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Metal-Enhanced Fluorescence: A Novel Approach to Ultra-Sensitive Fluorescence Sensing Assay Platforms

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Abstract

We describe the development of a novel generic approach to fluorescence sensing based on metal-enhanced fluorescence (MEF). This work follows our initial reports of radiative decay engineering (RDE), where we experimentally demonstrated dramatic signal enhancements of fluorophores positioned close to surface-bound silver nanostructures. The attractive changes in spectral properties of fluorophores includes increased rates of excitation, increased quantum yields, decreased fluorescence lifetimes with an increased photostability, and drastically increased rates of multi-photon excitation. In this report we present a new class of fluorescent biomarkers which are strongly enhanced by metallic particles. This has afforded the development of a novel generic approach for ultra-sensitive fluorescence assay technology. The assay platform utilizes metal particles deposited on glass/quartz surfaces, covered with sub-nanometer layers of a fluorescent biomarker. As such the fluorescence signal of the composite is strongly enhanced. This readily allows easy, quantitative and inexpensive fluorescence detection of minimal traces of specific antigens. We also explore different sensing geometries, such as using evanescent wave excitation.

1. INTRODUCTION

Fast and sensitive detection of physiological markers, deadly pathogens and/or chemicals, is a key element for preventive medicine, as well as an early alert system for national and personal security. For this reason novel biochip devices are of increasing importance for the rapidly emerging market of diagnostics for chronic and pathogenic diseases, as well as genetic analysis. Most of the promising detection systems, such as DNA microarrays, make use of the hybridization of an immobilized target polynucleotide with oligo- or polynucleotide probes that are labeled with reporter fluorophores. The most challenging present goals are: detection of minimal traces of physiological markers (like troponin or myoglobin in case of cardiac arrest), detection of low-abundance DNA, RNA transcripts, or proteins specific for target organisms. A successful sensing technology should offer high sensitivity and selectivity, and ideally not require any chemical or biological amplification steps like with PCR or ELISA.

Recently we have begun to apply a new fluorescence technology that enables substantially enhanced fluorescence signals. 1–3 This novel approach relies on the long-range interactions of fluorophores with sub-wavelength sized metallic islands and colloids. We call this technology Metal Enhanced Fluorescence (MEF). We have predicted that fluorescence signal enhancements may be over million-fold. 1–3 We speculate that with the proper development and tuning, this technology should provide the equivalent sensitivity of PCR or ELISA, without any chemical or biological amplifications steps.

The assay platform utilizes metal particles deposited on the glass/quartz surface covered with sub-nanometer layers of a fluorescent biomarker. The fluorescence signal of the composite is enormously enhanced, which allows easy, quantitative and inexpensive fluorescence detection of minimal traces of specific antigens. We exploit and indeed report different formats like evanescent wave excitation, directional emission and/or multi-photon excitation.

2. OPTICAL PROPERTIES OF METALLIC PARTICLES

The theoretical aspects of the effect of metal particles on fluorophores have been studied for many years [1–4 and refs therein]; however this phenomenon is still not widely recognized in fluorescence sensing. For this reason we briefly review the basic concepts. Metal particles (gold and silver) exhibit strong absorption bands which are absent in the bulk metal. These absorption bands, known as the surface plasmon resonances, result in strong wavelength selective absorption and scattering and create enhanced local electromagnetic fields near the surface of the particles. An example of gold colloid absorption is shown in Figure 1. The plasmon resonances are highly dependent on the size and shape of the metal nanoparticles and the dielectric properties of the surrounding environment.^{5,6} These near field enhancements have given rise to surface enhanced spectroscopies such as surface enhanced Raman spectroscopy (SERS) and surface enhanced resonance Raman scattering (SERRS). The enhancement effect for fluorescence near the metallic particles is related to surface enhanced Raman scattering. It is well known that SERS may enhance Raman signal of chromophore by a factor 10^5 or in some cases even more. ⁷

3. FLUORESCENCE NEAR METALLIC PARTICLES

Following excitation a fluorophore, in free space, can either emit a photon with a radiative rate, Γ , or return to ground state by a non-radiative deactivation rate, k_{nr} . For simplicity we are omitting transitions to triplet state which can be significant in some cases and chemical processes leading to photodegradation which are usually slower and not needed for describing the spectral properties. The quantum yield, Q , and lifetime, τ of the fluorophore are given by:

$$Q = \frac{\Gamma}{\Gamma + k_{nr}} \quad (1)$$

$$\tau = \frac{1}{\Gamma + k_{nr}} \quad (2)$$

The presence of nanometer-size metal particles in the proximity of a fluorophore can affect its spectral properties in two ways: firstly, illumination at the plasmon absorption of the nanometer sized metal particles creates enhanced near-field intensity, I_m that results in higher fluorescence due to increased rates of excitation. We define the excitation rate enhancement factor as a ratio of enhanced excitation intensity in the presence of metal particle, I_{enh} , and excitation intensity, I , in the absence of particle; $G_{exc} = I_{enh}/I$. Secondly, the interaction between the plasmon band and the fluorophore dipole influences both the non-radiative and radiative deactivation rates. The new radiative rate is given by: $\Gamma_m = N_r \Gamma$, and the non-radiative rate given by: $k_{nr}^m = N_{nr} k_{nr}$. We are not assuming any correlation between enhancement factors N_r and N_{nr} . The new fluorescence quantum yield, Q_m and lifetime τ_m in the presence of metal particle and subsequently be defined as:

$$Q_m = \frac{N_r \Gamma}{N_r \Gamma + N_{nr} k_{nr}} = \frac{\Gamma^m}{\Gamma^m + k_{nr}^m} \quad (3)$$

$$\tau_m = \frac{1}{N_r \Gamma + N_{nr} k_{nr}} = \frac{1}{\Gamma^m + k_{nr}^m} \quad (4)$$

The presence of the metal particle will change both the emission quantum yield and the fluorescence lifetime. The change in the quantum yield is described by the emission factor G_{qy} and the change in the fluorescence lifetime by the lifetime factor G_τ :

$$G_{qy} = \frac{Q_m}{Q} = \frac{N_r (\Gamma + k_{nr})}{N_r \Gamma + N_{nr} k_{nr}} = N_r \frac{\tau_m}{\tau} \quad (5)$$

$$G_\tau = \frac{\tau_m}{\tau} = \frac{\Gamma + k_{nr}}{N_r \Gamma + N_{nr} k_{nr}} \quad (6)$$

Both radiative N_r and non-radiative N_{nr} factors can have significant effects on the fluorescence emission. The relative contributions of these factors will depend on the separation and the relative orientation between the fluorophore and metallic particle. For very small separations $N_{nr} k_{nr}$ will dominate, and fluorescence quenching is expected. For larger separations $N_{nr} k_{nr}$ rapidly decreases and $N_r \Gamma$ is expected to become the dominant factor resulting in fluorescence enhancement. It is intuitive that the maximum value of quantum yield enhancement factor cannot exceed the value $G_{qy} = 1/Q$ (when $N_r \gg 1$, $Q_m \sim 1$) that corresponds to a decrease in fluorescence lifetime to zero. We expect significant emission enhancement factor for weak fluorophores ($Q \ll 1$) and much smaller effect for high quantum yield fluorophores.

In the case of both low and high quantum yield fluorophores the fluorescence intensity can be also increased by the change in excitation rate G_{exc} . It is important to stress that observed fluorescence enhancement ($G_{fl} = G_{exc} G_{qy}$) reflects the changes in both the excitation and decay rates (radiative and nonradiative).

$$G_{f1}^{(1)} = G_{exc} \cdot G_{qy} = G_{exc} N_r \frac{\tau_m}{\tau} \quad (7)$$

The increase in radiative decay rate, as well as increases in the excitation rate in the presence of metal particles, has until now been ill-explored in fluorescence sensing. However given the extent of fluorescence enhancement expected from these phenomena, 1–4 then was are faced with some new possibilities for the development of more sensitive ways of assay sensing.

Figure 2 schematically shows these three main phenomena that affect the fluorescence signal. For very short separation the dominant effect is quenching of fluorescence by nearby metal [1–4]. This quenching is essentially a dumping of the excitation energy of the dipole oscillators to the nearby metal. For fluorophore-metal separations larger than 50 X, the field enhancement and radiative decay increase dominates the fluorophore metal interaction. In the range 50 – 150 X the cumulative enhancement reaches a maximum and quickly decreases over the next few hundred angstroms. Fluorophores located within the enhancement layer typically show much stronger fluorescence signals. This enhancement contains a contribution from the field enhancement (increase in excitation rate) and an increase in the quantum yield. For separations larger than 200 X, the metal effects diminishes and one readily observes unmodified free space fluorescence from the fluorophore.

4. ASSAY PLATFORM DEVELOPMENT

Our experimental results to date 1–4, 8–10 indicate significant effects with silver island films, which are made by depositing sub-wavelength size silver particles on a flat glass surface. The rough silver-surface can be conveniently covered by a monolayer (or multilayer) of biotinylated-BSA or indeed other inert spacers (to elevate close-range quenching). The thickness of the spacer can be adjusted in such way that captured oligomers of DNA, or any biomolecules of interest, will be positioned within the enhancement distance. This can provide a population of fluorophore-labeled DNA/proteins localized at the optimal enhancement distance (about 7–15 nm from a silver island surface).

These thin layer samples can easily be excited in a front-face configuration. Figure 3 shows the front-face configuration employed. The sample sandwich consists of one glass (quartz) slide covered with a silver island film, and a second cover slide, essentially used to form a micro-cuvette. In an ideal scenario, the fluorescence sensor should be located in the enhancement layer close to the silver film. The buffer solution can be used to occupy the space between the slides. Our studies have shown that two high quality optical glass slides cannot be squeezed closer than ≈ 1 micron. The enhancement layer is thought less than 500 X, and is therefore much smaller than the bulk volume. A front-face excitation through the sample equally excites the space between the slides. In effect any background fluorescence will significantly contribute to the measured signal.

We have realized that a much better excitation choice for such a thin layer of fluorophores located close to a transparent glass surface, is using an evanescent wave. The evanescent

wave is formed between two media with different refraction indexes, when light enters the medium of the lower refraction index, under the angle greater than the critical angle, α_c .^{11,12} The intensity of an evanescent wave decreases exponentially with distance from the surface and limits the effective excitation to a couple hundred nanometers. Figure 4 shows the schematic of evanescent wave excitation.

5. ASSAY DESIGN

In designing the format of the assay, we will take advantage of the metal surface plasmon enhancement of fluorescence and the potential benefits of evanescent wave excitation. In this way we will obtain maximum excitation efficiency on the layer of fluorophores just above the metal-island film. The fluorescence signal will be enhanced due to the field effect near-to the island and an increase in quantum yield of the fluorophore. Also the evanescent wave will limit the excitation volume to the effective depth (sensing volume) below 1 micron. In the text below we now describe different assay designs using as an example, a TnI protein. The approach is general and applicable to other markers.

5.1. Labeled Marker Protein

The concept of a MEF surface based immunoassay for detecting TnI can be understood from the schematic in Figure 5 (top). The TnI specific antibodies (SAB) are immobilized on a surface layer. Fluorophore labeled TnI are binding to sites of the antibodies.

5.2. Sandwich Assay Format

The concept of the sandwich type immunoassay is also shown in Figure 5 (bottom). The unlabeled TnI specific antibodies (SAB) are immobilized in the layer above the silver island films. Addition of the sample containing natural TnI results in binding of TnI to the antibodies.

5.3. DNA Hybridization Format

The notion of using silver island films for DNA hybridization is shown in Figure 6. Basically a labeled fluorophore DNA strand is enhanced when binding to a complimentary strand immobilized on the silver surface.

5.4. Experimental Platform

Figure 6 and 7 shows the practical design of an assay cartridge which we consider would be of considerable use in MEF assay sensing. The cartridge has a transparent bottom (made from glass or plastic with high refractive index) that is coated with the silver island film. As shown in the insert on Figure 6, the island layer is covered with the layer of spacer with immobilized SAB. Prior to measurements the SAB are filled with labeled TnI. Upon addition of sample (blood/serum) natural TnI, if present in the sample, will exchange labeled TnI. Displaced labeled TnI will quickly diffuse from the enhancement layer and evanescent excitation field. This results in a drop in fluorescence signal that will be proportional to the concentration of TnI, and indeed time of exposure.

The analogical concept can also be used for a sandwich format assay cartridge. The cartridge with an immobilized layer of unlabeled antibodies (over the silver island film) will be filled with the sample. The background signal will be detected. After a predefined time period the sample is rinsed and the cartridge washed with the buffer containing known concentration of labeled antibodies. The labeled antibodies form a complementary layer over the bound TnI. This results in fluorescence signal increase proportional to the amount of bound TnI.

Figure 7 shows the schematic of a simple detection platform. The detection platform consists of a cylindrical lens made from a high refractive index glass or plastic. The refractive index of the lens has to be greater than the refractive index of the liquid sample in the cartridge (blood or serum). The laser beam from a laser diode is directed to the flat (horizontal) surface of the lens under the angle greater than the limiting angle, to form an evanescent wave. The cartridge can be tightly placed on the platform. When the refractive index of the lens and bottom of the cartridge are matched, the evanescent wave will form over the sensor layer. For the blank measurements the cartridge can be filled with the reference solution that does not contain marker. This will effectively zero the reading. After filling the cartridge with the real sample containing markers, one expects to observe the signal decreasing. The extent of the drop will be proportional to the marker level.

Figure 8 shows only the qualitative principles of the sensor. It is easy to realize that such a measurement can be calibrated to be quantitative, but may be subject to optical artifacts. The most important considerations will be the auto-fluorescence of the different samples and ambient light.

6. CONCLUSIONS

In this short paper we have reviewed the favorable spectral effects that can be obtained by employing fluorophore metal particle combinations. We have additionally shown how this new phenomenon, metal-enhanced fluorescence can be applied to surface assay platforms. Further work developing these assay platforms is underway in our laboratories and will be reported in due course.

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Figure 1.
Concept of metal plasmon absorption and calculated plasmon absorption spectra for different particle sizes.

Figure 2.
Effect of a metallic particle on the fluorescence intensity as a function of distance from the nano-particle.

Figure 3.
Front-face configuration for excitation of a thin layer samples.

Figure 4.

Top. View of experimental configuration for measurements in total internal reflectance geometry. **Bottom.** Conditions for total internal reflection excitation. Insert shows the dependence of the excitation field intensity as a function of distance from the interface.

Figure 5.
The “layer” (top) and “sandwich” (bottom) format immunoassay based on labeled TnI-specific antibodies (SAB)

Figure 6.
DNA hybridization using evanescent wave excitation and MEF.

Figure 7.
Schematic of cartridge immunoassay system.

Figure 8.
Surface enhanced immunoassay platform.