Published in final edited form as:

Proc SPIE. 2012 February 9; 8234: 82340B-. doi:10.1117/12.924603.

Fluorescence enhancement using silver-gold nanocomposite substrates

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Abstract

Metal-enhanced fluorescence (MEF) is a newly emerging phenomenon in which the near-field interactions of fluorophores with the plasmons in metallic nanostructures can lead to substantial fluorescence enhancements. In the present study, we have investigated the use of silver-gold nanocomposite (Ag-Au-NC) structures, prepared by the galvanic replacement reaction of silver with gold, as plasmonic substrates for MEF. We have observed significant enhancement in the fluorescence intensities and decrease in the fluorescence lifetimes of two commonly used dyes, ATTO655 and Cy5, using the fabricated Ag-Au-NC substrates. Interestingly, the fluorescence enhancement depends on the amount of residual silver present in the substrates after the galvanic replacement reaction. Our results show that the galvanic replacement reaction is a very facile and powerful route to prepare Ag-Au-NC substrates that can be suitable for various MEF based applications.

Keywords

Silver-gold nanocomposites; plasmonics; metal enhanced fluorescence; galvanic replacement; plasmon controlled fluorescence

1. INTRODUCTION

Fluorescence is one of the dominant spectroscopic tools for the current research in biological and chemical sciences. Today fluorescence spectroscopy and microscopy require minimal sample volumes, often in the range of femtoliters, and extremely low concentrations of fluorophores. The fluorescence signals observed from such samples are low and may not be detectable with low quantum yield fluorophores. Remarkable progress has been made in the development of bright fluorescent probes whose optical cross sections or extinction coefficients are within a factor of two of their physical cross-sections. At present we have probably reached the practical sensitivity limit of fluorescence. This means that the amount of light absorbed by the fluorophore cannot be increased further by modification of its chemical structure. So there is a growing need to find alternative methods for improving fluorescence signals. In this regard, the coupling of fluorophores with plasmons provides us with ample opportunities. Metallic nanoparticles or nanostructures have the potential to substantially increase the local electromagnetic fields in their vicinity and this enhanced local field can result in increased rates of excitation of nearby fluorophores. More importantly, the proximity of fluorophores to metal particles can increase the radiative decay

rates of the fluorophores. The increased radiative rate results in higher quantum yields, decreased lifetimes and potentially improved photostability that can enhance the capabilities of present fluorescence methodologies [1, 2]. Hence, a lot of attention is being focused on the interactions of fluorophores with plasmonic nanostructures or nanoparticles.

The metal-enhanced fluorescence (MEF) is largely dependent on the plasmonic properties of the metallic nanostructure or nanoparticles. For example, silver, one of the most widely studied plasmonic substrates, is known to enhance the fluorescence of probes emitting in the wavelength range of 380–800 nm, while gold provides enhancement only beyond 500 nm. Below 500 nm, the inter-band transitions of gold leads to fluorescence quenching. On the other hand, aluminium nanostructures are effective for MEF in the UV region [1–3]. So in order to have the desired MEF effects, it is necessary to appropriately tune the properties of the metal nanostructures. Further, in order to be useful for practical applications, the metallic substrate should also be robust; it should have good chemical stability and should be easy to fabricate in a cost-effective manner. A large number of methods have been investigated for the fabrication of MEF substrates, starting from simple vapor deposition to more exotic nanofabrication techniques [3–5]. Bimetallic and multilayer substrates have also been studied to obtain better fluorescence enhancements [6–9]. In this work, we present the facile and convenient fabrication of silver-gold nanocomposite (Ag-Au-NC) substrates by the galvanic replacement reaction of silver by gold. Our studies indicate that these substrates are not only easy to fabricate but also provide excellent fluorescence enhancements for some of the widely used dyes such as ATTO655 and Cy5. As expected, the fluorescence enhancements are accompanied by decrease in the fluorescence lifetimes thus providing support for the fluorophore-plasmon coupling mechanism. Interestingly, the residual silver left after the galvanic replacement reaction of silver with gold is found to play an important role in the MEF effect of the Ag-Au-NC substrates.

2. EXPERIMENTAL SECTION

Materials

Silver wire (99.999%), gold(III) chloride trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$), biotinylated bovine serum albumin (BSA-Bt) and phosphate buffer (pH 7.4) were purchased from Sigma-Aldrich. The streptavidin conjugated dyes; ATTO655-SA and Cy5-SA were procured from Invitrogen. Ultrapure water (with a resistivity of $18.2 \text{ M}\Omega\text{-cm}$) purified using a Millipore Milli-Q gradient system was used in the preparation of aqueous solutions. Glass microscope slides were obtained from VWR.

Silver-gold nanocomposite substrates preparation

Glass slides were cleaned by soaking them in “piranha solution” (35% $\text{H}_2\text{O}_2/\text{H}_2\text{SO}_4$, 1:3) overnight (*Caution:* piranha solution reacts strongly with organic compounds and should be handled with extreme caution. Do not store the solution in a closed container.). Subsequently the slides were washed thoroughly with distilled deionized water and dried with air stream. Metallic layers were deposited on the cleaned slides using an Edwards Auto 306 Vacuum evaporation system under high vacuum ($< 5 \times 10^{-7}$ Torr). First, an adhesion layer of chromium was deposited on the slides, which was followed by the deposition of gold (~5 nm) and silver (~600 nm) films, without breaking vacuum. The deposition rate (~1.0 nm/min) was adjusted by the filament current and the thickness of the metal film was measured with a quartz crystal microbalance. Each metal deposited slide was cut into four equivalent smaller pieces (for convenience in the fluorescence studies) and immersed in 10 ml of 6 mM HAuCl_4 solution for the galvanic replacement reaction. The slides were removed from the solution at different times and the resulting Ag-Au-NC substrates were then washed with water, dried and used for further experiments. Scanning electron

microscope (SEM) images of these substrates were recorded with a Hitachi SU-70 SEM instrument. An Oxford Energy-dispersive X-ray spectrometer (EDS) with silicon drift detector (SSD) attached on the SEM was used for micro-chemical analysis. Surface morphologies were studied using an atomic force microscope (AFM), Witec Instruments, model alpha300.

Surface immobilization of ATTO655-SA and Cy5-SA

The fabricated Ag-Au-NC substrates and glass slides (used as control) were covered with 100 μ l of 1mg/ml BSA-Bt solution in phosphate buffer and placed in a humid chamber for overnight at 4°C. Following this step, the slides were washed thrice with phosphate buffer and placed again in a humid chamber. About 60 μ l of the dye solutions (ATTO655-SA or Cy5-SA, 2 μ M in buffer) was then added on the BSA-Bt coated surfaces and incubated for 2 hours at room temperature. Finally the samples were washed multiple times with buffer. The resulting dye monolayers on glass or the Ag-Au-NC surfaces were used for fluorescence measurements. A Schematic representation of the immobilization process of the dye on the substrates through the BSA-Biotin-streptavidin chemistry is shown in Scheme 1. The surfaces were always kept in the wet condition while performing the experiments to avoid protein unfolding or denaturation by drying. All comparative studies between the fabricated Ag-Au-NC substrates and the glass slides were made under identical experimental conditions. The biotin-streptavidin assembly, used in the present case not only helps in the immobilization of a monolayer of fluorophore on the surfaces but also places the fluorophore at a suitable distance ($\sim$ 9 nm) from the metallic surface for efficient MEF [10].

Fluorescence measurements

Fluorescence spectra were recorded using a Varian Cary Eclipse Fluorescence Spectrophotometer using front face illumination geometry. Bandpass filters were used to minimize the scattered light at the excitation wavelength. Time-resolved fluorescence intensity decays were recorded using a PicoQuant Fluotime 100 time-correlated single-photon counting (TCSPC) fluorescence lifetime spectrometer. The excitation at 635 nm was obtained using a pulsed laser diode (PicoQuant PDL800-B) with 20 MHz repetition rate. The Instrument Response Function (IRF) is about 300 ps. The excitation was vertically polarized and the emission was recorded through a polarizer oriented at 54.7° from the vertical position. A bandpass filter at 685 $\pm$ 35 nm (Chroma Inc.) was used in the collection path to record the intensity decays. The FluoFit software from PicoQuant was used for the analysis of the observed fluorescence decays. With the present TCSPC set-up, Fluorescence lifetimes as short as 60 ps could be recovered from the reconvolution analysis.

2. RESULTS AND DISCUSSION

The galvanic replacement reaction is reported to be a very effective strategy for preparing various metallic nanostructures with applications in catalysis and as substrates for surface enhanced Raman spectroscopy [11–15]. With this perception we have used the galvanic replacement reaction for the simple one-step fabrication of Ag-Au-NC substrates from thin silver films coated on glass slides. Our interest is to improve the stability and biocompatibility of silver by combining it with gold nanoparticles and to tune the plasmonic properties of the substrate by mixing the optical properties of gold and silver so that they can be more suitable for MEF applications.

The galvanic replacement reaction of silver with gold is driven by the difference between the reduction potentials of $AuCl_4^-/Au$ (0.99 V vs standard hydrogen electrode, SHE) and Ag^+/Ag (0.8 V vs. SHE). So the immersion of the silver coated slides into the $HAuCl_4$ solution leads to the spontaneous oxidation of elemental silver and the uniform deposition of

nanoscale Au particles on the surface of the sacrificial silver substrate, according to the following reaction

The progress of the reaction was clearly observed by the change in the color of the films from shiny silver to brown-grey. The slides were removed at suitable intervals from the HAuCl_4 solution to terminate the reaction. Figure 1 shows representative SEM images of the slides before and after the galvanic replacement reaction at different times. A change in the nature of the substrate from a smooth surface to a rough and structured morphology is immediately evident. The particle sizes were observed to gradually increase with increase in the reaction time.

To determine the composition of the fabricated substrates, we carried out EDS measurements. In accordance with reaction 1, the EDS analysis yielded an elemental composition of Ag, Au and Cl, with the percentage of Au increasing with increased reaction times (Fig. 1C and Table 1). The presence of Cl is due to the formation of AgCl as a by-product in the galvanic replacement reaction (eq. 1). Since AgCl is insoluble in the aqueous solution, it is co-deposited as microcrystals on the substrate along with the Au nanoparticles [11, 16]. So the percentage of Ag obtained from the EDS analysis is due to the contribution of both AgCl, as well as any residual unreacted Ag that remains from the starting Ag film (*cf.* Table 1). As will be discussed later, this residual Ag is found to play an important role in the fluorescence enhancement obtained with the fabricated Ag-Au-NC substrates. It is possible to remove the AgCl from the substrates by washing with concentrated NaCl solution [16]. However, no significant differences were observed in our subsequent fluorescence studies using the Ag-Au-NC substrates, with or without the treatment with NaCl.

In addition to the SEM imaging that gives information on the lateral dimensions of the Ag-Au-NC substrates, AFM was used to determine the axial dimensions and morphology of the nanostructures (Fig. 2). The line profile across the AFM images shows that the average height of the nanostructures increases from about 15 nm, for the starting Ag film to ~300 nm after the reaction with HAuCl_4 for 15 minutes and ~500 nm after the reaction with HAuCl_4 for 90 minutes. This is in accordance with the results obtained from the SEM studies.

The Ag-Au-NC structures thus fabricated were used to immobilize the dye molecules (Scheme 1) for fluorescence studies. Figure 3A shows the fluorescence spectra of ATTO655-SA on the fabricated Ag-Au-NC substrates, the starting Ag-film before the galvanic reaction and on bare glass slides, used as the control. The fluorescence spectra were similar on all the substrates with maximum around 683 nm. The fluorescence intensities were observed to be higher on the Ag-Au-NC substrates in comparison to glass and the starting Ag film; however, there was a distinct variation in the fluorescence enhancement factors on the substrates depending on the time for which the galvanic replacement reaction had been carried out (Fig. 3B and Table 1). The maximum enhancement was observed to be ~15-fold for the Ag-Au-NC substrates with 15 minutes reaction time, after which the fluorescence enhancement gradually decreased. This could be due to the changes in the plasmonic properties of the Ag-Au-NC substrates, as explained below.

The increase in the fluorescence intensities on the fabricated Ag-Au-NC substrates suggests that these substrates are suitable for MEF. To have a better understanding of the MEF effect and the interesting variation in the fluorescence enhancement factors with the reaction times, we carried out time-resolved fluorescence measurements. Figure 3C shows the fluorescence intensity decay traces of ATTO655 on glass and on the Ag-Au-NC substrates for two

different reaction times. The average fluorescence lifetimes were substantially shorter on the nanocomposite substrates (~ 0.1 ns for 15 min and ~ 0.18 ns for 90 min of the galvanic reaction) in comparison to glass (~ 0.5 ns). The reduction in the fluorescence lifetimes of the probe on the Ag-Au-NC substrates in spite of the increase in the fluorescence intensities supports our claim that the observed fluorescence enhancement with the Ag-Au-NC substrates is due to the near-field fluorophore-plasmon coupling effect [1–3]. In classical far-field fluorescence, an increase in the fluorescence intensity is always accompanied by an increase in the fluorescence lifetime. However, the near-field metal-fluorophore interaction leads to an increase in the radiative decay rate and subsequently an increase in the fluorescence intensity is accompanied by a decrease in the fluorescence lifetimes, as in the present case. Accordingly, it is expected that the larger the fluorescence enhancement, more should be the reduction in the fluorescence lifetime. Indeed the substrate, which shows the largest fluorescence enhancement (15 min reaction, 15-fold enhancement) also leads to the largest reduction in the fluorescence lifetime of the probe (~ 0.1 ns, Table 1).

The variation in the fluorescence enhancement effects of the Ag-Au-NC substrates prepared with different reaction times can be explained in terms of the amount of residual Ag present in the nanocomposite substrates. At shorter reaction times the fluorescence enhancements on the Ag-Au-NC substrates are observed to be larger than the starting Ag-film which shows a 5-fold enhancement in the fluorescence intensity of the probe in comparison to glass. This means that the Ag-Au-NC structures are better than the starting Ag-substrates for MEF applications. However, as the reaction progresses, the enhancement factors on the Ag-Au-NC substrates gradually decreases and falls below that of the starting Ag-film. This change corresponds to the decrease in the percentage of Ag present on the Ag-Au-NC substrates (Table 1). We believe that in addition to the Au nanostructures formed by the galvanic replacement reaction, the residual Ag also plays an important role in the fluorescence enhancements obtained using the fabricated substrates. As the percentage of the residual silver decreases, the MEF effect of the substrates also decreases. It is known that Ag particles show suitable plasmonic properties for MEF. So the residual Ag in conjunction with the Au nanostructures leads to the superior performance of the fabricated Ag-Au-NC substrates. A favorable effect of the residual Ag in dealloyed nanoporous gold has also been recently reported by Zhang *et al.* for surface enhanced Raman scattering, a phenomenon closely related to MEF [17].

The decrease in the fluorescence enhancement of ATTO655 on the Ag-Au-NC substrates with increasing reaction times proves that the enhancement is not due to an increase in the surface areas or an increase in the concentration of dyes immobilized on these substrates. If the enhancement was related to the increase in the surface area, the substrates prepared with longer reaction times and having more surface roughness (Fig. 1 and Fig. 2), should have led to maximum fluorescence enhancements. This is contrary to our observation. So the increased fluorescence intensities on the Ag-Au-NC substrates are actually due to the fluorophore-metal coupling interactions.

To further investigate the suitability of the fabricated Ag-Au-NC nanostructures as MEF substrates we carried out fluorescence studies with another dye that is commonly used in fluorescence applications, namely Cy5. In this case also we observed a significant fluorescence enhancement (~ 9 -fold) and a reduction in the fluorescence lifetime (~ 0.35 ns in comparison to ~ 0.8 ns on glass). Representative fluorescence spectra and intensity decay traces of Cy5-SA on the Ag-Au-NC substrates and on the control glass slide are shown in Fig. 4. This result suggests that the MEF effect of the fabricated Ag-Au-NC substrates is a general, through space effect and is not dependent on the specific nature of the fluorophore. Thus the Ag-Au-NC substrates can be used for MEF studies with a wide variety of fluorophores.

4. CONCLUSION

In this article we have demonstrated that the galvanic replacement reaction is a very convenient and cost-effective method to fabricate Ag-Au-NC substrates that are suitable for MEF applications. We believe that this approach is a very elegant way of combining the optical properties of gold and silver and for the generation of robust and reproducible surfaces that will be well suited for biophysical and bioimaging studies based on MEF.

Acknowledgments

SDC acknowledges the Indo-US Science and Technology Forum (IUSSTF) for the grant of the IUSSTF Fellowship. We also acknowledge the support of the Maryland NanoCenter and its FabLab. This work was supported by the National Institutes of Health (NIH), Grants R01HG002655 and K25AI087968.

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Scheme 1.

Immobilization of dyes on Ag-Au-NC substrates using the biotin-streptavidin assembly.

Figure 1. SEM images of thermally evaporated Ag film before (A) and after reaction with HAuCl_4 for 15 min (B) and 90 min (C) and their respective EDS spectra (D).

Figure 2. AFM images ($10\mu\text{m} \times 10\mu\text{m}$) of thermally evaporated Ag film before (A) and after reaction with HAuCl_4 for 15 min (B) and 90 min (C) and their respective line profiles (D).

Figure 3.

(A) Fluorescence emission spectra of ATTO655-SA on glass, the thermally evaporated Ag film before the galvanic reaction and the different Ag-Au-NC substrates prepared with reaction times of 5, 15, 30, 45 60 and 90 minutes (1–6). (B) Variation in the fluorescence enhancement factor of ATTO655 on the Ag-Au-NC substrates with different reaction times. (C) Fluorescence decay traces of ATTO655-SA on glass and Ag-Au-NC substrates with reaction times of 15 min (1) and 30 min (2)

Figure 4. Fluorescence emission spectra (A) and fluorescence decay traces (B) of Cy5-SA on glass and the Ag-Au-NC substrate.

Table 1

The EDS elemental composition, fluorescence enhancement factors and average fluorescence lifetimes of ATTO655-SA in the Ag-Au-NC substrates at different reaction times with HAuCl₄. The parameters in the control samples (glass and Ag-film before the galvanic reaction) are also presented.

Substrate	Reaction Time (min)	Ag (wt. %)	Au (wt. %)	Cl (wt. %)	Fluorescence Enhancement Factor	Average Lifetime (ns)
glass	-	-	-	-	1	0.50
Ag-film	0	100	-	-	5	0.25
Ag-Au-NC	5	70	20.5	9.5	11	0.14
Ag-Au-NC	15	65.5	24.5	10	15	0.10
Ag-Au-NC	30	62.5	27	10.5	13	0.15
Ag-Au-NC	90	58	29	13	2	0.18