-wavelength surface roughness which is devoid of defects is ideal to achieve MEF using this method. The advantage of this method over other NSL based substrates is that we get a large surface area which can be readily used for enhancing fluorescence of the whole field from any bio-chemical sample by just illuminating normal to the substrate with a broad band source filtered at the excitation wavelength of the sample.

Though the fabrication of the metal film over nanosphere is well known and is extensively used in SERS, such substrates have seldom been used for enhancing fluorescence. In short, our substrate acts like a 2D metallic grating or 2D plasmonic crystal, because of the uniformity and periodicity of the patterns. Hence, the observed enhanced fluorescence emission could be attributed to the local field enhancement in addition to excitation of surface plasmon polaritons along the grating surface. The fabrication of such substrates are less expensive, consumes less time and are highly reproducible as compared to other reported methods of fabrication.

Conclusions

In conclusion, we have simulated the nanosphere substrate using FDTD to determine the plasmon resonance wavelength and map the local field distribution. Closely packed

2D array of PS nanospheres were used to fabricate metallic grating like substrate which can be an ideal substrate for metal enhanced fluorescence. Twelve times increase in fluorescence intensity was obtained from silver coated nanospheres as compared to regions with silver coating alone. The application of the nanosphere based substrate for metal enhanced fluorescence presented here is straight forward, requiring no special hardware other than the commonly available standard fluorescence microscope. Proposed method of fluorescence enhancement is readily applicable in molecular biology and materials science for imaging.

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