RELEASING THE SELF-QUenching OF FLUORESCENCE NEAR SILVER METALLIC SURFACES

(57) Abstract: Materials, systems and methods for reducing self-quenching due to homo resonance energy transfer when biomolecules are labeled with more than one fluorophore. The materials include a fluorophore labeled biomolecule conjugate and a metallic particle, wherein the labeled biomolecule conjugate comprises multiple fluorophores per biomolecule. The fluorophore-biomolecule conjugate may be bound to the metallic particle or positioned at a distance from the metallic particle, wherein the position of the fluorophore labeled biomolecule conjugate to the metallic particle reduces self-quenching due to homo resonance energy transfer. The methods include detecting a presence of or quantifying an amount of a biomolecule. The fluorophore labeled biomolecules and metallic particles can be used to obtain ultra-bright reagents for use in immunoassays, imaging, arrays and other applications.

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RELEASE OF THE SELF-QUENCHING OF FLUORESCENCE NEAR SILVER METALLIC SURFACES

CROSS REFERENCE TO RELATED APPLICATIONS

[01] This application claims benefit of priority of U.S. provisional application number 60/426,778, entitled "RELEASE OF THE SELF-QUENCHING OF FLUORESCENCE NEAR SILVER METALLIC SURFACES", filed on November 15, 2002, which is incorporated by reference herein in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[02] The work leading to this invention was supported in part by the U.S. Government under grant number RR-08119 awarded by the NIH National Center for Research Resources. This work was also supported by the National Institute for Bioimaging and Bioengineering, NIH-EB00682 and EB-00980, and The Human Genome Institute, HG-002655. Therefore, the U.S. Government may have certain rights in this invention.

BACKGROUND OF THE INVENTION

[03] Fluorescein (FI), rhodamine, cyanines and other fluorophores are widely used as fluorescent probes, for example in microscopy, biotechnology and clinical assays. These probes have the favorable spectral properties of visible absorption and emission wavelengths, high extinction coefficients and reasonable quantum yields. An unfavorable property of these probes is the small Stokes' shift and thus overlap of the absorption and emission spectra. These probes display the undesired property of self-quenching due to homo resonance energy transfer, which results in decreased intensity as the macromolecule is labeled with more than one fluorophore.


[06] One wide use of fluorescein is in fluorescence immunoassays, which are widely used in clinical diagnostics and research (Visor, G. C., and Schulman, S. G.,


[08] It is desirable that the labeled biomolecules be as bright as possible for maximal sensitivity and observability over sample autofluorescence. However, the obvious approach to increasing the molecule brightness by increasing the extent of labeling is not useful due to self-quenching.
BRIEF SUMMARY OF THE INVENTION

[09] In the present invention, it was discovered that most self-quenching can be partially eliminated by proximity of the labeled biomolecule to metallic silver particles. Thus, heavily labeled proteins and metallic colloids can be used to obtain ultra-bright reagents for use in immunoassays, imaging, and other applications.

[10] The present invention includes a material comprising a fluorophore labeled biomolecule conjugate and a metallic particle, wherein said labeled biomolecule conjugate comprises multiple fluorophores per biomolecule and said fluorophore-biomolecule conjugate is bound to said metallic particle. The material may also comprise a substrate with the at least one metallic particle on the substrate. The present invention also includes a system comprising a multi-fluorophore labeled biomolecule conjugate and a metallic particle, where the labeled fluorophore-biomolecule conjugate is positioned at a distance from said metallic particle, wherein the position of said fluorophore labeled biomolecule conjugate to said metallic particle reduces self-quenching due to homo resonance energy transfer.

[11] The present invention includes methods for detecting a presence of or quantifying an amount of a biomolecule, including labeling a biomolecule with multiple fluorophores to form a fluorophore labeled biomolecule conjugate, contacting said fluorophore labeled biomolecule conjugate with a metallic particle and irradiating the substrate to detect the presence of or to measure the amount of said fluorophore labeled biomolecule conjugate in said solution.

[12] The present invention also includes methods for detecting a presence of or quantifying an amount of a biomolecule, including labeling a biomolecule with multiple fluorophores to form a fluorophore labeled biomolecule conjugate, positioning said
fluorophore labeled biomolecule conjugate at a distance apart from a metallic particle, irradiating the substrate to detect the presence of or to measure the amount of said fluorophore labeled biomolecule conjugate in said solution, wherein said position of said fluorophore labeled biomolecule conjugate to said metallic particle reduces self-quenching due to homo resonance energy transfer.

[13] The present invention includes positioning of a biomolecule adjacent to a metal particle or positioning a metal particle adjacent to a biomolecule in the disclosed embodiments.

[14] The present invention provides a method for detecting a biomolecule including the steps of positioning a metal particle and a biomolecule at a distance apart sufficient to manipulate the electromagnetic emission from the biomolecule, exposing the biomolecule to an amount of exciting radiation, and detecting the electromagnetic emission from the biomolecule. The present invention also provides a method for increasing the fluorescence intensity of a fluorescently labeled biomolecule including the steps of labeling a biomolecule with a fluorophore, positioning the labeled biomolecule at a distance apart from a metallic particle such that in response to an amount of exciting radiation, the fluorophore emits radiation.

[15] The biomolecule may be a DNA molecule.


**BRIEF DESCRIPTION OF THE DRAWINGS**

[17] Figure 1. Emission spectra of FITC labeled HSA solutions. The samples had the same fluorescein absorption at 490 nm.
[18] Figure 2. Frequency-domain intensity decays of FITC-HSA with L = 1 and L = 7.

[19] Figure 3. Self-depolarization (top) and self-quenching of FITC-HSA observed in a cuvette.

[20] Figure 4. Absorption spectrum of a SIF on a poly-lysine treated slide.

[21] Figure 5. Absorption spectra of FITC-HSA adsorbed to poly-lysine-coated quartz slides with L = 1 to L = 9.

[22] Figure 6. Emission spectra of FITC-HSA with the degree of labeling L = 1 (top) and L = 7 (bottom) on quartz (Q) and SIFs (S).

[23] Figure 7. Intensity enhancement of FITC-HSA for various degrees of labeling.

[24] Figure 8. Relative intensities of FITC-HSA on quartz (○) and SIFs (●) for various degrees of labeling. Intensities are corrected to the same protein concentration.

[25] Figure 9. Emission spectra of FITC-HSA recorded without emission filter (● ● ●) and through the emission filter used for the time-resolved measurements (———).

[26] Figure 10. Frequency-domain intensity decays of FITC-HSA (L = 1) on quartz (Q, top) and SIFs (S, bottom).

[27] Figure 11. Frequency-domain intensity decays of FITC-HSA (L = 7) on quartz (Q, top) and SIFs (S, bottom). The solid lines represent the multi-exponential fits (Table I). The dashed lines represent the data for L = 1 (Figure 10).
Figure 12A. Emission spectra of a monolayer of FITC-HSA L = 1 (top panel) and L = 7 (bottom panel) containing 0.25 μM rhodamine B between the quartz plates (Q) or one SIF (S).

Figure 12B. Schematic of sample of Figure 12A with bound fluorescein and free rhodamine B.

Figure 13. Photograph of fluorescein-labeled HSA (molar ratio of fluorescein/HSA near 7) on quartz (left) and on a SIF (right) as observed with 430 nm excitation and a 480 nm long pass filter (Corning 3-71).

Figure 14. Experimental sample geometry.

Figure 15. Structures of Fl-DNA and Fl-DNA(Fl)₄. In the right bottom panel is also shown a sample geometry.

Figure 16. Jablonski diagram for free fluorophores (top) and on SIFs (bottom). The fluorescence occurs from a relaxed lowest excited singlet state (S₁).

Figure 17. Top: BSA-avidin monolayer deposited on SIFs. Fl-DNA or Fl-DNA(Fl)₄ were tethered to avidin using the complementary biotinylated oligonucleotide. Bottom: Absorption spectrum of SIFs used in this experiment.

Figure 18. Emission spectra of Fl-DNA and Fl-DNA(Fl)₄ solutions in a cuvette. The absorption of both samples were matched at the excitation wavelength of 495 nm (top). The emission anisotropies are 0.13 and 0.085 for Fl-DNA and Fl-DNA(Fl)₄, respectively. The middle panel shows the emission spectra of Fl-DNA and Fl-DNA-(Fl)₄ with
intensity decays. These lifetimes were measured in frequency-domain. The parameters of decays are in Table I.

[36] Figure 19. Fluorescence emission spectra of Fl-DNA tethered to the BSA-avidin monolayer.

[37] Figure 20. Fluorescence emission spectra of Fl-DNA(Fl)₄ tethered to the BSA-avidin monolayer deposited on quartz and SIFS. Also shown is a photograph of Fl-DNA(Fl)₄ fluorescence.

[38] Figure 21. Time-domain representations of intensity decays of Fl-DNA (top) and Fl-DNA(Fl)₄ (bottom) on quartz (Q) and SIFS (S). These lifetimes were measured in frequency-domain. The decays parameters are in Table I.

[39] Figure 22. Photostability of Fl-DNA(Fl)₄ tethered to the BSA-avidin monolayer deposited on quartz and SIFS. Top: measured with the same excitation power (514 nm, 5 mW focused to a diameter near ~100 μ). Bottom: Measured with the excitation intensities adjusted to yield the same emission intensity.
DETAILED DESCRIPTION OF THE INVENTION

[40] The present invention includes a new approach to increasing the brightness of heavily labeled macromolecules. To illustrate the present invention, experiments with human serum albumin (HSA) labeled covalently with fluorescein-5-isothiocyanate (Isomer I) (FITC) were conducted. HSA was used in testing because of its low cost, stability and affinity for surfaces. In the present invention, other albumin, such as bovine albumin, ovalbumin, and etc. may be used. Other immunoglobulins may also be used.

[41] The emission spectral properties of DNA oligomers labeled with one and multiple fluorescein residues was examined. The emission intensity of the more highly labeled oligomer was decreased due to self-quenching. The self-quenching was mostly eliminated when this oligomer was held about 90 Å from the surface of metallic silver particles. The intensities increased 7 and 19-fold for the oligomers with 1 or 5 fluorescein, respectively. The increased intensity did not result in increased photobleaching. These results suggest the use of substrates coated with silver particles for increased sensitivity on DNA arrays or for DNA analysis.

[42] For DNA as the biomolecule, two or more fluorophores per DNA may be used. Also, 2, 3, 4 or 5 may be used. 5 or more may also be used.

[43] Since the phenomenon of homo RET is a through-space interaction, the results obtained using HSA will be comparable with antibodies and other labeled macromolecules. Fluorescein-labeled HSA when bound to glass (quartz) and to silver island films (SIFs) was examined. The SIFs are sub-wavelength size particles of metallic silver on a glass substrate. The binding of fluorescein-labeled HSA to SIFs was found to eliminate much of the self-quenching. This effect is based on interactions of
the fluorophore with free electrons in the metal, the plasmon absorption, which can result in increased quantum yields and increased rates of radiative decay (Lakowicz, Radiative decay engineering: Biophysical and biomedical applications, Anal. Biochem., 298:1-24 (2001) and Lakowicz et al, Radiative decay engineering 2. Effects of silver island films on fluorescence intensity, lifetimes, and resonance energy transfer, Anal. Biochem., 301:261-277 (2002)). The dramatic difference in the intensity of heavily labeled HSA on glass and on SIFs is shown pictorially in Figure 13 (un-retouched photograph). The effect is dramatic as seen from the nearby invisible intensity on quartz (left) and the bright image on the SIFs (right). Thus, the use of SIFs, including colloidal silver, can be used to obtain dramatically increased intensities of fluorescein-labeled macromolecules.


[45] U.S. Appln. No. 10/073,625 discloses compositions and methods for increasing fluorescence intensity of molecules, including extrinsic fluorophores, which are added to allow molecules that do not ordinarily fluoresce or do not fluoresce at previously commercially useful levels to be detected. U.S. Appln. No. 10/073,625 discloses metal particles and biomolecules positioned at a distance apart sufficient to adjust intrinsic emission of electromagnetic radiation from the biomolecule in response to an amount of exciting electromagnetic radiation.

[46] The term "fluorophore" means any substance that emits electromagnetic energy such as light at a certain wavelength (emission wavelength) when the substance is illuminated or irradiated by radiation of a different wavelength (excitation wavelength).
Extrinsic fluorophores refer to fluorophores bound to another substance. Intrinsic fluorophores refer to substances that are fluorophores themselves. U.S. Appln. No. 10/073,625 (incorporated by reference in its entirety above) discloses exemplary fluorophores.

[47] Exemplary metals include, but are not limited to, rhenium, ruthenium, rhodium, palladium, silver, copper, osmium, iridium, platinum, and gold. Metal particles or metal films are known and can be produced using known methods. U.S. Appln. No. 10/073,625, which is incorporated by reference in its entirety, discloses examples of preparing metal particles and metal films. Silver metal particles and silver colloids are preferable types of metallic particles.

[48] Human serum albumin (HSA) which contained 1 to 9 covalently linked fluorescein molecules per molecule of HSA were examined. The occurrence of homo resonance energy transfer for labeling ratios greater than 1 were confirmed by decreases in the relative quantum yields, anisotropies and lifetimes.

[49] The present invention also relates to a new approach to avoid self-quenching and to increase the brightness of highly labeled oligomers. DNA 23-mers labeled with one or five fluorescein residues were examined. These labeled oligomers were hybridized with a complementary biotinylated oligomer, which in turn was bound to an albumin-biotin-avidin-coated quartz substrate. The double stranded oligomers were examined on glass and on glass coated with metallic silver particles. The sub-wavelength size silver particles were deposited by chemical reduction of silver. These particles are referred to as silver island films (SIFs) and are commonly used in surface enhanced Raman scattering (SERS) (Vo-Dinh et al, Surface-enhanced Raman scattering (SERS) method and instrumentation for
genomics and biomedical analysis, J. Raman Spectroscopy, 30:785-793 (1999), Kneipp, Surface-enhanced Raman scattering: A New tool for biomedical spectroscopy, Current Sci., 77(7):915-924 (1999) and Sokolov et al, Enhancement of molecular fluorescence near the surface of colloidal metal films, Anal. Chem., 70:3898-3905 (1998)). In recent studies it was found that SIFs could result in increased intensities and increased photostability of nearby fluorophores (Lakowicz et al, Radiative decay engineering 2. Effects of silver island films on fluorescence intensity, lifetimes, and resonance energy transfer, Anal. Biochem., 301:261-277 (2002) and Lakowicz, Radiative decay engineering: Biophysical and biomedical applications, Anal. Biochem., 298:1-24 (2001)). In the present invention, it was discovered that the emission intensities were increased 7 and 19-fold for the oligomer labeled with 1 or 5 fluoresceins, respectively. Photostability was also increased near the silver particles. The use of silver particles or silver colloids on DNA array substrates can be used for increased sensitivity.

[50] An array may comprising a substrate and at least one of the inventive fluorophore labeled biomolecule conjugate provided on said substrate at a selected location, a known position. The at least one fluorophore-biomolecule conjugate is may be attached directly to a metallic particle or positioned at a distance from a metallic particle. The metallic may be coated on a surface of said substrate. The biomolecule may be an oligonucleotide. The biomolecule may comprise DNA.

[51] The array may comprise multiple fluorophore labeled biomolecule conjugates wherein each of said fluorophore labeled biomolecule conjugates may be the same or different. And wherein they may have affinity for the same or different analytes. Thus, the array may analyze for or detect multiple analytes within the same sample or may
be used to analyze multiple samples by placing the multiple samples at different selected locations on the array.

**MATERIALS AND METHODS**

**Labeling of HSA:**

[52] 2 mg of human serum albumin (HSA; Sigma) was dissolved in 1ml of 0.1M bicarbonate buffer (pH 9.2) and was mixed with 5 - 90 µl of fluorescein-5-isothiocyanate (FITC; Molecular Probes) solution in DMSO (2 mg FITC/ 200 µl DMSO). The reaction mixture was incubated for 2 hours at room temperature and the labeled protein was separated from the unreacted probe by passing over a Sephadex G-25 column equilibrated with 0.1x PBS.

**Determining the degree of labeling:**

[53] The ratio FITC/HSA in stock solution of labeled protein was determined by independent measurements of dye and protein concentrations, respectively. The amount of FITC was calculated using absorbance of FITC-HSA conjugates in 0.1M bicarbonate buffer (pH 9.2) at 495 nm and molar extinction coefficient of FITC $\varepsilon(495$ nm) = 76,000 M$^{-1}$cm$^{-1}$. The HSA concentration was determined by using Coomassie® Plus Protein Assay Reagent (Pierce, IL, USA). For fluorescence measurements we used the samples with averages FITC/HSA ratios of 1, 3, 7 and 9.

**Preparation of silver island films:**

[54] Silver island film (SIF) on quartz slides was prepared as described previously (Lakowicz et al, Radiative decay engineering 2. Effects of silver island films on fluorescence intensity, lifetimes, and resonance energy transfer, Anal. Biochem., 301:261-277 (2002)). Before SIF deposition quartz slides were covered with poly-lysine
(0.01% poly-lysine in 0.1x PBS buffer spin-coated at 3000 rpm) and only half of each slide was coated with SIF.

**FITC-HSA deposition:**

[55] 250 µl of 10 µM FITC-HSA solution in 0.1x PBS was deposited and on each quartz slide (half coated with SIF) and placed in humid chamber at 5°C overnight. Next, slides were washed 3 times with 0.1x PBS and covered with one part of 0.5 mm demountable cuvette filled up with 0.1x PBS. As judged by absorbance measurement at 490 nm of 0.004 for a layer of FITC-HSA (L = 9), and using ε(490 nm) = 42,500 M$^{-1}$ cm$^{-1}$ for fluorescein in 0.1x PBS the coverage of protein on surface was about 10 pmole/cm$^2$. This density of protein is consistent with a monolayer of protein with an individual diameter of 4 nm.

**Fluorescence Measurements:**

[56] All measurements were performed in using front-face geometry in a 0.5 mm demountable cuvettes between quartz slides (Figure 14). For measurements on quartz and silver, one side of a demountable cuvette was prepared as described above. The cuvette was filled with buffer. HSA binds spontaneously to the quartz surface, and appears to bind to a similar extent to the SIF sample.

[57] Emission spectra were measured using an SLM 8000 spectrofluorometer. For the time-resolved frequency-domain measurements, the 514 nm excitation was obtained from a mode-locked argon ion laser, 76 MHz repetition rate. The emission was observed through a combination long pass 530 nm filter and a 540 nm interference filter, which reduced scattered and/or background to less than 1% of the sample signal. Excitation and emission polarizers were oriented vertically and 54.7° from the vertical, respectively.
[58] The frequency domain (FD) intensity decay were analyzed in terms of the multi-exponential model:

\[ I(t) = \sum_i \alpha_i \exp\left(-t/\tau_i\right) \]  \hspace{1cm} (1)

where \( \tau_i \) are the lifetimes with amplitudes \( \alpha_i \) and \( \sum \alpha_i = 1.0 \). Fitting to the multi-exponential model was performed as described previously (Laczko et al, A 10-GHz frequency-domain fluorometer, Rev. Sci. Instrum., 61:2331-2337 (1990) and Lakowicz et al Analysis of fluorescence decay kinetics from variable-frequency phase shift and modulation data, Biophys. J., 46:463-477 (1994)). The contribution of each component to the steady state intensity is given by:

\[ f_i = \frac{\alpha_i \tau_i}{\sum_j \alpha_j \tau_j} \]  \hspace{1cm} (2)

where the sum in the denominator is over all the decay times and amplitudes. The mean decay time is given by:

\[ \bar{\tau} = \sum_i f_i \tau_i \]  \hspace{1cm} (3).

[59] The amplitude-weighted lifetime is given by:

\[ <\tau> = \sum_i \alpha_i \tau_i \]  \hspace{1cm} (4).

RESULTS

Spectra Properties of FITC-HSA in Solution
While it is widely known that fluorescein bound to proteins is self-quenched, there is surprisingly little published data on this effect (Hemmilä, I. A., Ed., Applications of Fluorescence in Immunoassays, (see. p. 113) John Wiley & Sons, New York, (1991)). Hence we first examined the spectral properties of FITC-HSA with molar labeling ratios (L) ranging from 1-to-1 (L=1) to 1-to-9 (L=9). Emission spectra are shown in Figure 1 for samples which had the same optical density at 490 nm. The relative intensity decreased progressively with increased labeling. The insert in Figure 1 shows the intensities normalized to the same amount of protein, so that the relative fluorescein concentration increases 9-fold along the x-axis. It is important to notice that the intensity per labeled protein molecule does not increase, and in fact decreases, as the labeling ratio is increased from 1 to 9.

Self-quenching of fluorescein is expected to decrease the lifetimes, at least under some conditions. Frequency-domain intensity decays are shown in Figure 2 for labeling ratios of 1 (top) and 7 (bottom). A decreased lifetime is evident from the frequency response shifted to higher frequency. The amplitude-weighted lifetime decreased nearly 4-fold for the higher labeling ratio L = 7 (Figure 2, bottom). Additionally, the intensity decay becomes more heterogeneous, as can be seen from the need for these decay times to fit the data (Table I). Fluorescence depolarization is one of the most reliable indicators of homo transfer (Jablonski, J., Self-depolarization and decay of photoluminescence of solutions, Acta. Physica. Polonica, XIV:295-307 (1955), Jablonski, J., Depolarization of fluorescence of isotropic solutions produced by excitation transfer between luminescent molecules, Acta Physica. Polonica, A41:86-90 (1972), Knox, R. S., Theory of polarization quenching by excitation transfer, Physica, 39:361-386 (1968), Bojarski, C., Theory of concentration depolarization of

**Emission Spectra of FITC-HSA on SIFs**

[62] The effects of metallic silver were examined using SIFs on quartz. The slides were first coated with poly-lysine, which resulted in shorter wavelength plasmon absorption maximum and a less wide absorption then obtained previously on quartz without poly-lysine (Lakowicz et al, Radiative decay engineering 2. Effects of silver island films on fluorescence intensity, lifetimes, and resonance energy transfer, Anal. Biochem., 301:261-277 (2002)) (Figure 4). Additionally, the silver particles adhered more strongly to the poly-lysine treated slides than to untreated slides.

[63] The absorption spectra of FITC-HSA on quartz was examined to estimate the amount bound. HSA is known to bind passively to glass surfaces and to form essentially a complete monolayer (Sokolov et al, Enhancement of molecular fluorescence near the surface of colloidal metal films, Anal. Chem., 70:3898-3905 (1998)). For quartz coated with labeled HSA the fluorescein absorption increased roughly linearly with the degree of labeling (Figure 5). By measuring the absorbance of 0.004 for L = 9 we estimate an absorbance near 0.0004 for a monolayer of FITC-HSA with L = 1, confirming the presence of a bound monolayer on the quartz surface.
The emission spectra of FITC-HSA on quartz and SIFs was examined (Figure 6). The emission intensity for $L = 1$ was increased about 4-fold, and for $L = 7$ the intensity was increased 17-fold. The enhancement factor ($I_L/I_0$) increased as the degree of labeling increased (Figure 7). These results show that proximity of FITC-HSA to metallic silver particles can partially eliminate the self-quenching of fluorescein.

[64] The proximity to metal may be controlled by film spacer layers. See U.S. Appln. No. 10/073,625 incorporated by reference above.

[65] Consider the intensity per labeled protein molecule (Figure 8) in the above, in the absence of SIFs the intensity per protein molecule for FITC-HSA bound to quartz decreases as the extent of labeling increases ($o$) with the maximum intensity seen for an average labeling of fluorescein per HSA molecule $L = 1$. For FITC-HSA bound to a SIF the intensity is greater than on quartz for labeling ratios from 1 to 9. The maximum intensity was found for 3 to 6 fluorescein per HSA molecule. The intensities which can be expected for FITC-HSA in the absence of self-quenching were considered (Table II). For FITC-HSA on quartz the measured intensity decreases to 3% of the expected value for $L = 9$. For FITC-HSA on SIFs the measured intensities do not match the expected values in the absence of self-quenching. However, a significantly larger percentage of the expected intensity is found on the SIFs, 47% at $L = 3$ and 12% at $L = 9$. In the present experiments HSA is directly bound to the SIFs without an inert spaces. It is possible that a larger percentage of the maximum intensity, or less self-quenching, may be obtained with modification of the surface chemistry and/or distance to the surface.

[66] The intensity decays of FITC-HSA on the surfaces was examined. To be certain that scattered light did not contribute to the intensity decays the emission spectra under
the precise conditions used for the frequency-domain measurement were recorded (Figure 9). More specifically, we recorded the emission spectra through the filters used for the time-resolved measurements and scanned through the 514 nm excitation wavelength. These spectra showed that scattered light contributed less than 1% to the intensity decay measurements.

[67] Frequency-domain intensity decays are shown for FITC-HSA for L = 1 in Figure 10 and for L = 7 in Figure 11. For L = 1 the amplitude-weighted lifetime is reduced 11-fold on the SIFs as compared to quartz. For L = 7 the amplitude-weighted lifetime is reduced 5-fold relative to quartz. The smaller reduction in lifetime for L = 7 suggests a reason for the inability to obtain the expected intensities (Table II). As the result of a reduced lifetime for the heavily labeled sample the increase in the radiative rate does not completely compensate for the shorter lifetime. This is supported by consideration of the relationship between quantum yields and lifetimes. It is well known that the

\[ Q = \frac{\Gamma}{\Gamma + k} \]  

(5)

quantum yields \( Q \) and lifetime \( \tau \) of a fluorophore are given by:

\[ \tau = \frac{I}{\Gamma + k} \]  

(6)

where \( \Gamma \) is the radiative decay rate and \( k \) is the sum of the non-radiative decay rates.

The radiative decay rate can be obtained from:

\[ \frac{Q}{\tau} = \Gamma \]  

(7).
Assume that Equations 5-7 describe the spectral properties of FITC-HSA on quartz (Q) and that a similar set of equations describe the spectral properties on SIFs (S). Then the ratio of the radiative decay rates on silver ($\Gamma_s$) and quartz ($\Gamma_Q$) can be obtained from

$$\frac{\Gamma_s}{\Gamma_Q} = \frac{(Q/\tau)_s}{(Q/\tau)_Q} \quad (8).$$

This equation assumes the values of $k$ are not altered by the silver. Using the data in Table II, and using the intensities as quantum yields, the radiative decay rate increases 51-fold for $L = 1$ and 87-fold for $L = 9$ were calculated. This indicates that the metal-induced increases in the rate of radiative decay does not completely offset the decreases in lifetime at higher degrees of labeling. Conversely, if the rate of radiative decay can be increased further then one may obtain the higher expected intensities summarized in Table II.

DISCUSSION

It is informative to consider how silver-enhanced fluorescence, particularly of a heavily labeled sample, can be used for improved assays. Figure 12 shows emission spectra of a quartz plate coated with FITC-HAS, with the concentration of rhodamine B (RhB) (0.25 μM) adjusted to result in an approximate 1.5-fold larger RhB intensity. One can consider the RhB to be sample autofluorescence on any other interference signal. When the same conditions are used for FITC-HSA on silver with $L = 1$ the fluorescein emission is now 2 to 3-fold higher than of RhB, (Figure 12, top). When using the heavily labeled sample ($L = 7$) the fluorescein emission becomes dominant (Figure 12, bottom).

Labeling of DNA:
DNA 23-mers labeled with one or five fluorescein residues were examined. These labeled oligomers were hybridized with a complementary biotinylated oligomer, which in turn was bound to an albumin-biotin-avidin-coated quartz substrate. The double stranded oligomers were examined on glass and on glass coated with metallic silver particles. The sub-wavelength size silver particles were deposited by chemical reduction of silver. These particles are referred to as silver island films (SIFs) and are commonly used in surface enhanced Raman scattering (SERS) (Vo-Dinh et al., *Surface-enhanced Raman scattering (SERS) method and instrumentation for genomics and biomedical analysis*, J. *Raman Spectroscopy*, 30:785-793 (1999); Kneipp, *Surface-enhanced Raman scattering: A New tool for biomedical spectroscopy*, *Current Sci.*, 77(7):915-924 (1999) and Sokolov et al, Enhancement of molecular fluorescence near the surface of colloidal metal films, *Anal. Chem.*, 70:3898-3905 (1998)). In recent studies we found that SIFs could result in increased intensities and increased photostability of nearby fluorophores (Lakowicz et al, *Radiative decay engineering 2. Effects of silver island films on fluorescence intensity, lifetimes, and resonance energy transfer*, *Anal. Biochem.*, 301:261-277 (2002) and Lakowicz, *Radiative decay engineering: Biophysical and biomedical applications*, *Anal. Biochem.*, 298:1-24 (2001)). In the present invention, the emission intensities were increased 7 and 19-fold for the oligomer labeled with 1 or 5 fluoresceins, respectively. Photostability was also increased near the silver particles. The use of silver particles or silver colloids on DNA array substrates may be used for increased sensitivity.

**MATERIALS AND METHODS**

**Sample Preparation**
All oligonucleotides were obtained from the Biopolymer Core Facility at the University of Maryland School of Medicine (FIGURE 15). Silver island films on quartz slides were prepared as described previously (Sokolov et al, Enhancement of molecular fluorescence near the surface of colloidal metal films, Anal. Chem., 70:3898-3905 (1998) and Lakowicz et al, Radiative decay engineering 2. Effects of silver island films on fluorescence intensity, lifetimes, and resonance energy transfer, Anal. Biochem., 301:261-277 (2002)). Briefly, the quartz slides were soaked in a 10:1 (v/v) mixture of H₂O₂ (30%) overnight before the deposition, washed with distilled water, and air-dried prior to use. Silver deposition was carried out in a clean beaker equipped with a Teflon-coated stir bar. Eight drops of fresh 5% NaOH solution were added to a fast stirring silver nitrate solution (0.22g in 26 ml of water). Dark-brownish precipitates were formed immediately. Less than 1 ml of ammonium hydroxide was then added drop by drop to redissolve the precipitate. The clear solution was cooled to 5°C in an ice bath, followed by placing the clean quartz slides in the solution. At 5°C, a fresh solution of D-glucose (0.35 g in 4 ml of water) was added. The mixture was stirred for 2 min at that temperature. The beaker was removed from the ice bath and allowed to warm up to 30°C. As the color of the mixture turned from yellow-greenish to yellow-brown the color of the slides became greenish. The slides were removed and rinsed with water and bath sonicated for 1 min at room temperature. After rinsing with water the slides were stored in water prior to the experiments. Each slide (12.5 mm x 45 mm, half coated with silver island film) was covered with 250 µl of 10 µM biotinylated bovine serum albumin (BSA-biotin, Sigma) aqueous solution and placed in a humid chamber for 20 hours (5°C, cold room). After washing 3 times with water the slides were placed again in the humid chamber. 250 µl of 5 µM avidin (egg white, Molecular Probes) in 0.1 x PBS
buffer was deposited on each BSA-biotin-coated surface for 40 min at room temp. Slides were then washed 3 times with 0.1 x PBS buffer. This procedure is thought to result in a monolayer of surface bound BSA coated with a monolayer of avidin (Sokolov et al, Enhancement of molecular fluorescence near the surface of colloidal metal films, *Anal. Chem.*, 70:3898-3905 (1998) and Malicka et al, Effects of fluorophore-to-silver distance on the emission of cyanine-dye labeled oligonucleotides, *Anal. Biochem.*, in press (2002)).

**[73]** Solutions of double-stranded DNA (ds-DNA) samples (F1-DNA, Fl-DNA-(Fl)₄ and biotin-DNA) were prepared by mixing complementary oligonucleotides in 5 mM Hepes (pH 7.5), 0.1 M KCl and 0.25 mM EDTA buffer to final concentration 2 μM, followed by very slow cooling after incubation at 70°C for 2 min. 250 μl solution of ds-DNA was deposited on each BSA-biotin-avidin-coated surface for 1 hour at room temp. The slides were then washed and placed in the buffer solution (5 mM Hepes (pH 7.5), 0.1 M KCl and 0.25 mM EDTA) for 15 min. To protect from scratching during measurements we covered each sample (ds-DNA on a layer of BSA-biotin avidin) with a 0.5 mm demountable cuvette filled up with above buffer, as shown on Figure 15, bottom right. The cuvette was mounted on a translation stage.

**Fluorescence measurements**

**[74]** Emission spectra were collected in front face geometry on SLM 8000 spectrofluorometer with excitation 514 nm from argon ion laser or 495 nm from a Xenon lamp. Lifetimes were measured on 10 GHz frequency-domain fluorometer (Laczko et al, A 10 GHz frequency-domain fluorometer, *Rev. Sci. Instrum.*, 61:2331-2337 (1990)) using mode-locked argon ion laser 514 nm, 76 MHz repetition time, 120 ps pulse width. Excitation and emission polarizers were in the magic-angle orientation. Emission was
selected with combination of 520 nm long pass liquid chromate filter (CrO$_4^{2-}$/Cr$_2$O$_7^{2-}$, 0.3 M, pH 8) placed in 2 mM, 1" x 1", quartz cuvette and interference filter at 540 nm. Such combination of filters rejects efficiently scattered light and has minimal internal luminescence. We estimated ratios of the emission signals from samples without and with fluorescein fluorophores (background). The background from BSA-avidin coated SIF containing unlabeled biotin-DNA was less than 1% for both Fl-DNA and Fl-DNA(Fl)$_4$. Similarly, the backgrounds on quartz were less than 2% and 1% for Fl-DNA and Fl-DNA-(Fl)$_4$, respectively.

75] The FD intensity decay were analyzed in terms of the multi-exponential model. See Equation 1. Fitting to the multi-exponential model was performed as described previously (Laczko et al, A 10 GHz frequency-domain fluorometer, Rev. Sci. Instrum., 61:2331-2337 (1990)).

**Effect of SIFs on Fluorescence**

76] In a close proximity, up to about 50 Å fluorophore emission is strongly quenched by metallic surface. The emission of fluorophores near SIF but outside the quenching region depends on two major factors, enhanced local field and an increase of intrinsic decay rate of the fluorophore. The first factor provides stronger excitation rates. The second factor changes quantum yield and lifetime of the fluorophore. The observed fluorescence enhancement

\[ G \sim G_{ex} G_{QY} \]

77] where \( G_{QY} = Q_m / Q_0 \) is the increase in quantum yield of fluorophore near SIF.

78] Figure 16 shows the energy diagrams for molecules in the absence and presence of SIF. In the absence of SIF, the quantum yield and lifetime are given by:

\[ Q_0 = \Gamma / (\Gamma + k_{ar}) \]
\[ \tau_0 = \frac{1}{\Gamma + K_{nr}} \]

where \( \Gamma \) is a radiative rate and \( K_{nr} \) is non-radiative rate. In the presence of SIF, the quantum yield and lifetime are given by

\[ Q_m = \frac{\Gamma}{\Gamma + \Gamma_m + K_{nr} + K_m} = \frac{\Gamma}{\Gamma (1 + \gamma) + K_{nr} + K_m} \]

\[ \tau_m = \frac{1}{\Gamma + \Gamma_m + K_{nr} + K_m} = \frac{1}{\Gamma (1 + \gamma) + K_{nr} + K_m} \]

where \( \Gamma_m \) and \( K_m \) are radiative and nonradiative rates induced by metal.

[79] Increase in radiative rate near SIF results in increased quantum yield and decreased lifetime. For more details see (Lakowicz, Radiative decay engineering; Biophysical and biomedical applications, Anal. Biochem., 298:1-24 (2001)).

RESULTS

Sample Configuration

[80] The labeled oligomers are shown in Figure 15. The first oligomer had one fluorescein residue on the 3′-end (Fl-DNA). The second labeled oligomer contained a fluorescein residue at the same 3′-end plus 4 additional fluorescein residues (Fl-DNA (Fl)4) distributed along the sequence. These oligomers were tethered to the avidin-coated substrate by a complementary oligomer containing a biotin residue on the 5′-end.

[81] Following hybridization of the fluorescein and biotin labeled oligomers the double stranded DNA was bound to the slides via a double layer of proteins (Figure 17). The slides were first coated with a monolayer of biotinylated BSA ((Sokolov et al, Enhancement of molecular fluorescence near the surface of colloidal metal films, Anal. Chem., 70:3898-3905 (1998)) followed by avidin. The half of the slides coated with
silver displayed the expected plasmon absorption (Figure 17, bottom). From previous studies we found roughly the same surface concentration of albumin on the quartz and SIF surfaces (Sokolov et al, Enhancement of molecular fluorescence near the surface of colloidal metal films, Anal. Chem., 70:3898-3905 (1998) and Malicka et al, Effects of fluorophore-to-silver distance on the emission of cyanine-dye labeled oligonucleotides, Anal. Biochem., in press (2002)). The absorptions of high extinction cyanine dyes linked to BSA-Avidin coated, silvered and unsilvered surfaces were examined and no significant difference was noticed. Hence it is highly likely that roughly the same amounts of double stranded DNA are bound to both surfaces. The observed DNA should only be the fluorescein-labeled double stranded DNA. Single stranded biotin-DNA would not contribute to the signal and single stranded fluorescein labeled DNA without biotin is not expected to bind to the surfaces.

[82] The albumin-avidin layer deposited on SIF and on quartz provides a spacer between metal and probe. The thickness of this layer about 90Å gives us the opportunity to place our dye in the area of maximum enhancement (Malicka et al, Effects of fluorophore-to-silver distance on the emission of cyanine-dye labeled oligonucleotides, Anal. Biochem., in press (2002)). Direct deposition of our fluorescein-labeled DNA on SIF could result in partial quenching, which occurs, up to 50Å from the metallic particles.

Spectral Properties of the Fluorescein-Labeled Oligomers in Solution

[83] Prior to examining surface-bound DNA, the double stranded DNA in solution was examined. The emission spectra of FI-DNA(Fl)4 showed a two-fold decrease in intensity as compared to FI-DNA (Figure 18, top). For these spectra the optical densities at the excitation wavelength of 495 nm were identical. If the concentrations
of the samples were adjusted to the same amount of DNA, the Fl-DNA (Fl)₄ would show only two fold higher brightness than Fl-DNA, instead 5 fold expected for unquenched system (Figure 18, middle). This result shows that one cannot significantly obtain increased intensities from fluorescein-labeled DNA by increasing the extent of labeling for short oligonucleotides. The steady state anisotropies of these oligomers were 0.13 and 0.085, Fl-DNA and Fl-DNA-(Fl)₄, respectively. This loss of anisotropy is consistent with RET between the fluorescein residues. Following the homo-transfer act, emission occurs from fluorophores with different orientation of transition moments, e.g. fluorescence is depolarized. The loss of anisotropy is considered as a manifestation of energy transfer between identical molecules in rigid medium (Jablonski, Self-depolarization and decay of photoluminescence of solutions, Acta. Physica. Polonica, XIV:295-307 (1955), Jablonski, Depolarization of fluorescence of isotropic solutions produced by excitation transfer between luminescent molecules, Acta Physica. Polonica, A41:86-90 (1972), Knox, Theory of polarization quenching by excitation transfer, Physica, 39:361-386 (1968), Bojarski, Theory of concentration depolarization of photoluminescence and intermolecular energy transfer in rigid solutions, J. Luminescence, 5:413-429 (1972), Dale and Bauer, Concentration depolarization of the fluorescence of dyestuffs in viscous solution, Acta. Physica. Polonica, A40:853-882 (1971) and Kawski, Excitation energy transfer and its manifestation in isotropic media, Photochem. Photobiol., 38(4):487-508 (1983)).

[84] The intensity decays of the double stranded oligomers in homogeneous solution was also examined. The amplitude-weighted lifetime of Fl-DNA was near 3.6 ns, as expected for an isolated fluorescein residue. For Fl-DNA-(Fl)₄ the mean lifetime was
reduced to 1.5 ns (Figure 18, bottom), an indication of self-quenching between the fluorescein residues.

**Spectral Properties of Fluorescein-Labeled Oligomers Bound to Quartz and Silver Island Films**

[85] The emission spectra of FI-DNA and FI-DNA(Fl)$_4$ when bound to quartz and silver island films were examined, Figures 19 and 20, respectively. When normalized, the spectra on SIF and quartz are essentially identical. For the oligomer with a single fluorescein residue the intensity increases 7-fold on SIF as compared to quartz. This intensity increase may be due to a combination of both an increased quantum yield of FI-DNA on the SIF surface, with a possible increased rate of excitation due to an enhanced local field near the metal particles (Lakowicz, Radiative decay engineering: Biophysical and biomedical applications, Anal. Biochem., 298:1-24 (2001)). The quantum yield of FI-DNA in hepes buffer, pH 7.5 is about 0.44. The increased quantum yield can be responsible for about 2.2 fold enhancement of FI-DNA emission on SIFs. A more dramatic intensity increase of 19-fold was observed for FI-DNA-(Fl)$_4$ on the SIF as compared to quartz (Figure 20). The larger intensity increase observed for FI-DNA-(Fl)$_4$ as compared to FI-DNA, is consistent with the lower solution quantum yield of the more heavily labeled oligomer. Note that a mean lifetime of FI-DNA-(Fl)$_4$ on quartz is about 3.5 fold shorter than for FI-DNA (Table III). The increased intensity is nicely shown by a color photograph of the labeled DNA when illuminated with a laser beam at 440 nm (Figure 20, top). The laser beam was positioned either on the SIF (Figure 20, left) on the quartz (Figure 20, right), or on both (Figure 20, center image). This photograph has not been adjusted for intensity or contrast.
In previous reports we noticed that the lifetimes of fluorophores near quartz surfaces are usually lower than the lifetimes in solution (Lakowicz et al, Radiative decay engineering 2. Effects of silver island films on fluorescence intensity, lifetimes, and resonance energy transfer, Anal. Biochem., 301:261-277 (2002), Malicka et al, Metal-enhanced emission from indocyanine green: A new approach to in-vivo imaging, J. Biomed Optics, in press (2003) and Geddes et al, Metal-enhanced fluorescence (MEF) due to silver colloids on a planar surface: potential applications of indocyanine green to in vivo imaging, J. Phys. Chem., in press (2003)). We believe the effect is partly due to the higher refractive index of quartz for part of the space near the fluorophore. Also, the density of labeled DNA could be higher on surface monolayer than in solution resulting in interaction with fluoresceins from other DNAs, which increases a homotransfer. The lifetimes of both labeled oligomers on quartz were examined (Figure 21, solid lines). The amplitude-weighed lifetimes were reduced about 2-fold relative to the lifetimes in solution (Table III).

The increase in intensity on SIFs is believed to be due at least in part to increase in the radiative decay rate near the silver particles (Lakowicz et al, Radiative decay engineering 2. Effects of silver island films on fluorescence intensity, lifetimes, and resonance energy transfer, Anal. Biochem., 301:261-277 (2002)), which would result in decreased lifetimes. For the singly labeled oligomer the lifetime decreased 10-fold on silver as compared to quartz (Figure 21, top). For the heavily labeled oligomer the amplitude-weighed lifetime decreased 6-fold (Figure 21, bottom) as compared to quartz (Table III). A larger relative decrease in lifetime is expected for the higher quantum yield sample. This is because the same increase in the radiative decay rate
will have a smaller effect when competing with the larger non-radiative rate for a more highly quenched fluorophore.

[88] As described previously (Lakowicz et al, Radiative decay engineering 2. Effects of silver island films on fluorescence intensity, lifetimes, and resonance energy transfer, *Anal. Biochem.*, 301:261-277 (2002)), decreases in lifetimes are expected to result in increased photostability. This effect occurs because the shorter lifetimes result in less time in the excited state, and hence less time for photochemical reaction. The decreased lifetime is not expected to change the probability of photobleaching per unit time, but it will reduce the probability per excitation-relaxation cycle. Hence the emission intensity with continuous illumination should be more constant for a shorter lifetime but otherwise comparable fluorophore. On the other hand, fluorophores near SIF are exposed to the enhanced local field and should be more prompt to the photobleaching. The measured photostability will be affected by these two factors.

[89] The steady state emission intensity of the highly labeled oligomer with continuous illumination was examined (*Figure 22*). When observed with the same incident intensity the relative emission intensity for FL-DNA-(Fl)_4 decreased more rapidly on the SIF than on quartz (top). However, intensity on the SIF remained greater at all times up to 300 sec. Suppose the increased intensity seen on the SIF was due to an increased rate of excitation rather than an increase in quantum yield. In this case the rate of photobleaching would increase by the same factor as the intensity increased, which did not occur. The number of photons detectable per fluorophore is expected to be proportional to the area under the curves in *Figure 22*. This suggest that for FL-DNA-(Fl)_4 the total detectable signal per fluorophore is increased 12-fold or more.
[90] An alternative approach for examining photostability is to compare the samples on SIFs and quartz illuminated with adjusted laser power to weld the same emission intensity at time zero. This comparison was accomplished by attenuating the intensity incident on the brighter region of the slide with the SIF. We found roughly equivalent relative rates of photobleaching (Figure 22, bottom). Since the same signal level is obtained with a lower excitation intensity, it is probable that background signal from the instrument and optics, or autofluorescence from the sample distant from the surface, will be decreased by the same attenuating factor. Hence, the SIFs can be used to increase the signal-to-background level on DNA arrays.

DISCUSSION

[91] The results described in the previous paragraph show that it is possible to significantly increase a brightness of the sample near the SIF. Assuming arbitrarily the brightness of Fl-DNA on quartz as a 1, the brightness of Fl-DNA (Fl)₄ on quartz is only 2 instead of expected 5, due to self-quenching. Taking into account measured enhancement, the brightness in SIFs are 7 (1x7) and 38 (2x19) for Fl-DNA and Fl-DNA-(Fl)₄, respectively. Assuming that all 5 fluoresceins in Fl-DNA-(Fl)₄ were equal, the maximal brightness would be 5x7 = 35.

[92] We believe that fluorophore - SIFs spacing used in our experiment is close to optimal. In our earlier study, we investigated the dependence of fluorescence enhancement of SIF-cyanine dyes separation by building consecutive BSA-Avidin layers (Malicka et al, Effects of fluorophore-to-silver distance on the emission of cyanine-dye labeled oligonucleotides, Anal. Biochem., in press (2002)). The strongest enhancement for one BSA-Avidin spacer, e.g. for fluorophore-SIF distance was about 90 Å.
The present invention can be immediately applied to DNA arrays. A wide variety of approaches are available to deposit silver on surfaces, including illumination of silver nitrate solutions or passage of current between silver electrodes (Abid et al, Preparation of silver nanoparticles in solution from a silver salt by laser irradiation, Chem. Commun., 7:792-793 (2002), Pastoriza-Santos et al, Self-assembly of silver particle monolayers on glass from Ag⁺ solutions in DMF, J. Colloid and Interface Surf., 221:236-241 (2000), Bell and Myrick, Preparation and characterization of nanoscale silver colloids by two novel synthetic routes, J. Colloid Interface Sci., 242:300-305 (2001) and Geddes et al, Photodeposition of silver can result in metal-enhanced fluorescence, Appl. Spectroscopy, in press. (2002)). Furthermore, we found that silver deposition occurs more rapidly on surfaces coated with amino groups (Geddes et al, Photodeposition of silver can result in metal-enhanced fluorescence, Appl. Spectroscopy, in press. (2002)), which are commonly used in DNA arrays. Additionally, amine-coated surfaces spontaneously bind silver colloids from suspensions, and the bound colloids also enhance fluorescein. These procedures are not expensive. Therefore, with the present invention, silver coated substrates may be widely used for DNA analysis.

In conclusion, silver particles or silver colloids, when bound to heavily labeled fluorophores, can provide significantly higher intensities due to a decrease in the content of self-quenching.

Although the invention has been described with respect to specific embodiments, the details are not to be construed as limitations, for it will become apparent that various embodiments, changes and modifications may be resorted to without departing from the spirit and scope thereof, and it is understood that such equivalent embodiments are intended to be included within the scope of this invention.
[96] All of the patent documents, references and journal articles cited are hereby incorporated by reference herein in their entirety.
### Table I. Multi-exponential decay analysis of FITC-HSA

<table>
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<tr>
<th>Conditions</th>
<th>L</th>
<th>$\langle \tau \rangle$ (ns)</th>
<th>$\tau$ (ns)</th>
<th>$a_i$</th>
<th>$f_i$</th>
<th>$\tau_i$ (ns)</th>
<th>$\chi^2$</th>
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<td>Cuvette</td>
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<td>2.37</td>
<td>3.12</td>
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<td>0.91</td>
<td>0.8</td>
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<td>7</td>
<td>0.64</td>
<td>1.25</td>
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<td>0.087</td>
<td>0.16</td>
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<td>0.517</td>
<td>0.457</td>
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<td></td>
<td>0.135</td>
<td>0.456</td>
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<td>Quartz</td>
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<td>7</td>
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<td>0.907</td>
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<td></td>
<td>0.085</td>
<td>0.270</td>
<td>0.242</td>
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<td></td>
<td>0.008</td>
<td>0.161</td>
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<td>SIF</td>
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<td>0.006</td>
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### Table II. Summary of measured and calculated intensities for FITC-HSA

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<tr>
<th>Labeling</th>
<th>$I_s/I_Q$</th>
<th>$&lt;\tau_Q&gt;/&lt;\tau_S&gt;$</th>
<th>Quartz</th>
<th>Silver</th>
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<tbody>
<tr>
<td></td>
<td>Measured</td>
<td>Max</td>
<td>%</td>
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<td>1</td>
<td>4.47</td>
<td>11.34</td>
<td>1,600</td>
<td>1,600</td>
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<tr>
<td>3</td>
<td>10.63</td>
<td>-</td>
<td>950</td>
<td>4,800</td>
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<td>7</td>
<td>16.80</td>
<td>4.81</td>
<td>640</td>
<td>11,200</td>
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<tr>
<td>9</td>
<td>16.84</td>
<td>-</td>
<td>475</td>
<td>14,400</td>
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Table III. Multi-exponential analysis of fluorescein labeled DNA frequency-domain intensity decay

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<tr>
<th>Compound/conditions</th>
<th>$&lt;\tau&gt;$ (ns)</th>
<th>$\overline{\tau}$ (ns)</th>
<th>$\alpha_i$</th>
<th>$f_i$</th>
<th>$\tau_i$ (ns)</th>
<th>$\chi^2_{R}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>FI-DNA, cuvette</td>
<td>3.58&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.188</td>
<td>0.024</td>
<td>0.46</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>0.812</td>
<td>0.976</td>
<td>4.30</td>
<td>1.0 (72.6)&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>FI-DNA(Fl)&lt;sub&gt;4&lt;/sub&gt;, cuvette</td>
<td>1.47</td>
<td>2.79</td>
<td>0.308</td>
<td>0.022</td>
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<td>0.363</td>
<td>0.810</td>
<td>3.28</td>
<td>1.1 (511.5)</td>
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<td>2.081</td>
<td>3.517</td>
<td>0.286</td>
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<td>0.206</td>
<td>0.069</td>
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<td>0.508</td>
<td>0.920</td>
<td>3.766</td>
<td>1.0 (325.6)</td>
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<td>0.596</td>
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<td>2.0 (1018.4)</td>
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<td></td>
<td>0.012</td>
<td>0.177</td>
<td>1.610</td>
<td>1.1 (582.3)</td>
</tr>
</tbody>
</table>

<sup>a</sup> $<\tau> = \sum_i \alpha_i \tau_i$

<sup>b</sup> $\overline{\tau} = \sum_i f_i \tau_i$, $f_i = \frac{\alpha_i \tau_i}{\overline{\alpha}_{subj} \overline{\tau}_j}$

<sup>c</sup> For the best single decay time fit.
What is claimed is:

1. A material comprising
   a fluorophore labeled biomolecule conjugate and
   a metallic particle,
   wherein said labeled biomolecule conjugate comprises multiple fluorophores per
   biomolecule and said fluorophore-biomolecule conjugate is bound to said metallic
   particle.

2. A material comprising
   a substrate,
   at least one metallic particle on a surface of said substrate,
   a fluorophore labeled biomolecule conjugate coated on said at least one metallic
   particle and a fluorophore labeled biomolecule conjugate coated on said surface of said
   substrate
   wherein said labeled biomolecule conjugate comprises multiple fluorophores per
   biomolecule.

3. The material of claim 1 or 2, wherein said fluorophore is selected from the
   group consisting of a fluorescein, rhodamine and a cyanine.

4. The material of claim 3, wherein said fluorophore is fluorescein-5-
   isothiocyanate.

5. The material of claim 1 or 2, wherein said biomolecule is selected from
   the group consisting of a protein, an antibody and an immunoglobulin.

6. The material of claim 5, wherein said biomolecule is albumin.

7. The material of claim 6, wherein said albumin is human serum albumin.
8. The material of claim 1 or 2, wherein said fluorophore labeled biomolecule conjugate is fluorescein-labeled human serum albumin.

9. The material of claim 1 or 2, wherein said metallic particle is coated on a surface of a substrate.

10. The material of claim 9, wherein said metallic particle is coated on said substrate from a metallic colloid.

11. The material of claim 9, wherein said substrate comprises quartz or glass.

12. The material of claim 9, wherein said substrate is treated with poly-lysine.

13. The material of claim 1 or 2, wherein said metallic particle comprises silver.

14. The material of claim 1 or 2, wherein said fluorophore labeled biomolecule conjugate comprises 2 to 9 fluorophores per biomolecule.

15. The material of claim 1 or 2, wherein said fluorophore labeled biomolecule conjugate comprises 3 to 6 fluorophores per biomolecule.

16. The material of claim 1 or 2, wherein said fluorophores are covalently bound to said biomolecule.

17. A system comprising

   a fluorophore labeled biomolecule conjugate and
   a metallic particle,

   wherein said labeled biomolecule conjugate comprises multiple fluorophores per biomolecule and said labeled fluorophore-biomolecule conjugate is positioned at a distance from said metallic particle, wherein the position of said fluorophore labeled
biomolecule conjugate to said metallic particle reduces self-quenching due to homo
resonance energy transfer.

18. The system of claim 17, wherein said fluorophore is selected from the
group consisting of a fluorescein, rhodamine and a cyanine.

19. The system of claim 18, wherein said fluorophore is fluorescein-5-
isothiocyanate.

20. The system of claim 17, wherein said biomolecule is selected from the
group consisting of a protein, an antibody and an immunoglobulin.

21. The system of claim 20, wherein said biomolecule is albumin.

22. The system of claim 21, wherein said albumin is human serum albumin.

23. The system of claim 17, wherein said fluorophore labeled biomolecule
conjugate is fluorescein-labeled human serum albumin.

24. The system of claim 17, wherein said metallic particle is coated on a
surface of a substrate.

25. The system of claim 24, wherein said metallic particle is coated on said
substrate from a metallic colloid.

26. The system of claim 24, wherein said substrate comprises quartz or glass.

27. The system of claim 24, wherein said first substrate is treated with poly-
lysine.

28. The system of claim 17, wherein said metallic particle comprises silver.

29. The system of claim 17, wherein said fluorophore labeled biomolecule
conjugate comprises 2 to 9 fluorophores per biomolecule.

30. The system of claim 17, wherein said fluorophore labeled biomolecule
conjugate comprises 3 to 6 fluorophores per biomolecule.
31. The system of claim 17, wherein said fluorophores are covalently bound to said biomolecule.

32. A method of detecting a presence of or quantifying an amount of a biomolecule in a solution comprising

   labeling said biomolecule with multiple fluorophores to form a fluorophore labeled biomolecule conjugate,
   contacting said fluorophore labeled biomolecule conjugate with a metallic particle and
   irradiating the substrate to detect the presence of or to measure the amount of said fluorophore labeled biomolecule conjugate in said solution.

33. A method of detecting a presence of or quantifying an amount of a biomolecule in a solution comprising

   labeling said biomolecule with multiple fluorophores to form a fluorophore labeled biomolecule conjugate,
   positioning said fluorophore labeled biomolecule conjugate at a distance from a metallic particle,
   irradiating the substrate to detect the presence of or to measure the amount of said fluorophore labeled biomolecule conjugate in said solution,
   wherein said position of said fluorophore labeled biomolecule conjugate to said metallic particle reduces self-quenching due to homo resonance energy transfer.

34. The method of claim 32 or 33, wherein said fluorophore is selected from the group consisting of a fluorescein, rhodamine and a cyanine.

35. The method of claim 34, wherein said fluorophore is fluorescein-5-isothiocyanate.
36. The method of claim 32 or 33, wherein said biomolecule is selected from the group consisting of a protein, an antibody and an immunoglobulin.

37. The method of claim 36, wherein said biomolecule is albumin.

38. The method of claim 37, wherein said albumin is human serum albumin.

39. The method of claim 32 or 33, wherein said fluorophore labeled biomolecule conjugate is fluorescein-labeled human serum albumin.

40. The method of claim 32 or 33, wherein said metallic particle is coated on a surface of a substrate.

41. The method of claim 40, wherein said metallic particle is coated on said substrate from a metallic colloid.

42. The method of claim 40, wherein said substrate comprises quartz or glass.

43. The method of claim 40, wherein said first substrate is treated with polylysine.

44. The method of claim 32 or 33, wherein said metallic particle comprises silver.

45. The method of claim 32 or 33, wherein said fluorophore labeled biomolecule conjugate comprises 2 to 9 fluorophores per biomolecule.

46. The material of claim 1 or 2, wherein said biomolecule is DNA.

47. The material of claim 46, wherein said fluorophore is fluorescein.

48. The material of claim 48, wherein said fluorophore labeled biomolecule conjugate comprises 2, 3, 4 or 5 fluorophores per biomolecule.

49. The material of claim 48, wherein said fluorophore labeled biomolecule conjugate comprises 5 fluorophores per biomolecule.
50. The system of claim 17, wherein said biomolecule is DNA.

51. The system of claim 50, wherein said fluorophore is fluorescein.

52. The system of claim 51, wherein said fluorophore labeled biomolecule conjugate comprises 2, 3, 4 or 5 fluorophores per biomolecule.

53. The system of claim 51, wherein said fluorophore labeled biomolecule conjugate comprises 5 fluorophores per biomolecule.

54. The system of claim 51, wherein said fluorophore labeled biomolecule conjugate is positioned 90 Å from the surface of said metallic particle.

55. The method of claim 32 or 33, wherein said biomolecule is DNA.

56. The method of claim 55, wherein said fluorophore is fluorescein.

57. The method of claim 56, wherein said fluorophore labeled biomolecule conjugate comprises 2, 3, 4 or 5 fluorophores per biomolecule.

58. The method of claim 56, wherein said fluorophore labeled biomolecule conjugate comprises 5 fluorophores per biomolecule.

58. An array, comprising:

a substrate; and

a series of fluorophore labeled biomolecule conjugates provided on said substrate,

wherein each of the fluorophore labeled biomolecule conjugates is positioned at a selected location on said substrate and

wherein each of said fluorophore labeled biomolecule conjugates comprises multiple fluorophores per biomolecule and each of said fluorophore-labeled biomolecule conjugates is attached to a metallic particle.
59. The array of claim 58, wherein the metallic particles are on a surface of said substrate.

60. The array of claim 58, wherein each of said fluorophore labeled biomolecule conjugates is attached directly to said metallic particle.

61. The array of claim 58, wherein each of said fluorophore labeled biomolecule conjugates is attached to said metallic particle through a spacer or a spacer layer.

62. The array of claim 58, wherein the biomolecule is an oligonucleotide.

63. The array of claim 58, wherein the biomolecule comprises DNA.

64. The array of claim 58, wherein each of said fluorophore labeled biomolecule conjugates may be the same or different.

65. A method for analyzing a sample for at least one analyte therein, comprising:

   contacting the array of claim 58 with a sample containing at least one analyte therein, wherein at least one biomolecule of the array has an affinity for said at least one analyte so as to bind said analyte to said biomolecule; and

   irradiating the resulting array to detect the presence of or to measure the amount of said at least one analyte.

66. The method of claim 65, wherein each of said fluorophore labeled biomolecule conjugates may be the same or different and each may have affinity for different analytes.
Figure 1

FITC-HSA in PBS Buffer, pH 7.2

INTENSITY vs WAVELENGTH (nm)

L = 1, 3, 7, 9
Figure 2

FITC-HSA in Cuvette

L=1
\( \langle \tau \rangle = 2.37 \text{ ns} \)

Exc. 514 nm
Em. 540 nm

L=7
\( \langle \tau \rangle = 0.64 \text{ ns} \)
Figure 3
Figure 4

SIF on Poly-lysine coated slide
Figure 5
Figure 6
Figure 7

\[
\frac{I_S}{I_Q}
\]

FITC-HSA
Exc. 490 nm

Labeling
Figure 8

FITC-HSA
Exc. 490 nm

INTENSITY

LABELING

0 1 2 3 4 5 6 7 8 9 10
FITC-HSA, L=7
SIFs

Combination of IF 540 nm and LWP 530 nm

Figure 9
Figure 10

- FITC-HSA, L=1
- $\langle \tau \rangle = 1.27\text{ ns}$
- Exc. 514 nm, Em. 540 nm

- PHASE ANGLE (degrees) or MODULATION (%)
- FREQUENCY (MHz)
**Figure 12A**

FITC-HSA (L=1) monolayer plus 0.25 μM Rhodamine B solution

- Silver
- Quartz

**Figure 12B**

Diagram showing the distribution of FITC-HSA (L=7) monolayer plus 0.25 μM Rhodamine B solution with S and Q labels.
Excitation
or 514 nm (Argon Ion Laser)
490 nm (Xenon Lamp)

Observation
or to SLM 8000
10 GHz Fluorometer

Figure 14
biotin-5'-TCC ACA CAC CAC TGG CCA TCT TC-3'
Fi-3'-AGG TGT GTG GTG ACC GGT AGA AG-5'

Fl-DNA

biotin-5'-TCC ACA CAC CAC TGG CCA TCT TC-3'
Fi-3'-AGG TGT GTG GTG ACC GGT AGA AG-5'  
Fi  Fi  Fi  Fi

Fl-DNA(Fi)$_4$

biotin-5'-TCC ACA CAC CAC TGG CCA TCT TC-3'
3'-AGG TGT GTG GTG ACC GGT AGA AG-5'

DNA

Figure 15
Figure 16
Figure 18
Figure 19
Figure 20
Figure 21

Figure 22