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Strategy for photostable proximity bioassays using lanthanides

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Abstract

We report initial findings for research aimed at creating photostable lanthanide chelate reporters for proximity assays. These reporters take advantage of the nanometer scale distance dependence of fluorescence enhancement for molecules in the vicinity of noble metal nanoparticles and also capitalize on some unique properties of lanthanide chelates. This approach promises to lead to proximity assays that do not suffer from photobleaching and offer very high on/off enhancement ratios. Results for lanthanide chelates on silver island films and in colloidal suspensions are reported. Enhancement factors range from 1 to 2 orders of magnitude, with larger enhancements for strongly quenched lanthanides.

1. Introduction

The discovery of surface enhanced Raman scattering (SERS)¹ in the late 1970s soon led to the discovery of a related phenomenon, surface enhanced fluorescence (SEF).² Early research in surface enhanced spectroscopy focused on theoretical aspects of the phenomena and elucidation of the mechanism(s) involved in enhancement. SERS yields the highest enhancement when the molecules contact the metal. For SEF, the fluorescence is quenched when the fluorophore contacts the metal. The enhancement for SEF outstrips the quenching when there is a distance of roughly 10 nm between the fluorophore and metal surface.²⁻⁴ Although the phenomena differ in this respect, it has been concluded that SERS and SEF both derive from the amplified local electric fields that develop at the surface of noble metal nanoparticles or nanoscale roughness features due to surface plasmons.

In the early 1980s, Weitz et al. demonstrated that a large increase in radiative rate accompanied surface enhanced fluorescence of a europium chelate on a silver island film.⁵ More recently, the Lakowicz group has shown that surface enhancements are greater for fluorophores with lower quantum efficiencies.⁶ This stronger enhancement arises because the increase in radiative rate allows radiative relaxation to compete more effectively with non-radiative decay routes such as quenching by solvent. Furthermore, the increased radiative rate that accompanies SEF gives rise to other benefits including increased photostability, decreased self-quenching and improved energy transfer between donor-acceptor pairs. Researchers in the SEF field have focused on the design of nanostructured substrates which have yielded enhancement factors typically between 20 and 200.^{7,8} Some of the largest enhancements have been observed on electrochemically produced fractal structures.⁹ This is not surprising considering that surface enhanced spectroscopy was originally discovered on electrode surfaces. Where applications are concerned, Lakowicz¹⁰⁻¹² and others^{13,14} have made a good start toward deploying SEF for biomedical applications, including immunoassays and hybridization assays.

Lanthanide chelates have long been used as reporters in bioassays.^{15,16} Traditionally, the long-lived excited states of lanthanide chelates have been exploited in assays based on time-resolved fluorescence measurements. However, these reporters are also attractive because they are photostable, have large Stokes shifts and have narrow emission bands making them amenable to multiplexing. We have been investigating the use of lanthanide chelates as upconverting labels.^{17,18}

When used in conjunction with surface enhancement, lanthanide chelates have potential as reporters in proximity assays or as photostable molecular beacons with very high on/off ratios. Previous work on surface enhancement of lanthanides^{5,19,20} has been limited to the conventional lanthanide chelate methods using europium or terbium in conjunction with energy transfer from an organic ligand. These methods have good efficiency so the level of enhancement is limited. Less efficient lanthanides such as samarium and praseodymium, which are more strongly quenched in aqueous solution, theoretically should enjoy very large surface enhancement near noble metal nanoparticles due to the large radiative rate increases.

This paper presents the results of studies of surface enhanced fluorescence of lanthanide chelates on silver island films and silver colloids that demonstrate the potential for development of a new class of photostable reporters for proximity assays.

2. Experimental Methods

A. Preparation of Silver Island Films

Silver island films were prepared by two different methods. The first involved reduction of a silver-ammonia complex by glucose. First, glass slides were cleaned in piranha solution (3:1 mixture of concentrated sulfuric acid and 30% hydrogen peroxide). The silver ammonia complex was prepared by dissolving 0.22 g of silver nitrate in 25 mL of water in a 30 mL beaker and then adding 8 drops of 5% sodium hydroxide to produce a brown silver hydroxide precipitate. The brown precipitate was then titrated drop by drop with concentrated ammonium hydroxide until the precipitate cleared away. Typically the precipitate cleared with 10-20 drops. The resulting solution containing the silver ammonia complex was chilled to 5 °C in an ice bath. The clean slides were placed in the chilled solution for approximately 5 minutes before adding 0.35 g of glucose in 4 mL of water. Two minutes after adding glucose, the solution was warmed to 30 °C in a warm water bath. As the solution warmed up, the color of the solution changed slowly from faint yellow to golden yellow then to green. As the solution became darker, it became murky and opaque. At the point when the solution was opaque, the slides were removed. The slides had a greenish tinge from the solution clinging to them. The slides were then immersed in distilled water and allowed to develop a light orange film. As the film on the slide developed the solution turned cloudy. The orange slides were then sonicated for about one minute before being rinsed in distilled water. The slides were stored in distilled water until they were used. Some silver coated slides were subsequently over-coated with silica using the Stöber method,²¹ in which ammonia catalyzes the hydrolysis and condensation of tetraethyl orthosilicate (TEOS) in a solution of ethanol and water to form a silica layer.

The second technique for preparing silica coated silver was a thermal vacuum deposition method.²² Silica-coated silver island films were electron-beam deposited using a cryopumped thin film deposition system. The system pressure during deposition was approximately 2×10^{-6} Torr. Clean microscope slides were baked in vacuum at 80 °C overnight followed by the deposition while the slides were still at temperature. The silica overlayer was deposited over the silver immediately following the silver deposition. A quartz crystal microbalance was used to monitor the deposition rate and thickness of both the silver and the silica; the measured deposition rate for both materials was $\sim 1 \text{ \AA/sec}$. Silver

and silver/silica substrates were characterized by visible near-infrared (VIS/NIR) absorption spectroscopy using an Ocean Optics USB2000 fiber spectrometer. Selected silica coated silver and bare silver films were also characterized by atomic force microscopy (Nano-R AFM).

B. Preparation of Colloids

Colloids were prepared by reduction of AgNO_3 with citrate under reflux at about 90°C. A silver nitrate solution (9 mg in 49 mL) was heated to 90 °C. A sodium citrate solution (1 mL, 38.8 M) was added drop by drop over a period of ~2 minutes with stirring. As the reaction progressed the solution turned from yellow to deep amber. At that point the reaction was removed from the heat. The suspension was dark, but had no sign of murkiness. Dilution of the amber solution produced a clear golden yellow suspension. The suspension was centrifuged at 4000 rpm to remove any large aggregates that may have been present. The resultant colloidal suspensions were examined with dynamic light scattering (Horiba LB-550 Dynamic Light Scattering Particle Size Analyzer) to determine the size range of colloidal particles. The plasmon frequency was determined in the same manner used for island films. The change in plasmon frequency with protein adsorption was monitored to determine optimum protein/colloid concentrations.

C. Preparation of Lanthanide Complexes

Lanthanide chelates were prepared by mixing stoichiometric amounts of a lanthanide salt, typically a hydrated nitrate or chloride with chelating agent in aqueous or ethanol solution. Chelates used included beta diketones [naphthoyl trifluoro acetate (NTA), thenoyl trifluoro acetate (TTA) and 2,4 pentadione (acetyl acetone or acac)] and simple or bifunctional multidentate ligands such as ethylenediaminetetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid conjugated to biocytin (DTPAbiocytinamide). For example, a 10 mM solution of Eu(NTA)_3 would be prepared by adding 100 micromoles of europium nitrate and 300 micromoles of NTA to 10 mL of ethanol. Serial dilutions of 10-100 mM stock solutions were performed to obtain the desired solutions for any given experiment. The ethanol solutions (5 microliters or less) were drop coated onto the silver films and bare glass slides. A 5 microliter drop spreads out to about 4 cm² circular spot on both silver films and bare glass.

D. Spectroscopic Methods

Surface enhanced fluorescence spectra were obtained using either a conventional fluorimeter (Perkin Elmer Model 650-10S), or a custom fluorescence measurement system consisting of an argon ion laser for excitation and a Spex single (Model 270M) or double (Model 1402) monochromator with a cooled PMT for detection at the output slit. The laser is chopped in order to use lock-in detection. The lock-in amplifier signal or fluorimeter signal is digitized with a National Instruments DAQPad 6020E for USB in conjunction with a BNC 2120 interface using LabVIEW. Spectra are plotted using IGOR Pro plotting software.

Lifetimes were obtained using pulsed laser excitation (frequency-doubled Nd:YAG pumped dye laser) and a gated photomultiplier tube for detection. The time resolved signal was acquired by a digital oscilloscope (Tektronix TDS 380) interfaced to a PC also using LabVIEW software.

3. Results and Discussion

A. Island Film Studies

Silver island film studies were conducted first, leading eventually to silver colloid studies. Chemical preparation of island films resulted in films with a range of optical densities and

surface plasmon absorption maxima. Figure 1 shows absorption spectra for two films made on the same day under similar conditions. Among the factors influencing the outcome are ammonia concentration, temperature, and reaction time. The ammonia titration step was not perfectly reproducible leading to excess ammonia in some cases. Excess ammonia slows down the reduction while higher temperatures increase the reduction rate. Slower reaction allows for better control of film thickness and optical density. The variability in the films aided in finding film properties that were conducive to greater enhancements. For experiments in which samples were applied directly to the silver film, it was found that lower optical densities (~ 0.25) were far superior for enhancement. This is because these films have more surface area between silver islands where enhancement rather than quenching prevails. Higher optical density films that did not produce significant enhancement still induced significant radiative rate increases for europium chelates, suggesting that a solvent quenched lanthanide would exhibit higher enhancement. In cases where a silica coat was applied to the silver film, higher optical densities (0.4-0.5) could be tolerated because the silica provided the separation between the lanthanide chelates and the metal surface. Without the silica layer, large separations between islands of silver on the lower optical density films allowed for having molecules in a region on the slide where enhancement dominated over quenching. Higher sample coverage yielded greater enhancement factors suggesting that the enhancement zone can accommodate a large number of molecules. This effect has been observed by others²³ as well. Examination of a silica coated silver film by atomic force microscopy showed that the silica coated silver islands were on average 150-100 nm in diameter and 75-100 nm high.

When a europium chelate was applied to both a silica coated island film and a bare glass slide, the fluorescence intensity on the silver film was approximately five times greater than that on the bare glass as can be seen in Fig. 2(a). The radiative rate is also dramatically shortened [Fig. 2(b)]. The relatively modest enhancement is a consequence of the good quantum efficiency of $\text{Eu}(\text{TTA})_3$, reported by Weitz to be ~ 0.4 .⁵ A lanthanide chelate with a low quantum efficiency due to solvent quenching is expected to be enhanced to a much greater extent by the Ag film. As noted above, the enhancement derives from the fact that the greatly increased radiative rate allows fluorescence emission to compete more effectively with non-radiative pathways. This expectation is borne out for a samarium chelate as the spectra in Figure 3 demonstrate. Here the enhancement factor appears to be on the order of 10^2 .

We have found samarium chelates to be approximately 100 times less efficient than their europium counterparts; that is, emission from solutions of equal concentration was two orders of magnitude greater for europium as compared with samarium. Lifetime measurements have shown Sm chelates in solution to have lifetimes on the order of 10 microseconds, more than an order of magnitude shorter than Eu chelates. Therefore, the greater enhancement factor for Sm compared to Eu does not necessarily produce a fluorophore that is brighter, but one that is both comparable and would provide a higher enhancement factor in a proximity assay based on silver nanoparticles.

In addition to radiative decay increases, surface enhancement of excitation rate by the lanthanide chelate also occurs, particularly if the absorption frequency coincides with the surface plasmon frequency. This is the situation for terbium chelates excited with an Ar^+ ion laser tuned to 488 nm. Although terbium is similar to europium in that it enjoys relatively high quantum efficiency, greater enhancements are observed for Tb when directly excited at 488 nm rather than excited through energy transfer from an organic ligand (see Figure 4). It should be noted that in the case of $\text{Tb}(\text{acac})_3$, there is no sensitization by the ligands as there is for the europium and samarium chelates discussed above. Consequently, an unenhanced signal is not detectable above the noise.

B. Colloid Studies

Proximity assays will involve single nanoparticles or small clusters of silver or gold nanoparticles in suspension. Molecular probes bound to the nanoparticles and to the lanthanide chelates will bring the particles and chelates into the proper proximity for enhancement of the lanthanide chelate fluorescence. To simulate this situation, we prepared particles coated with streptavidin that would bind specifically with a terbium chelate attached to biotin, namely TbDTPAbiocytinamide.

Dynamic light scattering measurements showed that the particle suspension contained particles with an average diameter of 50 nm as well as 10 nm seed particles. Based on the total amount of silver present and the size of the particles determined by dynamic light scattering, the particle concentration was estimated to be 10^{-9} molar.

The particles were titrated with protein while monitoring the surface plasmon frequency. Figure 5 shows how the surface plasmon frequency shifts as the Ag particles are coated with protein. This particular sample of particles appears to have been completely coated when the protein concentration had reached 10 micrograms per milliliter. This information was used in the design of the surface enhanced fluorescence experiments.

As noted above, the terbium chelate used in our SEF experiments with Ag colloids was TbDTPAbiocytinamide. This chelate features a Tb^{+3} ion held in a DTPA multidentate ligand attached to two biotin moieties. These biotins can bind to streptavidin (SA), which is bound to the biotinylated BSA layer on the Ag nanoparticles. Figure 6 depicts the protein layers with a top layer of specifically bound TbDTPAbiocytinamide. The spectra shown in Figure 6 are for colloid samples with and without the streptavidin layer. The colloids without streptavidin are in solution with ten times more Tb chelate yet the signal is only twice that of the sample with SA. The expected result was to see greater signal from a colloid suspension with streptavidin because the Tb chelate would be brought into the enhancement zone of the particles when the biotin-streptavidin binding took place. This is indeed what was observed. A five-fold increase in signal for the sample with streptavidin is actually very encouraging considering the number of Tb chelates per particle in samples.

We estimate that only 1% of Tb chelates could be specifically bound to the particles. The estimate is based on the relative concentrations of particles and Tb chelates and the estimated number of protein molecules on each particle. This suggests that the enhancement factor for specifically bound Tb chelates is in the vicinity of 400. The streptavidin coated particles showed evidence of aggregation as might be expected since the TbDTPAbiocytinamide chelates have two biotins per molecule. The SA coated suspension began to settle out while the BSA only coated suspension did not. It is conceivable that aggregation of particles could have led to this very high enhancement by creating hot spots where Tb chelates were optimally positioned between particles.

4. Conclusion

The initial experiments have yielded promising results that bode well for development of photostable reporters for proximity assays. The effect of streptavidin on emission from Tb DTPA biocytinamide in the presence of protein coated Ag colloids serves as a proof of principle for exploiting surface enhancement in proximity assays. Future research will focus on increasing enhancement by utilizing lanthanides for which both enhanced absorption and the radiative rate increase advantage are operative. Praseodymium appears to be a good candidate considering that it is a more strongly quenched lanthanide, similar to samarium, and it absorbs near 488 nm, similar to terbium. Also, design and optimization of

homogeneous immunoassays and DNA hybridization assays will constitute a major part of future research efforts.

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Fig. 1.
Absorption spectra for two silver films prepared under similar conditions.

Fig. 2. Comparison of europium emission on a silver island film and glass showing (a) enhancement of emission intensity and (b) increased radiative rate.

Fig. 3. Spectra for a samarium chelate showing enhanced emission on silver island films as compared with a glass surface.

Fig. 4. Spectra for a terbium chelate showing enhanced emission on silica coated silver island film as compared with a glass surface.

Fig. 5. Shift in surface plasmon peak with increasing protein concentration; (a) individual spectra, (b) peak positions.

Fig. 6.

(a) schematic representation of protein coated silver particles; (b) spectra illustrating enhancement of terbium emission on silver colloid.