Increased Sensitivity of Fluorescence Detection

Using Metallic Nanoparticles

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A new technique called radiative decay engineering can be used to modify fluorescence emissions by changing the free space conditions around the fluorophores.

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The high sensitivity and convenience of fluorescence detection has resulted in its widespread use in biotechnology, drug discovery and genomics. It is well known that fluorophores with the highest absorption coefficients and quantum yields provide the highest sensitivity detection. In using fluorescence probes, typically we accept the spectral properties of the fluorophores emitting into free space, occasionally adjusting the solution conditions to provide increased intensity or increased photostability. This situation is changing with the emergence of a technique called radiative decay engineering (RDE) for modifying the emission by changing the free space conditions around the fluorophores. The use of RDE can increase sensitivity dramatically due to increases in quantum yields and photostability, and the potential application of directional emission (1).

Fundamental Principles

The potential of RDE for increased sensitivity is best understood with a review of some fundamental principles. All fluorophores have an intrinsic rate of emission, called the radiative decay rate (Γ). The radiative decay rate is a transition probability that depends on the absorption or extinction coefficient of the fluorophore (2). Molecules with high extinction coefficients have high emissive rates due to the high transition probability.

The spectral properties of fluorophores usually are described not in terms of the radiative decay rate, but rather in terms of the quantum yields (Q) and lifetimes (τ). The lifetime is determined by the sum of the radiative (Γ) and non-radiative (k) decay rates and is given by

$$\tau = \frac{1}{\Gamma + k} \qquad [1]$$

This equation shows the lifetime of a fluorophore is decreased from the natural lifetime ($\tau_N = 1/\Gamma$) by the non-radiative decay.

The quantum yield is determined by the competition between radiative and nonradiative decay. One can see intuitively that the fraction of absorbed photons that is emitted is given by

$$Q = \frac{\Gamma}{\Gamma + k} \qquad [2]$$

As currently practiced, the researcher has no control of Γ, which is only weakly dependent on the sample conditions. Increased quantum yields are accomplished by removal of quenchers or decreasing the temperature, both of which result in less quenching and decreased rates of radiationless decay.

The potential of radiative decay engineering results from the ability to modify the radiative decay rate or even modify Γ in a desired direction. The rate of emission depends on the photonic mode density around the fluorophore. The photonic mode density can be modified by nearby conducting metallic surfaces or particles, primarily by metallic silver. Simply stated, binding of fluorophores near silver particles can result in dramatically increased intensities, increased fluorophore photostability, modified rates of fluorescence resonance energy transfer (FRET) and increased multi-photon excitation (3–5).

Applications in DNA and Protein Analysis

The usefulness of RDE can be demonstrated by a simple example (6). We examined DNA oligomers labeled with YOYO-1 (Figure 1). Twenty-three mers of biotinylated,

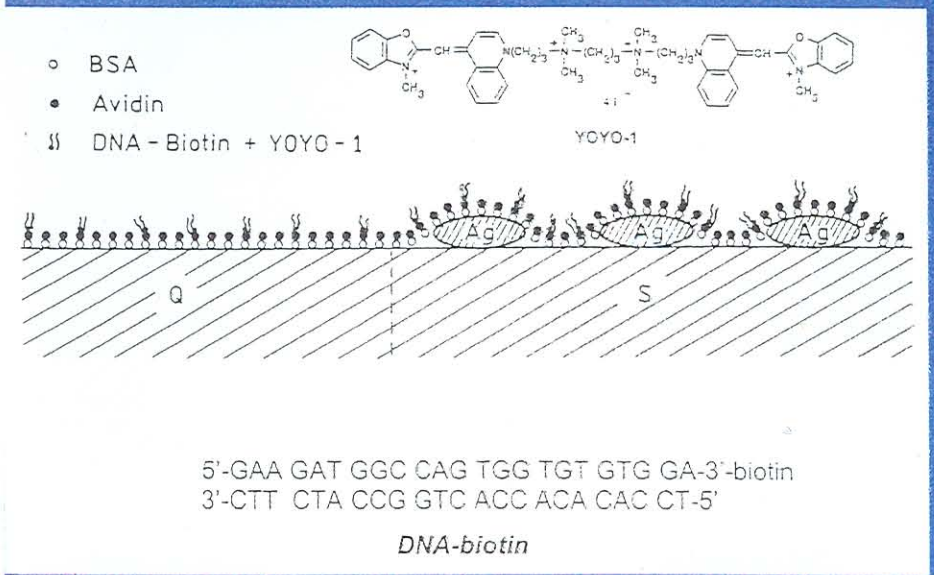

Figure 1. Schematic of surface, sequence of the DNA oligomers and structure of YOYO-1.

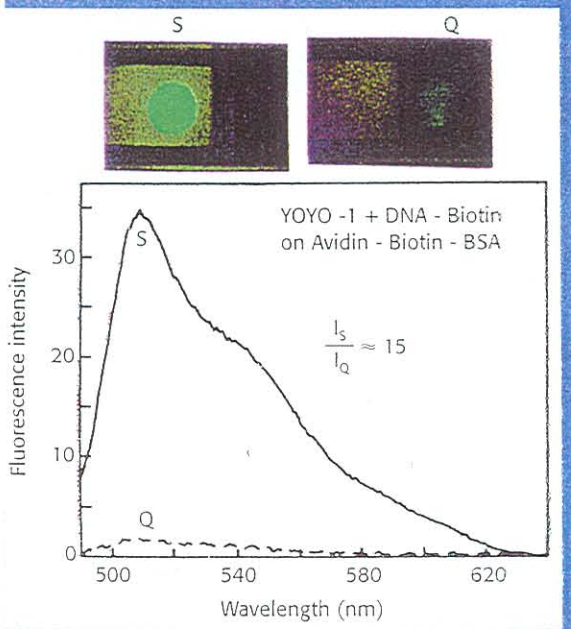

Figure 2. Emission spectra of YOYO-labeled DNA bound to the quartz (Q) and silver island films (S). The upper panels show a real-color photograph of labeled DNA spotted in the silver (left) and quartz (right) surfaces.

double-stranded DNA were bound to a surface that first was coated with bovine serum albumin and then with avidin. The surfaces were either quartz alone or quartz coated with silver particles — the so-called silver island films (SIFs), which often are used in surface-enhanced Raman scattering. These films consist of subwavelength-size particles of silver formed by chemical reduction, probably with an irregular size and shape. The surface-bound oligomers were then labeled with YOYO-1, which binds strongly to double helical DNA.

The dramatic effect of silver on the emission of YOYO-1-DNA can be seen in Figure 2. The top panel shows a photograph of the DNA-coated slide, with (S) and without (Q) silver. The emission is visually brighter on the SIF; the emission spectra shows that the intensity increased 15-fold on the SIFs. Examination of Figure 1 shows that the labeled DNA is bound both to the silver particles and between the silver particles. This suggests that the increased intensity seen in Figure 2 is due to only a fraction of the surface-bound DNA that is on or near the silver particles. We expect to observe larger enhancements for labeled DNA when bound only to the silver particles. From time-resolved measurements we know the lifetime of YOYO-1-DNA is decreased on the SIF. This demonstrates the possibility of increasing the radiative decay rate of a fluorophore by a silver surface.

In some applications, such as fluorescence microscopy and single molecule detection, the detection limit is determined by the photostability of the fluorophore. More photostable fluorophores undergo more excitation cycles prior to bleaching. Typically, we have little control over photobleaching. Remarkably, however, RDE can increase the number of excitation and emission cycles. This effect can be seen in Figure 3, where we examined the emission intensity of YOYO-1-DNA with continuous illumination. The area under the photobleaching curves is proportional to the total number of photons emitted prior to photobleaching. The experiment can be conducted with the same illumination intensity on quartz and silver (top) or with the illumination intensity adjusted to yield the same emission intensity. In either case the result is the same: the signal per fluorophore is increased 17-fold or more by the silver particles. This is due to an increase in the radiative decay rate by a factor γ — a value $\Gamma_m = \gamma\Gamma$, larger than Γ. The lifetime near the metal (τ_m) is now given by

$$\tau_m = \frac{1}{\gamma\,\Gamma + k} = \frac{1}{\Gamma_m + k} \quad [3]$$

Because photochemistry occurs in the excited state, the shorter lifetime results in a smaller probability of photobleaching per excitation cycle. The effect of the particles on the fluorescence intensity can be explained, at least in part, by an increase of Γ to Γ_m. The quantum yield near the silver Q_m is given by

$$Q_m = \frac{\gamma\,\Gamma}{\gamma\,\Gamma + k} = \frac{\Gamma_m}{\Gamma_m + k} \quad [4]$$

The larger radiative decay rate results in a larger fraction of the fluorophores decay by the radiative pathway and, thus, a high quantum yield.

Fluorescence also is used in protein-based assays such as immunoassays (7–8). In such assays, it is desirable to obtain the highest possible signal per antibody molecule. One obvious approach to increased sensitivity is to label each antibody with multiple fluorophores. Unfortunately this usually results in decreased rather than increased intensities. This ef-

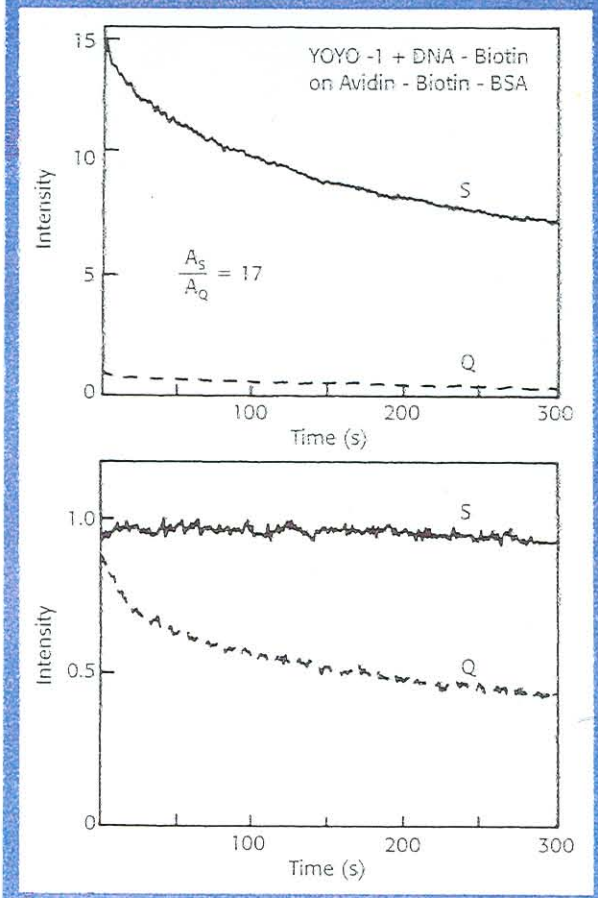

Figure 3. Photostabilities of YOYO-1-DNA on quartz and silver with the same incident power (top) and with the incident power adjusted to yield the same zero time steady-state intensity (bottom).

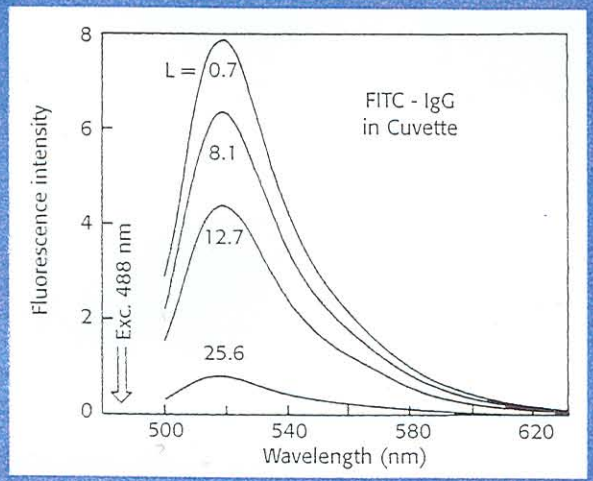

Figure 4. Emission spectra of FITC-labeled IgG in the molar ratio (L) of FITC to IgG. The optical densities were matched at the excitation wavelength of 488 nm.

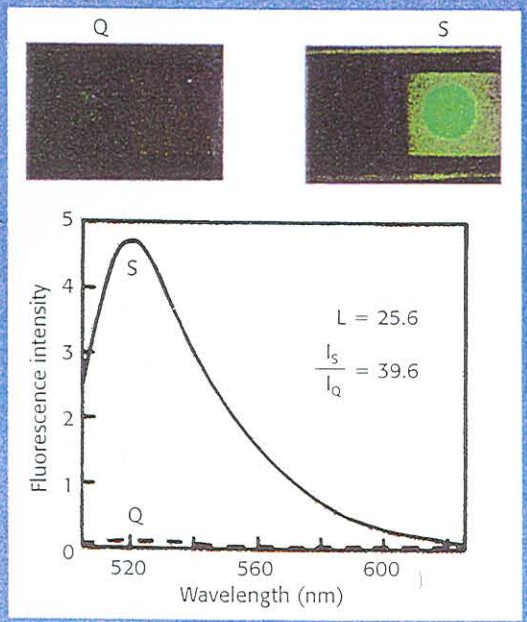

Figure 5. Emission spectra of FITC-IgG on quartz (Q) and silver (S) for L = 0 and 25.6. Excitation at 488 nm. The top panels are photographs of FITC-IgG (L = 8.1) on quartz and silver.

fect is shown in Figure 4, showing a 10-fold decrease in intensity with increased labeling of immunoglobulin G (IgG) with fluorescein isothiocyanate (FITC). When bound to SIFs, this self-quenching largely is reversed (Figure 5), resulting in a dramatically increased intensity (9).

Other Opportunities

The use of metallic surfaces to modify fluorescence is in its infancy, yet it might be useful to speculate about future developments. During the next year we expect rapid progress in three areas: directional emission, mechanical control of FRET and multi-photon excitation.

Fluorescence emission typically is isotropic in all directions. This occurs because the fluorophores have almost a random distribution of orientations, at least in fluid solutions where rotational diffusion is rapid. Another reason the emission is isotropic is the photonic mode density is the same in all directions. Metallic surfaces can be used to increase the photonic mode density and the radiative rate in a desired direction.

Why is directional emission desirable? Because of the limited size of light collection optics, it is only possible to collect a small fraction of the total emission. Suppose the fluorophore is positioned near a thin silver film. For detection, excited fluorophores close to the surface of the emission couple into the metal at the plasmon resonance angle for the emission wavelength (10–12). In effect, the emission is "proximity focused" toward the optical element. The optics could be further designed to focus the light to a detector. Fluorophores more distant from the metal will emit isotropically. As a result, the desired emission from the surface-localized signal can be selectively detected, with discrimination against unwanted signal from distant molecules. A recent publication described directional emission of rhodamine B into a silver-coated prism (13). In addition, researchers in the laboratory at the Center for Fluorescence Spectroscopy at the University of Maryland School of Medicine (Baltimore, Maryland, USA) are applying this phenomenon to biosensing.

Another opportunity might be found in modifying the extent of FRET by the design of the sample chamber, which would provide mechanical control of FRET. This seems possible because the rates of energy transfer can be altered in microcavities (14–16). From preliminary experiments in

the Center for Fluorescence Spectroscopy lab, it appears that the extent of FRET is decreased near one silver surface and increased between two silver surfaces (5). Suppose a sample, labeled with donors and acceptors, flows through a chamber designed to modify FRET. The chamber would contain regions with no silver, with a single silver surface and with two silver surfaces. As the sample flows through the chamber, the extent of FRET will change between the free-space value, the value near one surface and, finally, the increased value between two surfaces. One can imagine new assay formats based on this control of FRET. For example, the donors and acceptors might be separated too widely in a long DNA oligomer for FRET to occur. FRET would be observed for this donor–acceptor pair only in the two-silver surface part of the chamber. More closely spaced donor–acceptor pairs would be detected in the no-silver or one-silver surface parts of the chamber. Further research is needed to determine if such control of FRET is possible.

Finally, RDE provides new opportunities in multi-photon excitations. Two- and three-photon excitation are used widely in fluorescence microscopy and fluorescence correlation spectroscopy (17). Part of the increased emission seen near silver particles is thought to be due to an increased intensity of the incident light field near the particles. This increase can be rather large (10- to 100-fold) or possibly larger (1). Because the extent of two-photon excitation is dependent on the square of the light intensity, large enhancements of the extent of excitation are expected near metallic particles. In fact, reports of increased multiphoton excitations already have appeared near silver particles (4,18). This suggests the possibility of specially designed particles that provide localized multi-photon excitations. Near the metal particles, the emission would be dominated by fluorophores close to the particles. Such particles could provide a new class of probes for optical microscopy or a new class of contrast agents for medical imaging.

Acknowledgments

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