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Plasmonic Enhancement of Single-Molecule Fluorescence Near a Silver Nanoparticle

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Abstract In this short paper, we reported the enhanced fluorescence from a single fluorophore bound to a 50nm silver nanoparticle. We found that on average the Cy5 molecules bound to metal nanoparticles are approximately 15-fold brighter than that of free dyes, and that single molecule lifetimes are shorter as compared to free fluorophores. The increased emission rate is primarily the result of local plasmon enhancement. These results demonstrate that the use of fluorophore-metal interactions can increase the brightness and photostability of fluorophores for single molecule detection.

Keywords Single-molecule fluorescence · Metal-enhanced fluorescence · Silver nanoparticle · Lifetime · Plasmonic-controlled fluorescence · DNA · Radiative decay engineering

Introduction

In past years, single molecule detection has become an effective method to study complicated molecular complexes, including dynamic behaviours of biomolecules in vitro and in situ [1–3]. However, the accuracy and duration of the measurement are limited by the brightness, blinking and photostability of fluorescence probes. At present we have probably reached the practical sensitivity limit of fluorescence without amplification steps. The optical cross-sections or extinction coefficients of many modern fluorophores are within a factor of two of their physical cross-sections. This

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implies that the amount of light absorbed cannot be substantially increased by modification of the aromatic chemical structure. As a result, the sensitivity of fluorescence with entirely organic fluorophores cannot be substantially increased by synthesis of novel organic structures. Additionally, many detectors such as the single photon counting avalanche photodiodes (SPADs) have high quantum efficiencies so that the sensitivity of fluorescence cannot be very much increased with improved detectors. And finally, there are limits to the collection efficiency with practical optical configurations. Because of these sensitivity limits using typical fluorophores there are ongoing attempts to design new classes of probes such as the semi-conductor quantum dots (QDs) or the use of polymeric or phycobiliprotein probes which contain multiple fluorophores [4]. Nevertheless, it appears unlikely that the photophysical properties of typical fluorophores can be substantially improved.

We have performed ensemble experiments of the interactions of fluorophores with metallic surfaces and particles during the past years [5–7]. The changes in brightness and/or photostability are the result of interactions of the incident light with the metal and interactions of the excited fluorophores with the metal. A sub-wavelength metallic particle can enhance the local field near its surface. This local field can result in increased rates of excitation of nearby fluorophores. A second and perhaps more important effect is an increase in the radiative decay rate of fluorophores near metal particles [7]. This increased rate can result in higher quantum yields, decreased lifetimes and potentially improved photostability. The photostability can be increased because a shorter lifetime allows less time for adverse reactions to occur in the excited state and thus more excitation–emission cycles prior to photobleaching.

Recent advances in single molecule detection offer an opportunity to probe and understand the details that are

hidden in ensemble experiments by sample heterogeneity [8]. For our experiments we presented a novel configuration by binding a single fluorophores bound to silver nanoparticles. The metal particles were controlled to quantitatively succinimidylate with (2-mercapto-propionylamino) acetic acid 2,5-dioxo-pyrrolidin-1-ylester via ligand exchange, and one aminated single-stranded oligonucleotide was covalently bound selectively to a single silver particle via condensation between the terminal succinimidyl ester moiety on the silver particle and the amino moiety on the oligonucleotide (Fig. 1). Finally, the silver particle was fluorescently labeled by hybridization with a labeled complementary single-stranded oligonucleotide. The bound fluorophore was spatially separated from the metal core by the hybridized DNA duplex chain to control the distance between the particle and the probe. The ds-DNA chain contains 23 base pairs, which corresponds to the dye-nanoparticle separation $d = 8$ nm. Previous studies predicted that the fluorescence enhancement is maximized not at the plasmon resonance wavelength, but somewhat at a wavelength redshifted from it [9]. For this reason, we chose to work at a wavelength of 637nm, which is red-shifted from the resonance peaks of silver particles. A widely used near-infrared fluorophore Cyanine 5 (Cy5) was employed to label the complementary single-stranded oligonucleotide. In our experiments we selected individual Cyanine 5 dye molecules and measured their fluorescence intensity and lifetime distributions. We used this approach to quantitatively compare the emission properties of free fluorophores and fluorophore-silver nanoparticle pairs. The work presented suggests potential of increasing detectability of single molecules coupling to metal nanoparticles. High selectivity and ultra-high sensitivity offered by plasmonic-controlled fluorescence have significant potential in biological research. Enhanced feasibility and minimal collection times, when combined with improved resolution of modern confocal microscopes, could provide a unique platform for single molecule studies of living human cells.

Experimental

All reagents and spectroscopic grade solvents were used as received. The Cy5 labeled oligonucleotides were obtained from the Biopolymer Shared Service at the University of Maryland, School of Medicine.

Preparation of fluorophore/nanoparticle complex Silver nanoparticles were prepared by chemical reduction of silver nitrate using ascorbic acid as previously described [10, 11]. N-(2-mercapto-propionyl) glycine (abbreviated as tiopronin) were bound as ligands on the surfaces of metal cores though metal-sulfur interaction. The tiopronin-coated silver particles were then succinimidylated via ligand exchange [12]. Aminated oligonucleotides were chemically attached to the succinimidylated silver particles by condensation between the amino moieties on the oligonucleotides and the terminal succinimidyl ester moieties on the silver particles [13]. The fluorophore-labeled complementary oligonucleotides were bound to the metal particles by hybridization with the particle-bound oligonucleotides (molar ratio 1:1). The unbound oligo left in the solution was removed by centrifugation and washing. Transmission electron micrographs (TEM) of nanoparticles were taken using a side-entry Philips electron microscope at 120keV. Samples were cast from water solutions onto standard carbon-coated

Fig. 1 Schematic illustration of synthesizing fluorophore-nanoparticle complex. The succinimidylated silver particle was covalently bound with an aminated single-stranded oligonucleotide (1) and fluorescently labeled by a complementary single-stranded Cy5-labeled oligonucleotide (2). The insert on the left shows a typical transmission electron micrograph (TEM) image of silver metal cores

Aminated oligo: H₂N-3'-TCCACACACCACTGGCCATCTTG-5'
Labeled oligo: 3'-AGGTGTGTGGTGACCGGTAGAAC-5'-Cy5

(200–300Å) Formvar films on copper grids (200mesh) by placing a droplet of a 1mg/mL aqueous sample solution on grids. The size distribution of metal core was analyzed with Scion Image Beta Release 2 counting at least 200 particles.

Single molecule experiments All single molecule studies were performed using a time-resolved confocal microscopy (MicroTime 200, PicoQuant). Immobilization of nanoparticles on glass coverslips was achieved by adding 20μL of 100 times diluted Cy5-nanoparticle suspension onto an amino-silanized coverslip following by spin drying at 4,000rpm. A single mode pulsed laser diode (635nm, 100ps, 40MHz) (PDL800, PicoQuant) was used as the excitation light. The collimated laser beam was spectrally filtered by an excitation filter (D637/10, Chroma) before directing into an inverted microscope (Olympus, IX 71). An oil immersion objective (Olympus, 100×, 1.3NA) was used both for focusing laser light onto sample and collecting fluorescence emission from the sample. The fluorescence that passed a dichroic mirror (Q655LP, Chroma) was focused onto a 75 μm pinhole for spatial filtering to reject out-of-focus signals and then reached the single photon avalanche diode (SPAD) (SPCM-AQR-14, Perkin Elmer Inc). Images were recorded by raster scanning (in a bidirectional fashion) the sample over the focused spot of the incident laser with a pixel integration of 0.6ms. The excitation power into the microscope was maintained less than 1μW. Time-dependent fluorescence data were collected with a dwell time of 50ms. The data was stored in a time-tagged-time-resolved (TTTR) mode, which allows recording every detected photon with its individual timing information. Instrument Response Function (IRF) widths of about 300ps FWHM can be obtained in combination with a pulsed diode laser, which permits the recording of sub-nanosecond fluorescence lifetimes extendable to less than 100ps with reconvolution. Lifetimes were estimated by fitting to a χ^2 value of less than 1.2 and with a residuals trace that was fully symmetrical about the zero axis. All measurements were performed in a dark compartment at room temperature.

Results and discussion

The tiopronin-coated nanoparticles were observed to display good solubility in water and good stability in subsequent surface modifications. TEM images depict the *average* diameter of metal cores, which was *ca*. 50nm on the basis of at least 50 image counts. These particles appeared to be approximately homogeneous in the size distributions (Fig. 1). In order to avoid multiple labeling on the single metal particle, the molar ratio of succinimidyl-

ligand and metal particle was controlled to be 0.5. It assures that most of single nanoparticles are conjugated to a single fluorophore [13].

Typical fluorescence images of 5 × 5μm regions recorded for single molecules are illustrated in Fig. 2. Images shown were recorded primarily for locating single molecules for further time dependent and lifetime analysis. Each pixel has a 0.6ms dwell time and the total number of photons counted in that time was displayed in a colorized gray scale, ranging from dark (fewer integrated photons) to light. Typical background signals in these images are less 1 count per pixel. These images provide initial, qualitative information on local sample properties. Obviously silver nanoparticles gave rise to a strong fluorescence enhancement resulting in much brighter spots over the confocal image. The intensity of individual fluorescence spots varies in the image because the sample consists of molecules with different dipole orientation.

Figure 3 shows representative time dependent fluorescence traces for individual Cy5 molecules. Both single free fluorophores and single nanoparticle-dye pairs undergo irreversible photochemical destruction as evidenced by the abrupt and permanent drop in fluorescence intensity to the background level [14]. All our fluorescence data were checked for single-step photobleaching to ensure that only signals from single molecules were considered. For both samples, the characteristics of the time-dependent signals were observed to vary dramatically between individual molecules as well. The general appearance of the time trace is representative of more than half of those observed in the experiment. A particularly high emission rate is normally observed from fluorophore near silver nanoparticles. The fluorescence enhancement from silver nanoparticle-fluorophore pair is approximately 20-fold as shown in

Fig. 2 Typical 5×5 μm fluorescence images of single molecules. **a** Free Cy5 fluorophores; **b** Cy5 molecules attached to silver nanoparticles. The experimental setup is shown on right

Fig. 3a and b. A more quantitative view of the brightness of fluorophores from two species is obtained though statistical analysis of data recorded for numerous single molecules. Shown in Fig. 3c are histograms incorporating emission rate values recorded from more than 40 single-molecule time traces. It is clear from these data that there are two distinct intensity levels for the brightness. Signals from free fluorophores yield a mean value near 13kHz, while signals from nanoparticle-dye pairs display a mean emission rate of 200kHz. The fluorescence enhancement factor is around 15-fold for silver nanoparticles.

Once we identified single-molecules, we implemented the time-correlated single photon counting (TCSPC) measurement on a single fluorescent spot. Single-molecule lifetime was determined by recording the arrival time of each photon with respect to laser pulse. Fig. 4 shows examples of the recorded lifetime curves for free fluorophore and nanoparticle-dye pair, respectively. The typical single-exponential character of the plot depicted in Fig. 4a and its good signal-to-noise ratio allow us to determine the lifetime of this single molecule to be 2.33 ns. In the other example shown in Fig. 4b, we measured $\tau_{av}=0.43$ ns in the presence of a silver nanoparticle. The inserts in Fig. 4 display distributions of the recorded lifetimes for more than 40 single molecules in both sets. The distribution of the measured τ_{av} values follows a symmetric Gaussian function for free fluorophores (Fig. 4a insert). This implies that local

environments of immobilized dyes on glass surface are relatively homogeneous in the absence of silver nanoparticles. The distribution obtained from dye-nanoparticle pairs (Fig. 4b insert) clearly illustrates changes in fluorescence lifetime induced by the presence of metallic nanostructure.

We note that the interactions of fluorophores with metallic particles have been studied intensively [5, 15–21]. It is now agreed that the metal-enhanced fluorescence occurs via near field interaction of fluorophore with the metal substrate, which can be described as localizing a dipole fluorophore in the electric field near a metal particle [18, 19, 22]. The rate of photon emission by an excited fluorophore can be modified by changing the photonic mode density at the emitter position. Fluorophores near metallic particles may show the remarkable behaviors of increased quantum yields and decreased lifetimes due to the increased photonic mode density near the fluorophore. The enhancement of fluorescence intensity generally relies on the optimal distance of fluorophores from the surface and the nano-objective features. The emission can be quenched due to radiation energy transfer to the metal as molecules adsorbed directly on the surface. Shifting a fluorophore only a few nanometers away from the metallic surface may lead to an enormous emission change by orders of magnitude [19, 21]. In addition, the strong energy transfer from the excited molecules to a nearby metallic surface or an increase in the radiative decay rate of the fluorophore

can dramatically shorten the lifetime of the excited state. Assume that the metallic nanoparticles result in an increase in the radiative relaxation rate, the instant outcome of increased radiative rate will cause an increase in quantum yield. In free space, increases in quantum yield are typically accompanied by increased lifetimes due to decreased non-radiative decay rates. However, a metal-induced modification in the radiative rate would lead to a decreased lifetime near the metal and consequently, a fast de-excitation, which is consistent with our recorded data in this experiment. This effect increases the number of excitation cycles a molecule can survive until photobleaching. As a result, a dramatic increase in the number of photons can be observed from a single fluorophore. Hence, fluorophores nearby metallic objectives are expected to display better photostability than free fluorophores in the absence of metal. This is in good accord with our observations that, as shown in Fig. 3, the emissive time of the fluorophore attached to metal nano-particle was longer compared to that of a free fluorophore.

The fluorescence lifetime is sensitive to the local environment of the molecule. The skewed broadening in τ_{av} distribution displayed in Fig. 4b can have diverse origins due to various strengths of metallic electromagnetic fields, i.e. distributions in the orientation of the dye molecules with respect to the metallic surface as well as the size of the nanoparticles. The repulsion between the charged carboxylic moieties and the silver nanoparticles retains a preferential orientation of the dye, which is parallel to the silver surface. There could also be some uncertainties in the distance between the dye and the nanoparticle surface depending on whether the DNA chain is stretched radially or tangentially to the particle surface. In addition, TEM images indicate some distributions in particle shapes and sizes, which can play a role in fluorophore lifetime variations. The broad lifetime distribution near silver particles could arise from diverse near-field interactions that may be due to the assorted of metal particles.

In conclusion, we demonstrated the enhanced fluorescence and shortened lifetime of a fluorophore attached a metal nanoparticle with an approximate distance of 8 nm. The silver particles were synthesized by reduction salt using ascorbic acid and the core size was controlled to be roughly 50 nm. We found that on average the Cy5 molecules bound to metal nanoparticles are approximately 15-fold brighter than that of free dyes. Considering the divergences of the experimental conditions (i.e. distances, particle shapes and sizes), actual dipole orientations, and dielectric interface factors, this result is rational when comparing with the calculated fluorescence enhancement factors, which is about a range of 7–8 for gold and silver nanoparticles excited by 637 laser line [20]. By employing single molecule spectroscopic approaches, we experimentally provide evidence for near-field enhancement nearby a metal nano-objective. Since the spatial extension of the enhanced field can be tailored down to the nanometer-scale, these results are useful for future developments of nanosensors at molecular length scales. Lifetime measurements support the coupling mechanism between the fluorophore and metal particle. Our results show that plasmonic-controlled fluorescence can lead to a novel physical mechanism to enhance fluorescence intensity. These effects can increase the field incident on the fluorophore and cause the changes in quantum yields, lifetime and photostability. Thus, the use of these ultra-bright and stable fluorophore/metal complexes has great potential for applications in the fields of medical diagnostics and biotechnology which have high background emission.

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