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Fabrication and Characterization of Planar Plasmonic Substrates with High Fluorescence Enhancement

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Abstract

The use of plasmonic nanostructures for fluorescence signal amplification is currently a very active research field. The detection of submonolayers of proteins labeled with organic dyes is a widely used technique in surface-based immunoassays and DNA hybridization. There is a strong interest in the development of new optical and chemical methods to increase the signal from ultralow concentrations of dyes on the surface of sensor substrates. Herein, we have explored the possibility of using vacuum-deposited silver nanostructures on dielectric layers and silver mirrors as potential plasmonic substrates that effectively amplify fluorescence over a broad spectral range. By optimizing deposition parameters for dielectric layers and silver nanostructures and applying thermal annealing processes, we observed large fluorescence amplifications from three different dye-strept(avidin) conjugates: about 7-fold for a UV/blue dye AF350-Av, 49-fold for a blue-green dye AF488-SA, and up to 208-fold for red-emitting AF647-SA dye. The observed amplification factors for the ensemble of fluorophores are very promising for development of surface-based bioassays. These substrates can be prepared using simple vacuum deposition in which we circumvent using the expensive nanofabrication methods. In addition, unlike most nanofabrication methods, the present approach is appropriate for large scale fabrication of substrates with microscope slide surface area suitable for sensing applications.

Introduction

Metallic nanostructures of gold, silver and aluminum have been recently explored as promising materials for signal amplification of fluorescence from UV to NIR wavelengths. The plasmonic properties of metallic nanostructures (scattering, absorption, and creation of enhanced local electromagnetic fields) and their ability to enhance the fluorescence are highly dependent on the nanoparticle composition, size, shape, and surface density and the dielectric properties of the substrate and medium above the surface.¹ Many kinds of nanoparticle geometrical configurations and nanofabrication methods have been used to fabricate these metallic nanostructures that modify the excitation and emission processes and lead to enhanced fluorescence. A few of the most notable methods for nanostructure fabrications include using electrochemistry,^{2,3} chemical deposition,^{4,5} electron beam lithography,^{6–8} and nanosphere lithography.⁹ To maximize the enhanced signal from the nanoparticles, in some instances, a combination of solid films with nanoparticles was also used.^{10–12} Several excellent review articles describing the studies on the effect of metallic nanostructures on fluorescence were published previously.^{13–16}

Although several groups have demonstrated the potential of metallic nanostructures for signal amplification applications, the process of their fabrication remains difficult; it also is hard to obtain consistent enhancements. Consequently, the reported results widely differ in the factors of fluorescence enhancement, from fractional (quenching) to large amplification of above 100-fold. This is largely due to small changes in the particles' plasmonic features that result from the minute variations in the size, shape, composition, environment, and geometry of the composites used in the study. The size of the nanoparticles has a large effect on the enhanced intensity. For example, Au nanoparticles with 12 nm diameter displayed a quenching effect (reduced quantum yield and reduced lifetime) on Cy5 emission at distances from 2 to 16 nm from the particle surface.¹⁷ On the other hand, the Ag and Au nanoparticles with 80 nm particle diameter resulted in emission enhancement of AlexaFluor488 and Nile Blue by ~14- and ~8.5-fold, respectively.¹⁸ Even larger enhancements were obtained for Indocyanine Green (50-fold) using gold nanoshells that have varied composition and a large scattering cross section for which the plasmon spectra overlapped with the emission spectrum of the dye.¹⁹ In a recent study using silver nanoprisms and several dyes,²⁰ strong correlations between fluorescence enhancement and size of the nanoparticles and, hence, the localized surface plasmon spectra were also reported. Recent advances of using plasmonic nanostructures for improved photovoltaic devices²¹ provide additional insights for design of planar substrates that can be applied for highly efficient fluorescence enhancement. Although numerous fabrication methods are currently available, approaches for more consistent and reproducible metallic substrates with large surface area, suitable for bioassays or ensemble sensing applications, are still to be developed.

Accordingly, fabrication of metallic nanostructures or a robust nanofabrication procedure with reproducible plasmonic properties is one of the important aspects in surface-enhanced fluorescence. In this paper, we describe a simple method for the fabrication of nanostructure composites that display up to 210-fold fluorescence enhancement using a simple vacuum deposition process. First, we tuned the deposition effective thickness of Ag nanostructures, followed by thermal annealing. Second, we fabricated multilayered plasmonic substrates consisting of a silver mirror, dielectric layer, and silver nanostructures. The performances of substrates were evaluated using a broad spectral range from UV to NIR wavelength range.

Experimental Methods

Materials

Silicon monoxide and silver wire (99.999%) were purchased from Aldrich. Streptavidin (SA) or avidin (Av) conjugated dyes, Alexa Fluor 350 (AF350-Av), Alexa Fluor 488 (AF488-SA), and Alexa Fluor 647 (AF647-SA) were purchased from Invitrogen (Carlsbad, CA). Phosphate buffer saline (PBS) pH 7.4 and biotinylated bovine serum albumin (BSA-Bt) were from Sigma-Aldrich. Ultrapure water (>18.0 M Ω) purified using a Millipore Milli-Q gradient system was used in preparation of buffers and aqueous solutions. Glass microscope slides were purchased from VWR.

MEF Substrate Preparation

Glass slides were cleaned with "piranha solution" (35% H₂O₂/H₂SO₄, 1:3) overnight, rinsed with distilled deionized water, and dried with nitrogen before thermal vacuum deposition steps. Metallic and dielectric layers were deposited by thermal evaporation (Edward, model 306) or magnetron sputtering (AJA model ATC 1800-V). For thermal deposition, chromium (adhesion layer) and silver (mirror and outer layer) were evaporated from tungsten boats at 2×10^{-7} Torr and silicon monoxide at 5×10^{-6} Torr with a deposition rate of ~1.0 nm/min. After coating with silicon monoxide (or silicon dioxide), slides were silanized by immersion in a water solution of 1% of aminopropyl trimethoxysilane (APS), for 30 min. The silanized

slides were dried in air and used for deposition of a final thin layer of Ag followed by thermal annealing in air at various temperatures and various annealing times.

Protein-Dye Immobilization

The surfaces of annealed and nonannealed slides were covered with a self-adhesive silicone/rubber of thickness of 2 mm with wells of 2.5 mm diameter. First, the BSA-Bt solution (100 $\mu\text{g/mL}$) in sodium phosphate buffer (50 mM, pH, 7.2) was added into the wells (10 μL) and incubated for 1 h. This step facilitated a monolayer of BSA-Bt that provided the mean for immobilization of streptavidin–dye conjugates. The same procedure was used for preparation of control samples using bare glass slides. After incubation with dye–streptavidin conjugates (25 $\mu\text{g/mL}$), the wells were washed with PBS buffer to remove unbound dye streptavidin conjugates. Finally, the wells were filled with PBS and covered with a microscope coverslip for spectroscopic measurements. The schemes of multilayer substrates with immobilized dye–streptavidin conjugates are shown in Figure 1.

Spectroscopic Measurements

Absorption spectra were acquired with a Hewlett-Packard 8453 spectrophotometer. For baseline corrections, bare glass substrate and SiO_x coated glass slides were used. Steady-state intensities were measured on the multilayer substrates and compared with the signal of the respective samples on bare glass. Fluorescence enhancement was determined as the intensity ratio of the fluorescence signal measured on the multilayer substrate divided by the signal in respective reference sample on bare glass using identical experimental conditions. Fluorescence from surfaces was measured with epi-fluorescence configuration (see Figure 1) using a fluorescence microscope (Axiovert 135TV, Zeiss) with a 10 $\times$, NA 0.30 objective (UPlanFI, Olympus). The excitations were provided using UV LED (Nichia NSHU590E) with a peak wavelength at 374 nm, blue LED (Nichia NSPB500S) with a peak wavelength at 467 nm, and red LED (Nichia NSPR510CS) at 625 nm and emission observed at band-pass filters of 460/50 nm (AF350-Av), 535/50 nm (AF488-SA), and long pass filter above 655 nm (AF647-SA). Time-resolved data were measured using a phase-modulation fluorometer (K2 from ISS, Champagne, IL). The LEDs were modulated by applying a RF driving signal from a Marconi model 2022A frequency synthesizer (from Marconi Instruments, Allendale, NJ) to the LED.^{22,23} A more detailed description of the phase-modulation technique can be found elsewhere.²⁴ The fluorescence decays were analyzed using least-squares fit to multiexponential model

$$I(t) = \sum_{i=1}^n \alpha_i \exp(-t/\tau_i) \quad (1)$$

where α_i ($\sum \alpha_i = 1.0$) and τ_i are the amplitude and decay time, respectively. The average, amplitude-weighted lifetime $\langle \tau \rangle$, the value of which is proportional to the area under the normalized intensity decay, was calculated as

$$\langle \tau \rangle = \sum_{i=1}^n \alpha_i \tau_i \quad (2)$$

To correlate the intensity enhancements with lifetime changes, we measured the phase shifts at a single modulation frequency. The measured phase shift (ϕ) is related to the phase lifetime (τ_ϕ) as

$$\varphi = \tan^{-1}(\omega\tau_{\varphi}) \quad (3)$$

where ω is the circular frequency. In the case of single exponential intensity decay, the phase lifetime is equal to the fluorescence lifetime, irrespective of modulation frequency. For multiexponential intensity decay, the phase lifetime is dependent on the modulation frequency and composition of decay times.²⁴ Phase shift measurements at a single modulation frequency allow acquiring information on lifetime changes without extensive data acquisition and performing analytical computations. A single modulation frequency was used for sample comparisons: 100 MHz for AF488-SA and 130 MHz for AF647-SA.

Scanning electron microscopy (SEM) images were collected with a Hitachi SU-70 SEM instrument, and surface morphologies were studied using an atomic force microscope (AFM), model D3000 (from Digital Instruments, Inc.).

Results and Discussion

SEM and Absorbance Measurements

A set of silver films with nominal thicknesses 9, 15, 20, 23, 30, and 36 nm were prepared by thermal evaporation of silver (Ag) onto silanized SiO_x coated microscope slides. Part of the slide from each set was annealed for 3 h at 230 °C in air. SEM images and absorption spectra of annealed and nonannealed films are shown in Figures 2 and 3. The measurements of absorption spectra were performed for surfaces wetted with phosphate buffer, pH 7.2.

Thin, silver nanostructures with deposition thickness of 9 nm result in fine nanoparticles (with average particle size of ~10 nm) that display a well-defined surface plasmon absorption spectrum with peak maximum at 497 nm and with an extended absorption tail toward long wavelengths (Figure 2, top). The annealing process of the above Ag film changes the morphology of nanoparticles markedly. The size of nanoparticles increased to an average particle size of ~15 nm. It is also seen from the SEM image that the interparticle spacing also increased. The resulting surface plasmon absorption spectrum displays a sharp peak at 477 nm with significant reduction in the bandwidth.

Unlike with the 9 nm silver layer, the 15 and 23 nm thick films of silver show interconnected Ag nanostructures with heterogeneous shapes and sizes, including some short rods/wires. The absorption spectra were very broad without defined absorption peaks. However, when heated, the interconnections between Ag nanostructures break up, and isolated nanoparticles of various sizes and shapes have formed (Figures 2 and 3). The plurality of particle sizes, shapes, and interactions between particles resulted in a broad surface plasmon spectrum that is well extended to the long wavelengths. The trend in surface morphology changes due to heating for films with 20 (not shown) and 23 nm deposition thicknesses are similar to that of 15 nm; the larger the film thickness, the larger the particle size and broader the surface plasmon absorption spectrum (peak at 520–550 nm).

Deposition of Ag films with thicknesses of 30 and 36 nm resulted in practically continuous Ag films with small defects (Figure 3, not annealed). When heated, the mirror-like continuous films have been efficiently broken into large Ag islands/ nanoparticles, many of them large, rodlike particles. The absorption spectrum for 30 nm annealed film still displays the peak (very broad from about 640 to 740 nm), but for the film with a thickness of 36 nm, no such wavelength-dependent absorption peak was observed, even after annealing. It is interesting to note that the absorption is significant at short wavelength, despite the lack of

small nanoparticles on the surface, as seen from the SEM images, which are likely due to interactions between nanoparticles and possibly the transversal effects of elongated particles.

Generally, the absorption spectra of annealed Ag nanostructures with deposition thicknesses of less than ~25 nm are blue-shifted and with narrower bandwidth, which is in agreement with other reports.^{25,26} However, unlike in the other report,²⁶ the optical densities increased markedly upon the heating.

The above measurements using SEM imaging and UV/vis/NIR absorption spectroscopy reveal the relation between the morphology of Ag nanostructures deposited as thin nanostructured (thickness less than ~25 nm) or continuous films (thickness above ~30 nm) and the plasmon absorption spectra. The important fact is that the absorption spectra can be very informative of the surface morphology and changes after annealing process and guide toward applications using enhanced fluorescence.

In addition to the SEM imaging that gives the information on the lateral dimensions of the Ag nanostructures, we used atomic force microscope (AFM) for revealing information on the axial dimensions of nanostructures. We chose the Ag nanostructures with a deposition thickness of 20 nm, which we more extensively evaluated using mirrored substrates with various thicknesses of dielectric spacer layers in a broad spectral range. Figure 4 shows the absorbance spectra of annealed and nonannealed Ag layers of 20 nm effective thicknesses on the glass substrate, which were fabricated simultaneously with Ag mirrored substrates coated with dielectric layers. AFM images show that the annealing process results in substantial changes in the surface morphology, both lateral and axial dimensions of Ag nanostructures (Figure 4, inserts). The line profiles across the images indicate that smaller silver nanostructures are formed during the vacuum deposition, with an average height close to the effective deposition thickness of 20 nm. After annealing, the height of the particles increased up to ~60 nm and the lateral size also increased up to ~150 nm. Figure 4 shows the respective absorption spectra of nonannealed and annealed substrates and three spectral windows used for evaluation of fluorescence enhancements. The selected spectral windows overlap with the plasmon spectrum over a broad range of wavelengths representative of the fluorescent dyes commonly used in biotechnology applications.

Fluorescence Measurements

Effects of the above fabricated Ag nanostructures on the fluorescence were studied using three fluorophores (AF350, AF488, and AF647). The selected fluorophores have distinct spectral ranges representing coumarins (UV-blue), fluoresceins (blue-green), and cyanines (red), respectively. The selected dye-strept(avidin) conjugates display similar quantum yields: 0.55 (AF350-Av), 0.42 (AF488-SA), and 0.33 (AF647-SA).^{27,28} Similar quantum yields of fluorophores allow us more properly to characterize the plasmonic effects of Ag nanostructures on fluorescence enhancement over the broad spectral range. It is known that low quantum yield fluorophores undergo larger fluorescence enhancements compared with that of high quantum yield.⁵ Additional advantages of using Alexa Fluors with plasmonic nanostructures are their better photostabilities and less self-quenching when labeled with proteins compared with conventional dyes.^{27,28} The avidin and streptavidin conjugated dyes were immobilized on substrates that were precoated with BSA-Bt. The binding interaction between streptavidin and biotin is very strong and results in a stable monolayer of dye-streptavidin over the BSA. The layer of BSA-Bt serves also as a separation layer between the fluorophores and the silver surface. The average distance between fluorophores (bound to SA) and the surface is ~6–7 nm. This separation prevents fluorescence quenching when the dye is in direct contact with the metallic surface or in close proximity to the surface where the quenching effects are more dominant over the enhancement effects. All

investigated substrates, including control bare glass slides, were treated with the same protein concentrations to facilitate a direct comparison of fluorescence signals.

First, we investigated the effect of thickness of deposited Ag nanostructured film without and with thermal annealing. Figure 5 shows fluorescence enhancements for AF488-SA and AF647-SA on nonannealed and annealed glass substrates with various thicknesses of deposited Ag films. The most effective signal amplification was observed for Ag deposition, with the thicknesses ranging from 15 to 23 nm, with similar trends for blue/ green (AF488-SA) and red range (AF647-SA). It is interesting to note that the fluorescence enhancements for annealed Ag films significantly increase for AF488-SA, whereas the corresponding enhancements for AF647-SA are similar or slightly lower as compared with those on the nonannealed substrates. The observed fluorescence enhancements can be explained by close examination of the plasmon absorption spectra of the Ag nanostructures (Figures 2–4). The annealing process causes the Ag nanostructures to display stronger surface plasmon resonance in the blue-green range (450–550 nm); therefore, fluorescence enhancement observed for AF488-SA increased about 1.7-fold. This is because of favorable matching between the excitation wavelength, fluorophore emission spectrum, and silver nanostructure plasmon spectra (Figure 4).

The explanation of the difference between fluorescence enhancements for nonannealed and annealed Ag nanostructures in the red range (AF647-SA) is less clear than for AF488-SA. At lower Ag thicknesses (up to 23 nm), the deposited nanostructures display more favorable plasmonic properties for fluorescence enhancement in the red range than those after heating. However, for larger Ag thicknesses (above 23 nm), the annealing process improves the enhancement. These observations can be explained by considering the changes in plasmon spectra in the range of 600–750 nm before and after heating. A common observation is that absorbance values decrease in the red range after heating, regardless of the Ag nanostructure thickness. In the presence of heterogeneous and partially aggregated metallic nanostructures, one needs to consider the nature of the resonance plasmon spectrum. The extinction spectrum of the silver nanostructure consists of absorption and scattering components. The annealing process substantially changes the morphology of the Ag nanostructures and, therefore, the extent of absorptive and scattering contributions of the plasmon resonance spectra. Before annealing, the high absorbance is present at longer wavelengths; however, a substantial part of the absorbance values originates from the absorptive properties of mirrorlike Ag nanostructures. After annealing the substrates, we observed substantially smaller extinction values at longer wavelengths that predominantly originate from highly scattering properties of particle plasmons. The observed fluorescence enhancement for AF647-SA likely is due to the comparable balance between the spectral overlap (decreased after heating) and the scattering properties of annealed particles (increased) compared with that of the nonannealed Ag films.

The observed fluorescence enhancements for AF488-SA and AF647-SA on annealed and nonannealed substrates indicate that the simple correlation of spectral overlap between fluorophore and plasmon spectra may not always be appropriate. Although the excellent correlation between localized surface plasmon resonances of the single silver nanoparticles with the spectral properties of immobilized dyes were reported,^{19,20} a similar interpretation of fluorescence enhancements by regular particle arrays on planar surfaces is less clean.^{7,8} It should be noted that particle surface plasmon spectra are far-field observations and do not clearly reveal the near-field electromagnetic intensity, which is primarily responsible for observed fluorescence enhancements.

Overall, it is evident that the deposition of a Ag nanostructured film with an effective thicknesses from 15 to 23 nm results in the best fluorescence enhancement for AF488-SA as

well as for AF647-SA. The changes in the Ag nanostructure morphology induced by thermal annealing maximize the fluorescence enhancement for fluorescence in the blue-green range but have small (~25%) adverse effect for fluorescence enhancement in the red range.

Optimization of Annealing Process

Because the annealing process has a significant effect on the Ag film morphology (Figures 2–4) and on the overall fluorescence enhancements (Figure 5, top), we investigated the effect of annealing temperature and annealing time on the performance of Ag film-based substrates. For this study, silver films with a thickness of 30 nm were deposited on the glass substrates having a 25 nm SiO_x under layer and annealed at various temperatures for a fixed time (2 h). We selected a Ag thickness of 30 nm to possibly maximize the annealing effect on the fluorescence enhancement. Note that this Ag thickness is not optimal for enhancement (see Figure 5). The second set of substrates was annealed for different times at a fixed temperature (180 °C). The fluorescence enhancements for annealed and nonannealed films are shown in Figure 6. From these studies, we can conclude that the optimal annealing temperature is in a broad range from about 100 to 200 °C (Figure 6, bottom), and sufficient annealing time is ~60 min (Figure 6, top).

Fluorescence Enhancements Using Mirrored Substrates

The above measurements were performed with the epifluorescence optical configuration, in which the excitation and emission observation are achieved at the same side of the substrate, that is, above the protein layers (Figure 1). For the substrates with Ag nanoparticles on the glass, we found that excitation and observation at the other side of the glass (opposite to the protein–fluorophore layers) resulted in fluorescence signals comparable to those for direct excitation/observation conditions. This implies that for direct excitation/emission configuration, a significant amount of fluorescence is coupled into a glass substrate and radiated in the opposite direction to the detector. This is because the underlayer of glass has a higher permittivity than the aqueous solution above the silver nanostructures, and highly efficient scattering occurs into a glass substrate.²⁹ Implying a proper mirror and layer of dielectric will redirect the scattered light toward the observation path. The scattered light trapped in dielectric layer is reflected by the silver mirror which efficiently increases the excitation and redirect fluorescence into the observation path. The process of reflections within a dielectric layer will occur multiple times since the outer layer of silver nanostructures is semitransparent.

In design of plasmonic substrate for efficient fluorescence enhancement one need consider the interferences between incident and reflected excitation waves as well as interferences between fluorescence signals. Such interference effects have been studied using silver mirrors and variable thickness of alumina in the visible wavelength range.³⁰ The attempts of using semitransparent mirrors (50 nm thick) and silver islands separated by thin layer of silica of 2 nm thickness has been also reported.¹⁰ However, there were no considerations given to plasmon spectra of silver nanostructures, reflecting properties of Ag mirror and optimal thickness of dielectric layer.

First, we prepared glass slides with an adhesion layer of chromium (15 nm), a silver mirror layer (300 nm), and seven various thicknesses of silicon monoxide (SiO_x, from 5 to 150 nm) using the thermal vacuum deposition system (Edwards 306). We also prepared a second set of substrates with the same parameters for layer thicknesses using a magnetron sputtering system (AJA ATC 1800-V) with SiO₂ as th dielectric layers. The outer Ag nanostructured film thickness was 20 nm and achieved with the Edwards 306 system, the properties of which are shown in Figure 4.

Figures 7 and 8 show the fluorescence enhancements for three dyes, AF350-Av, AF488-SA, and AF647-SA on annealed and nonannealed mirrored substrates. The observed enhancements in fluorescence are dramatic, over 200-fold (AF647-SA). The enhancements strongly depend on several parameters: (1) the fluorophore excitation/emission spectral range, (2) the dielectric spacer layer between the mirror and the Ag outer film, and (3) annealing. The largest enhancement, ~208-fold, was obtained for AF647-SA on the annealed substrate with a dielectric spacer of 75 nm. To the best of our knowledge, such large fluorescence enhancements for an ensemble of surface bound molecules are observed for the first time using single photon excitation. Recently, a fluorescence enhancement of ~200-fold on colloidal silver nanoparticles was reported; however, only for selected “hot spots” on a gold mirror.¹² It is important to note that AF647-SA is one of the brightest organic fluorophores with a quantum efficiency better than 0.30 and high extinction coefficient of $239\,000\text{ M}^{-1}\text{cm}^{-1}$, which is spectrally equivalent to widely used Cy 5 fluorophore for biomolecule labeling.

The maximum enhancements observed for AF-488-SA and AF350-Av were markedly lower than for AF647-SA, of 49- and about 7-folds, respectively. However, the enhancement of 49-fold for AF488-SA can be regarded as one of the highest for the blue/green spectral range, compared with those previously reported.^{10,18,20} The lower fluorescence enhancements for the UV/blue spectral range for AF350-Av might be surprising in view of good spectral overlap between surface plasmons and excitation/emission spectra of AF350-Av. However, one should consider that the scattering contributions of Ag nanostructures in this wavelength range (350–450 nm, Figure 4) are much lower than the absorptive (quenching) contributions. This is an unlikely situation with the visible and NIR spectral range (above 500 nm, Figure 4).

Remarkably, the maximum of fluorescence enhancements is observed for a broad range of dielectric (silicon monoxide or dioxide) layer thicknesses. One would expect that the enhancements would appear at well-defined dielectric thicknesses, as was observed for mirrors.³⁰ The lack of a distinct enhancement peak is likely due to Ag nanostructures of the outer layer and multiple reflections of scattered light within the dielectric layer. The excitation in normal incidence to the surface (epifluorescence with low NA) is expected to create an excitation field parallel to the surface in the absence of scattering element. However, the presence of Ag nanostructures on the surface allows efficient excitation of fluorophores with transition dipoles perpendicular to the plane of the substrate surface. Because the emission from parallel and perpendicular orientations of the fluorophore are most effectively enhanced at distances that differ by about a quarter of the emission wavelength,³⁰ thus, the observed enhancement does not display a sharp dependence on the thickness of the dielectric layer. However, one can easily notice that there is a tendency of maximum enhancement with the spectral properties of fluorophores. For example, a maximum enhancement for AF350-Av is observed at dielectric thicknesses of ~40 nm for AF488-SA at 50–60 nm and for AF647-SA at ~75 nm. The reported optimal thickness for the Ag film and alumina layer ($n = 1.65$) is 58 nm for Cy3 (excitation 532 nm and emission 570 nm),³⁰ which corresponds well to our results for AF488-SA.

The lack of sharp layer-thickness-dependent enhancements is advantageous for the applications of our Ag film/dielectric substrates. This is because it allows relaxed conditions for reproducible fabrication. In addition, it allows use of the same substrate for a broad range of fluorophores, an important aspect for multiplexing approaches. Our results show that substrates fabricated using two different vacuum deposition systems, thermal and magnetron sputtering, resulted in a very similar performance. We believe that our approach will find immediate applications in surface-based sensor designs because of easy fabrication and the availability of a broad range of fluorescent probes for various biotechnological applications.

One can assume that the magnitude of fluorescence enhancements includes an effect of a larger amount of fluorophores on silver nanostructures compared with the planar glass. We estimated the excess of the surface area due to Ag nanostructures of less than 25% compared with the planar glass. We assumed an array of semispherical particles with a diameter of 40 nm and spacing (side-to-side) of 40 nm and found that particles will increase the total surface area by about 21%. Therefore, the observed large fluorescence enhancements are due to surface-enhanced phenomena and not the differences in the surface concentration of bound dye–streptavidin conjugates. In view of sensing applications, the increase in the sensing active area is desirable for improved sensitivity.

Fluorescence Lifetime

In metal-enhanced fluorescence, the fluorescence enhancement is accompanied by a decrease in the fluorescence lifetime due to the modified radiative rate of the fluorophore.^{5,12,31} Accordingly, the lifetime measurements provide valuable information about the mechanism of the enhancement. For example, if the intensity is enhanced but the lifetime is not changed, this means that the mechanism of intensity enhancement is due to an enhanced excitation intensity or an increase in the fluorophore concentration. However, if the fluorescence enhancement is accompanied by a lifetime decrease, the effect of the surface plasmons on the decay rates of the fluorophore needs to be considered. We used phase-modulation fluorometry to acquire data on lifetime changes at the same time as steady-state intensity measurements. The typical intensity decays using phase-modulation fluorometry are shown in Figure 9, in which the intensity decays of AF647-SA are shown in various environments. The average lifetime of AF647-SA in buffer solution is ~1.7 ns. When AF647-SA is bound to a glass surface with immobilized BSA-Bt, the lifetime is reduced (due to a higher refractive index near the glass and some quenching effects of biotin binding to streptavidin) to about 0.7 ns. The corresponding lifetime on the surface with silver nanostructures decreases to ~40 ps, which is on the lower time limit of excitation/detection of our LED/PMT system. Thus, because of the interaction of the excited fluorophore with the surface plasmons, AF647-SA displays an 18-fold shorter lifetime compared with that bound to the glass substrate and 41.5-fold compared with that in buffer solution.

The phase modulation technique provides a convenient way to observe the lifetime changes using a single modulation frequency in which the changes in phase shift and modulation allow the lifetime changes to be rapidly monitored, which can be very useful in the design of surface-based immunoassays.³² The shorter the lifetime, the smaller the phase shift and the larger the modulation values. We used a single modulation frequency for measurements of phase shifts and modulations for all samples in various configurations. We found that increases in fluorescence enhancements for all fluorophores were accompanied by decreases in the lifetime (decreases in phase shifts and increases in modulations).

Figure 10 shows the changes in phase shift (phase lifetime) for AF488-SA and AF647-SA on mirrored substrates with an annealed 20 nm Ag layer with various dielectric thicknesses. Decreases in the phase shifts for both fluorophores are very large compared with that on bare glass or in buffer solution. As can be seen from the data of phase changes in Figure 10 and fluorescence enhancements shown in Figure 8, there is a good correlation between an increase in fluorescence enhancement and a decrease in phase shift (decrease in lifetime). This indicates that the Ag mirror and dielectric layer play an active role in enhancing the fluorescence that also includes increasing the radiative decay rate.⁵ The increase in the radiative decay rate causes the quantum yield of fluorophore to increase; in our cases, up to 2–3 fold.

The intensity and lifetime data illustrate that fluorescence enhancement on multilayer substrates is due to several effects, including the reflective properties of mirrors with an optimal dielectric thickness layer, the surface plasmons of the outer layer of Ag nanostructures, and the increased surface area for protein binding. The important fact is that fluorescence enhancement due to Ag nanostructures strongly depends on the spectral range. Despite the three dye–streptavidin conjugates' displaying similar quantum yields, the fluorescence enhancements are markedly different. This suggests that the far-red (AF647) and possibly infrared wavelength ranges are the most promising for fluorescence enhancement with Ag nanostructures. The proper combination of layers and the annealing process provides a convenient method for the fabrication of planar substrates suitable for fluorescence enhancements over 200-fold, which can be applied for the design of low-cost detection instrumentation for proteomics and genomics applications.

Conclusions

Deposition of thin silver films and subsequent thermal annealing provides a means for the fabrication of silver nanostructures that significantly amplify the fluorescence. The enhancement of fluorescence is highly dependent on the fabrication parameters and the spectral range. An average enhancement from about 50-fold in blue-green and up to 210-fold in the red range has been observed. A noted advantage of the fabrication method is its simplicity. The substrates can be deposited by a vacuum process, followed by annealing in air. In addition, because of the simplicity of the method, a large number of substrates can be produced using simple microscope glass slides that accommodate a large area for enhanced biosensing and can utilize many available fluorescent readers and fluorophores. It is expected that high reproducibility of substrates for enhanced fluorescence will be achieved with the described fabrication process. This is because the critical thicknesses of the dielectric and Ag outer layers can be deposited with high accuracy using a vacuum deposition process. The two vacuum deposition system used resulted in very similar values of fluorescence enhancements for two dyes and a broad range of dielectric thicknesses. Moreover, the optimal fluorescence enhancement occurs for a relatively broad range of dielectric as well as Ag effective thicknesses, which further assures the reproducible performance of the substrates.

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Figure 1.

Schemes of geometrical configurations of multilayered substrates with an immobilized layer of dye–streptavidin conjugate: (a) control substrate using bare glass, (b) fluorescence enhancement substrate using a silver film deposited on glass, and (c) fluorescence enhancement substrate using a silver film deposited on a silica layer and Ag mirror. The specifics about the metal and dielectric layer thicknesses are within the text. The thicknesses in the figure are in not to scale. The excitation and observed emission configuration is also depicted. Red shapes illustrate fluorophores with progressively enhanced fluorescence.

Figure 2. SEM and absorption spectra of Ag nanostructures deposited at various effective thicknesses (9, 15, and 23 nm). SEM images are at magnification of 100 K $\times$ (500 nm scale bar is shown in the middle panel). Solid line, not annealed; dashed line, annealed Ag films.

Figure 3. SEM and absorption spectra of Ag nanostructures deposited at effective thicknesses of 30 and 36 nm. SEM images are at magnification of 25 K $\times$ (1000 nm scale bar is shown below images). Solid line, nonannealed; dashed line, annealed Ag films.

Figure 4.

Absorbance spectra of annealed (175 °C, 2 h) and nonannealed Ag films on glass in buffer condition. AFM images (top, inserts) and height profiles (bottom) of the Ag film deposited on a silicon dioxide layer of 75 nm over the Ag mirror of 300 nm. Deposition of the Ag film with 20 nm thickness on bare glass and Ag mirrored substrate were performed under the same deposition conditions. The arrows indicate the excitation wavelengths (374, 467, and 625 nm) and horizontal bars, the emissions (460/50 nm, 535/50 nm, and >655 nm) for AF350-Av, A488-SA, and AF647-SA, respectively.

Figure 5. Effect of Ag film thickness on fluorescence enhancement for AF488-SA (top) and for AF647-SA (bottom) for an annealed film at 230 °C, 3 h (triangles) and nonannealed Ag films (squares). The lines are drawn for guidance.

Figure 6. Effects of Ag film annealing conditions on the fluorescence enhancements of AF488-SA and AF647-SA. The lines are drawn for guidance.

Figure 7.

Fluorescence enhancement of AF350-Av on mirrored substrates with various thicknesses of silicon dioxide and a fixed effective thickness of the Ag outer layer of 20 nm. Lines are for guidance.

Figure 8.

Fluorescence enhancement of AF488-SA (top) and AF647-SA (bottom) on mirrored substrates with various thicknesses of silicon dioxide (squares) or silicon monoxide (triangles). Annealing was at 175 °C for 1 h. Data for a thickness of zero (dashed line) indicate the results for substrates without the Ag mirror.

Figure 9.

Intensity decays of AF647-SA in various environments. The lines represent the best fit to the biexponential model. Intensity decay of enhanced fluorescence was measured for an annealed sample with a silicone dioxide thickness of 75 nm. The values of the lifetimes were calculated on the basis of eq 2. The dashed line indicates the single modulation frequency (130 MHz) for monitoring the phase changes of other samples with various thicknesses of silicon dioxide and monoxide.

Figure 10.

Phase shifts for AF488-SA (top) and AF647-SA (bottom) on mirrored substrates with an annealed Ag layer (20 nm) and various dielectric layer thicknesses. Data points are for substrates with silicon monoxide (triangles) and silicon dioxide (squares). The dashed line shows the data points for the substrate without the Ag mirror. The phase shift measurements were at a single modulation frequency: 100 MHz for AF488-SA and 130 MHz for AF647-SA.