Metal-enhanced fluorescence of an organic fluorophore using gold particles

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Abstract

Particulate gold films were deposited on glass substrates by vapor deposition. Rabbit immunoglobulin G (IgG) was immobilized by physisorption and then Alexa Fluor anti Rabbit IgG was bound to the protein-coated surfaces. Fluorescence was enhanced with increasing the Au thickness and reached saturation at 30 nm when Alexa Fluor555 anti IgG was used. We also examined the effect of silica spacers between the gold film and the labeled protein. The maximum enhancement was dependent on the thickness of silica and reach maximum at 10 nm. The maximum increase in intensity was about 6-fold. We also bound Alexa Fluor-680 anti IgG to the protein-coated surface, and the maximum enhancement was about 10-fold.

Introduction

Fluorescence detection is a central technology of the biosciences. While fluorescence is a sensitive method there is a continuing need for increase sensitivity, as evidenced by the use of amplification methods such as ELISA [1–2] and PCR [3–4]. During the past five years there has been a growing interest in the use of metal particles for enhanced fluorescence [5–9], in particular silver colloids and silver island films (SIFs). A large number of experiments have shown that fluorescence intensities can be increased 10-fold or more when the fluorophores are in close proximity to the silver particles. The mechanism of this enhancement is, at least in part, due to an increased rate of radiative decay near the particles. Fluorophores in the excited state undergo near-field interactions with the metal particles to create plasmons. While the mechanism of metal-enhanced fluorescence (MEF) is not completely understood, an important factor is the ability of the plasmons to radiate away from the particle [10]. Given the electrodynamic nature of this interaction, rather than a chemical interaction, it is reasonable to question whether gold can cause MEF.

When considering gold there are known factors which argue for or against the existence of MEF. Arguing against MEF is the well known ability of gold to strongly quench fluorescence. Quenching occurs for a wide variety of fluorophores and quenching occurs over longer distances than for Forster transfer between two fluorophores [11–15]. Alternatively, one can also argue that MEF can occur with gold, particularly at longer wavelengths. According to radiating plasmons (RP) model for MEF the scattering component of metal particle extinction contributes to MEF and the absorption component contributes to quenching. For gold colloids
above 80 nm in diameter the light scattering yield is over 50% [16,17], which suggests that MEF may occur. A couple of reports have been presented from other laboratories [18–20].

There are many potential advantages of using gold in place of silver for MEF. Gold has high chemical stability and surfaces containing even small gold particles are expected to be stable indefinitely. The surface chemistry of gold is well understood, surface modification and binding of biomolecules is easily accomplished [21,22]. Additionally, there is now a large literature which describes batch synthesis of gold particles and shells of a variety of shapes [23–25].

In this report we describe studies of the MEF displayed by labeled proteins in close proximity to gold particles (Scheme 1). Particulate gold surfaces were made by vapor deposition. The particle size and/or spacing were controlled by the mass thickness and rate of deposition. The particulate gold surfaces were covered with a layer of silica. The silica layer was used to keep the fluorophores out of direct contact with the gold where quenching is expected to dominate over enhanced fluorescence. Because of the use of MEF in bioassays we chose a model immunoassay as the test system consisting of unlabeled rabbit immunoglobulin (IgG) and Alexa Fluor-labeled anti-rabbit IgG (Alexa-IgG). The silica surface, which covered the gold particles, was coated with unlabeled IgG by physioabsorption. The labeled protein was then bound to IgG resulting in a layer of labeled protein above the gold particles. In the case of silver, it is known that the extent of MEF depends on the size of the particles and the distance of the fluorophore from the metal surface. Accordingly, we varied the gold particle size and the thickness of the silica spaces to identify the optimum conditions for MEF to gold.

**Experimental method**

Silicon monoxide and gold wire (99.999 %) were purchased from Aldrich. Alexa Fluor-555 and Alexa Fluor-680 anti Rabbit IgG (2 mg/mL) were obtained from Molecular Probes. Rabbit IgG (5 mg/mL) and buffer components and salts (such as bovin serum albumin, glucose, and sucrose) were from Sigma-Aldrich. Nanopure water (>18.0 MΩ cm) purified using Millipore Milli-Q gradient system was used in experiments.

Gold films were deposited in a consolidated vacuum vapor deposition system (Model 306, Edward). Glass slides were pre-treated by air plasma under 1 × 10^{-4} mbar for 3 min before depositing the metal film to increase the stickiness of metal. The gold wire was melted on the filament and evaporated under a pressure < 1 × 10^{-7} mbar. The deposition rate was adjusted by the filament current and the thickness of film was measured with a quartz crystal microbalance. The silica layer (SiO_2) was grown on the gold film using silicon monoxide in the same vacuum chamber.

The slide was covered with a water-proof tape with wells of diameter 5 mm [26]. The IgG solution was diluted by sodium phosphate buffer (50 mM, pH = 7.4) to 17 g/mL. 25 μL of diluted IgG solution was added to each well. The slide was incubated for 4 h at room temperature in a humid chamber, and then rinsed with water and a washing solution (0.05 % Tween-20 in water). Blocking was performed by adding 25 μL of blocking buffer (1 % bovine serum albumin, 1 % sucrose, 0.05 % NaNO_3, 0.05 % Tween 20 in 50 mM Tris-HCl buffer, pH = 7.4) overnight at 4°C in humid chamber. After washing with water and washing solution (0.05 % Tween 20 in water), a diluted dye-labeled conjugate solution (10 mg/mL in blocking solution) was added to the well on the slide and incubated in a humid chamber at room temperature for 2 h. After incubation, the residual supernatant solution was collected to detect the fluorophore intensity change before and after the binding. The slide was then rinsed with water, washing solution (0.05 % Tween 20 in water), and water. Covered by blocking buffer, the slide was stored at 4°C before the measurement.
Absorption spectra were monitored with a Hewlett Packard 8453 spectrophotometer. Fluorescence spectra were recorded with Cary Eclipse Fluorescence Spectrophotometer. Surface fluorescence spectra were recorded with an excitation angle 90° and monitoring angle 45°. A combination of a protein-coated glass slide and a corresponding gold-coated slide worked as a control to correct for reflection. Lifetimes were recorded with a 10-GHz frequency-domain fluorometer [27] using mode-locked argon ion laser at 514 nm, 76 MHz repetition rate, and 120 ps pulse width. Excitation and emission polarizers were in the magic angle orientation. Emission was selected with combination of a 520 nm long-pass liquid chromate filter (CrO$_4^{2-}$/Cr$_2$O$_7^{2-}$, 0.3 M, pH 8) placed in a 2 mm, 1 in. × 1 in., quartz cuvette and an interference filter at 540 ± 10 nm. This combination of filters rejects efficiently scattered light and has minimal internal luminescence. AFM images were collected with Atomic Force Microscope (TMX 2100 Explorer SPM, Veeco), equipped with AFM dry scanner. Surfaces were imaged in air, in taping mode of operation, using the SFM non contact mode cantilevers (Veeco). Samples were freshly prepared prior to imaging. The AFM scanner was calibrated using standard calibration grid as well as gold nanoparticles 100 nm in diameter from Ted Pella. Images were analyzed using SPMLab software.

Results and discussion

Surface roughness is known to provide a mechanism for coupling incident light to surface plasmons and of creation of far-field light from the plasmons [28]. For this reason we used a slow gold deposition rate to result in a rough metal surface [6–7]. All gold films in the current experiment were grown at a relatively slow rate of 0.1 nm/10 s under a pressure of ca. 5 × 10$^{-7}$ mbar [29]. The surface morphology was controlled principally by the film thickness (2 – 100 nm). A thin Au film (2 nm) displayed a plasmon absorbance at 600 nm (Fig. 1), close to the wavelength of nano-size individual particle, indicating that the thin film was indeed composed of nano-size particles [30–32]. The plasmon wavelength was red-shifted to 680 nm with increasing the thickness to 5 nm, showing that the particle size became larger for the thicker gold film (Fig. 1). In the other words, the size of particle increased with the thickness of Au film. When the film thickness was over 10 nm, the plasmon absorbance disappeared, inferring that the metal film became continuous without the presence of individual particles.

The change of surface morphology with the thickness of gold film was characterized by AFM. A thin gold film (2 nm) was found to be composed of individual particles [Fig. 2(a)]. The average height was ca. 3 nm. Considering that the particles were packed separately on the solid substrate, this height was plausible for an average thickness of 2 nm measured by microbalance. With an increasing thickness of the metal film, it was shown that the particle size became larger. If the gold film was further increased, the individual particles were aggregated and the metal film became more and more continuous [Fig. 2(b)]. While this AFM image indicates the surface consists of separated particles the extinction spectrum in Fig. 1 suggests the film is continuous or that the particles are strongly interacting. The height was ca. 14 nm, close to the thickness measured by the microbalance.

In order to avoid a strong quenching due to the close proximity of fluorophores to the metal surface, and to protect the metal film, a silica layer was coated on the metal by the vapor deposition method in the same chamber [30]. Because a portion of silicon monoxide was oxidized to silicon dioxide by oxygen left in the chamber during the evaporation, the silica layer was supposed to be composed of silicon monoxide and dioxide mixture. The AFM image of 10 nm silica-coated gold film showed smooth regions different from the metal film, although there were obvious regions of roughness [Fig. 2(c)]. The average height of these defects was ca. 10 nm, which was consistent with the thickness of silica measured by microbalance.
We studied the effects of the gold surfaces and the thickness of the gold surfaces on the emission spectra of the AlexaFluor-555 labeled antibody (Alexa-IgG). All gold films were coated by 5 nm thick silica. Rabbit IgG was adsorbed by physisorption on the silica [26], and Alexa Fluor-555 anti Rabbit IgG was bound to the immobilized IgG. The concentration of bound fluorophore could be estimated quantitatively through the luminescence intensity change in buffer solution before and after binding. Because there were average 4.5 fluorophores on each antibody molecule, the antibody coverage on the silica was estimated to be ca. 1 × 10^{-12} mol/cm^2. It was known that one IgG molecule could bind 1 – 2 antibody molecules, so the coverage of IgG molecule was inferred to be less than 2 × 10^{-12} mol/cm^2. This coverage was found to be almost independent of the thickness of gold and silica.

Emission spectra are shown in Fig. 3. The fluorescence intensity on the 2 nm gold film was close to that on the glass substrate. The quenching effects of gold were not seen, possibly as a result of the 5 nm layer of silica. The intensity of the labeled IgG increase with increasing mass thickness of gold, reaching saturation above 30 nm (Fig. 3, insert). Remarkably, instead of quenching, the enhancement reached 6-fold for an approximate thickness of 30 nm. This enhancement is less than that reported silver, but the enhancement is substantial.

In this experiment, besides the thickness of silica layer, the thickness of IgG has to be considered. The IgG molecule can be described as a cylinder shape with a 4 nm diameter and a 10 nm height. So the thickness of protein layers is about 5 – 10 nm when it was adsorbed on the solid substrate vertically and horizontally. It means that there exists a spacer between the dye-labeled antibody and metal surface. In addition, because the thin metal film is not continuous, some fluorophores are adsorbed on the glass instead of the metal island. Hence, even though the protein layer is adsorbed directly on the metal without a separation by the silica, the fluorophores are not quenched. It is of interest to estimate the optimal distance for MEF. Combining the thickness of silica and the proteins, the distance of maximum MEF was about 15 – 20 nm. This distance seems to be slightly longer than that found optimal for MEF with silver, which is typically near 10 nm. At this time the data are not adequate to state with certainty the gold MEF occurs at larger distances than silver MEF, but this result seems reasonable given the quenching effect of gold at short distances.

It is interesting to know the role of localized surface plasmon (LSP) resonances or surface plasmon polariton (SPP) resonance in the observed fluorescence enhancement. There is no complete answer to this question because there is a continuous transition from LSP to SPP with increasing the metal thickness. Even with the imperfect separation of LSP and SPP, we believe the dominated cause of enhancement on LSP. This is because our samples remain rough even at the large gold thickness. The samples are not illuminated under the condition when SPP can be created on a smooth surface, so the created plasmon is mostly LSP for all the metal thickness.

An important property of metal-enhanced fluorescence is a reduction in lifetimes occurring simultaneously with increases in intensity. We measured the time-dependent decays of the surface-bound protein. We used the frequency-domain (FD) method. The FD data were analyzed in terms of the multi-exponential model [31]:

\[
I(t) = \sum_i \alpha_i \exp(-t/\tau_i)
\]

where \(\alpha_i\) are the amplitudes and \(\tau_i\) the decay times, \(\sum \alpha_i = 1.0\). The amplitude-weighted lifetime is given by:
The contribution of each decay component to the steady state intensity is given by

\[ <\tau> = \sum_i \alpha_i \tau_i \]  

(2)

The average lifetime is given by

\[ \tau = \sum_i f_i \tau_i \]  

(3)

The values of \( \alpha_i \) and \( \tau_i \) were determined by non-linear least squares impulse reconvolution with a goodness-of-fit \( \chi^2_R \) criterion. After fitting to the multiexponential model, the recovered parameters and lifetimes were listed in Table 1. We believe the shorter lifetimes are due to an increase in the radiative decay rate, but we cannot rule out quenching of some portion of the population. The amplitude-weighted lifetime displayed a tendency of increase with the thickness of the gold film. The lifetime on 10 nm silica was shorter than that on 5 nm silica when the thickness of gold film was 10 nm, indicating that the intrinsic decay rate became faster when the fluorophore was localized at a certain distance from the gold surface. The recovered intensity decays for Alexa 555-IgG from the parameters in Table I were plotted in Fig. 5. The intensity decays on 5, 10, and 20 nm thick gold films are more rapid than on glass. Short decay time components are seen on the 5 and 10 nm thick films, as expected for fluorophores near metal particles [5]. The short lifetime component is less prominent on the 20 nm film and the intensity decay becomes comparable to glass on the 50 nm film. The smaller changes in lifetimes means the thicker metal film is in agreement with recent theory for a fluorophore above a smooth metal film and with our own unpublished observations.

We also bound Alexa Fluor-680 anti-Rabbit IgG to the protein-coated surface with 5 nm silica. The fluorescence spectra of Alexa Fluor-680 displayed the maximum at 698 nm upon excitation at 610 nm (Fig. 6). The intensity was observed to increase more greatly than that of Alexa Fluor-555 under the same conditions. The maximum enhancement of Alexa Fluor-680 is about 10 fold (inset of Fig. 6), higher than that of Alexa Fluor-555 indicating that the near IR dye can be enhanced more efficiently on the Au film. This result consists with our previous observation on the silver [30].

**Discussion**

It is of interest to speculate why we observed enhanced fluorescence using gold where many other laboratories observed quenching. We speculate that the difference may be due to the different types of metal particle surfaces. Because of their widespread use in surface-enhanced Raman scattering (SERS) the silver island films (SIFs) are often used for MEF. In contrast, the ease of preparation of gold colloids has resulted in their widespread use, and we are not aware of methods to produce gold island films by chemical reduction. In unpublished experiments we generally observed weaker MEF with round silver colloids on surfaces than with SIFs. In studies of gold colloids on surfaces we observed either quenching or only modest...
enhancement. These observations suggest that MEF using gold is due to the irregular particles formed by vapor deposition. Irrespective of the cause, the observation of MEF using gold particles suggests the use of these stable particles in surface-localized bioassays. Additionally, we observed larger enhancements with aggregated silver colloids than with more isolated single colloids [30]. While less than reported for the silver particles, the gold is chemically stable with well defined surface chemistry, so that devices may be readily fabricated for long-term use.

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Fig. 1.
Absorbance spectra of Au films of varying thickness, 2 nm, 5 nm, and 10 nm, deposited at 0.1 nm/10 s on glass substrate.
Fig. 2.
AFM planar and 3D images of 2 nm Au film, 10 nm Au film, and 10 nm Au film coated by 10 nm silica.
Fig. 3.
Emission spectra of Alexa Fluor-555 labeled anti-Rabbit IgG on varying thickness gold films upon excitation at 514 nm. The inset represents the dependence of the enhancement factor on the thickness of gold film. All gold films were coated by 5 nm silica.
Fig. 4.
Dependence of enhancement factor on thickness of silica coated on a 10 nm gold film.
Fig. 5.
Recovered intensity decays for Alexa 555-IgG from the parameters in Table 1.
Fig. 6.
Emission spectra of Alexa Fluor-680 labeled anti-Rabbit IgG on a 50 nm gold film upon excitation at 610 nm. The inset represents the dependence of the enhancement factor on the thickness of gold film. All gold films were coated by 5 nm silica.
Scheme 1.
Fluoroimmunoassay model on a gold particle film.
Table 1
Lifetime data obtained using the multi-exponential model for the fluorophore coated on the glass substrate or metal surface.

| Au thickness (nm)/Silica thickness (nm) | \( \tau_1 \) (ns) | \( \alpha_1 \) | \( <\tau> \) (ns) | \( \chi^2 \) |
|--------------------------------------|------------------|--------------|-----------------|----------|
| Glass substrate                      | 0.776            | 0.486        | 0.49            | 1.10     |
|                                      | 0.291            | 0.339        |                 |          |
|                                      | 0.061            | 0.175        |                 |          |
| 5/5                                  | 0.09             | 0.806        | 0.25            | 1.3      |
|                                      | 0.90             | 0.194        |                 |          |
| 10/5                                 | 0.12             | 0.780        | 0.23            | 0.6      |
|                                      | 0.63             | 0.220        |                 |          |
| 20/5                                 | 0.29             | 0.944        | 0.33            | 0.7      |
|                                      | 0.97             | 0.056        |                 |          |
| 50/5                                 | 0.64             | 0.621        | 0.40            | 0.9      |
|                                      | 0.24             | 0.379        |                 |          |
| 10/10                                | 0.07             | 0.658        | 0.19            | 0.5      |
|                                      | 0.43             | 0.342        |                 |          |