Published in final edited form as:

J Phys Chem C Nanomater Interfaces. 2011 April 21; 115(15): 7298–7308. doi:10.1021/jp112255j.

Ensemble and Single Molecule Studies on the Use of Metallic Nanostructures to Enhance the Intrinsic Emission of Enzyme Cofactors

Mustafa H. Chowdhury, Joseph R. Lakowicz, and Krishanu Ray*

Center for Fluorescence Spectroscopy, Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, 725 West Lombard Street, Baltimore, MD, 21201, USA

Abstract

We present a strategy for enhancing the intrinsic emission of the enzyme cofactors flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and nicotinamide adenine dinucleotide (NADH). Ensemble studies show that silver island films (SIFs) are the optimal metal enhanced fluorescence (MEF) substrates for flavins and gave emission enhancements of over 10-fold for both FAD and FMN. A reduction in the lifetime of FAD and FMN on SIFs was also observed. Thermally evaporated aluminum films on quartz slides were found to be the optimal MEF substrate for NADH and gave a 5-fold increase in the emission intensity of NADH. We present finite-difference time-domain (FDTD) calculations that compute the enhancement in the radiated power emitting from an excited state dipole emitting in the wavelength range of NADH in close proximity to an aluminum nanoparticle, and a dipole emitting in the emission wavelength of flavins next to a silver nanoparticle. These calculations confirm that aluminum serves as the optimal MEF substrate for NADH and silver was the optimal MEF substrate for flavins. This is because the plasmon resonance properties of aluminum lie in the UV-blue regime and that of silver lie in the visible region. We also present the results of single molecule studies on FMN which show SIFs can both significantly enhance the intrinsic emission from single FMN molecules, significantly reduce their lifetimes and also significantly reduce FMN blinking. This is the first report of the observation of MEF from cofactors both at the ensemble and single molecule level. We hope this study will serve as a platform to encourage the future use of metallic nanostructures to study cofactors using their intrinsic fluorescence to directly monitor enzyme binding reactions without the need of extrinsic labeling of the molecules.

Keywords

enzyme cofactors; FAD; NAD⁺; NADH; metal-enhanced fluorescence; surface plasmons; plasmon-controlled fluorescence; aluminum; silver; nanoparticle; fluorescence; single molecule fluorescence; finite-difference time domain

1.0 Introduction

For the last 15 years there has been a growing interest in studies of biomolecules using single molecule detection (SMD) and fluorescence correlation spectroscopy (FCS). SMD and FCS have provided many insights into protein folding, biomolecule association reactions, and membrane dynamics, to name a few.¹⁻³ In almost all cases, SMD studies are

* krishanu@cfs.umaryland.edu .

performed with extrinsic probes which are usually placed on the surface of biomolecules and distant from the binding site or catalytic sites of enzymes in order to avoid perturbation of the native functionality of the proteins. As a result the probes report indirectly on the functioning of the molecules, as illustrated by the use of fluorescence resonance energy transfer (FRET) to study global conformational changes rather than events at the sites of interest. It would thus be valuable to perform SMD studies of enzymes using the intrinsic fluorescence from their cofactors. A cofactor is a non-protein chemical compound that is bound to a protein and is required for the protein's biological activity.¹⁻⁷ These proteins are commonly enzymes and cofactors can be considered "helper molecules" that assist in biochemical transformations. The most widely recognized cofactors are organic molecules such as nicotinamide adenine dinucleotide (NAD⁺ or NADH), flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN).¹⁻⁷ Since the cofactors participate directly in the enzyme catalyzed reactions, their emission can report directly on processes occurring at the active site. Additionally, an ability to detect single molecules of NADH or FAD could provide direct measurements of the catalytic events by a single enzyme molecule. Such experiments could provide a wealth of information about enzyme function, especially for studies of allosteric effects which are difficult to resolve using ensemble measurements.

With a few limited exceptions, all protein SMD measurements are performed using extrinsic probes such as Cy5 which have high extinction coefficients of $250,000 \text{ M}^{-1}\text{cm}^{-1}$, a quantum yield of approximately 0.27, good photostability, and narrow emission spectrum so that the signal can be easily separated from the broad background emission. However, NADH and FAD have low extinction coefficients of 6,200 and $11,300 \text{ M}^{-1}\text{cm}^{-1}$ respectively (over 20-fold smaller than Cy5) and low quantum yields.¹ Additionally all the three cofactors have wide emission spectra (see Figure 1), and hence detection over the entire emission spectrum results in background contributions from the same wide range of wavelengths. It has been reported that SMD of FAD is known to be compromised by extensive blinking^{4-5,8-12} which is probably due to transitions to the triplet state. SMD of flavoproteins is even more difficult because the emission of FAD is strongly quenched when bound to flavoproteins as shown by the shortened lifetimes and heterogeneous intensity decays.⁵⁻⁶ As a result there are relatively few publications of single molecule studies of flavoproteins. In contrast, NADH displays the favorable property of a 4-fold increase in intensity upon binding to dehydrogenases.¹ This occurs because in water the adenine moiety quenches the reduced nicotinamide group. The protein bound form of NADH is extended which prevents the adenine quenching. However, NADH requires UV excitation which typically results in high background emission and an inability to detect single molecules. Additionally, the protein binding constants for NADH are typically in the micromolar range which prevents the detection of protein bound NADH.

The goal of this study is to explore both experimentally and theoretically the possibility of using metallic nanostructures to enhance the intensity of the intrinsic emission of the cofactors NADH, FAD and FMN. We first present ensemble experimental results where we show that NADH, FAD and FMN on a thin particulate aluminum film (for NADH) or silver particulate substrates (for FAD and FMN), show significant increases in intrinsic emission intensity when compared to a control substrate. Next, we use finite-difference time domain (FDTD) calculations to explore the effects of nearby aluminum particles on NADH and of nearby silver particles on FAD and FMN.¹³⁻²³ The FDTD method is a rigorous computational electrodynamics method that can accurately describe plasmonic effects and the interaction of nearby dipoles with the plasmons. We have previously used this approach to describe MEF around single and dimers of silver and aluminum nanoparticles.¹³⁻¹⁵ Our calculations show that aluminum nanoparticles are very promising substrates for enhancing the emission of NADH and silver nanoparticles are efficient substrates for enhancing the emission of FAD and FMN. We show both incident light-induced near-field distributions

around the aluminum and silver nanoparticles and the fields induced by nearby fluorophores. Additionally we also show that these cofactors display increased radiative power when placed near the metal particles. Within the FDTD classical physics formalism the increase in radiated power corresponds to increases in the radiative decay rate as will be explained in greater detail in the Methods section below.^{13-15,23} These theoretical results corroborate our experimental observations that plasmonic metal nanostructures can be used for significantly enhancing the intrinsic emission from the enzyme cofactors. Since it is known that metal-enhanced fluorescence (MEF) is due to electrodynamic through-space interactions rather than chemically dependent contact interactions, our results suggest that MEF can be productively used for other cofactors such as the pyridoxal group, folates and thiamines. And finally, we show for the first time the capability of metallic nanostructures to enhance the single molecule emission of the cofactor FMN. We found that silver island films (SIFs) enhances the intrinsic emission from FMN by as much as 20-fold at the single molecule level. Additionally, the use of SIFs significantly reduces the lifetime of FMN molecules and thus also significantly reduces their blinking properties. This is significant because it can potentially ameliorate the extensive blinking problems associated with flavins that has severely hampered single molecule studies thus far.

2.0 Materials and Methods

2.1 Ensemble Experimental Study Details

Aluminum slugs, silicon monoxide, agarose, NADH, FAD, FMN, premium quality APS-coated (amine terminated) glass slides (75×25 mm), silver nitrate, ammonium hydroxide, sodium hydroxide, and glucose were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. Quartz slides were purchased from SPI supplies (West Chester, PA). Distilled water (with a resistivity of 18.2 MΩ-cm) purified using Millipore Milli-Q gradient system was used for sample preparation.

Silver island film (SIFs) were prepared on glass slides as described in earlier reports.²⁴ The SIFs slides were stored in Milli-Q water until they were used. We used the SIFs in our experiments within two days of forming them. After two days of storage in Milli-Q water we did not see a change in the surface plasmon resonance of the SIFs, both showing an extinction maximum at roughly $\lambda = 450$ nm (measured right after formation and after two days of storage in water), showing the absence of any detrimental effects of oxide layer formations on our SIFs.

10 nm thick aluminum film was deposited on quartz slides using an Edwards Auto 306 Vacuum Evaporation chamber under high vacuum ($<5 \times 10^{-7}$ Torr). In each case, the metal deposition step was followed by the deposition of 5 nm of silica via evaporation without breaking vacuum. This step served to protect the metal surface as well as it adds a spacer layer between the metal surface and fluorophore. The deposition rate was adjusted by the filament current and the thickness of film was measured with a quartz crystal microbalance.

An aliquot of approximately 500 μ l of 1 wt % aqueous solution of agarose containing separately dissolved FAD or FMN was spin coated at 3000 rpm (Specialty coating system Inc., Speedline Technologies, Indiana) on the surface of the SIFs and glass substrates. Similarly agarose containing NADH was spin coated on the surface of aluminum nanostructured or quartz slides.

Absorption spectra were collected using a Hewlett-Packard 8453 spectrophotometer. Fluorescence spectra of the cofactors on solid substrates were recorded using a Varian Cary Eclipse Fluorescence Spectrophotometer. Both the steady-state and time-domain lifetime measurements were carried out using front face illumination. Time-domain lifetime

measurements were obtained on a PicoQuant lifetime fluorescence spectrophotometer (Fluotime 100). The excitation source was a pulsed laser diode (PicoQuant PDL800-B) with a 20 MHz repetition rate. The Instrument Response Function (IRF) is about 300 ps. Intensity decays were measured through bandpass interference filters. Emission lifetimes were measured with vertically polarized excitation. Magic angle observation was used in the emission path for the time-domain measurements. This optical configuration reduced scattered light of the excitation wavelength and removed the effects of rotational diffusion without significant distortion of the spectra or lifetimes.

The fluorescence intensity decays were analyzed in terms of the multiexponential model as the sum of individual single exponential decays:¹

$$I(t) = \sum_{i=1}^n \alpha_i \exp(-t\tau_i) \quad (1)$$

In the above expression, τ_i are the decay times and α_i are the amplitudes. The fractional contribution of each component to the steady-state intensity is described by:¹

$$f_i = \frac{\alpha_i \tau_i}{\sum_j \alpha_j \tau_j} \quad (2)$$

the mean (intensity weighted) lifetime is represented by:¹

$$\bar{\tau} = \sum_i f_i \tau_i \quad (3)$$

and the amplitude weighted lifetime is given by:¹

$$\langle \tau \rangle = \sum_i \alpha_i \tau_i \quad (4)$$

The values of α_i and τ_i were determined using the PicoQuant Fluofit 4.1 (Professional Version) software with the deconvolution of instrument response function and nonlinear least squares fitting. The goodness-of-fit criterion was determined by the χ^2 value.

Portions of the aluminum or silver nanostructured sample were cut and mounted on an aluminum stub with conductive tape, and then observed in an Hitachi SU-70 scanning electron microscope (SEM) directly, i.e., without any further treatment of the sample. Samples were surveyed at low magnifications to see the general features and the homogeneity. Representative areas were selected for higher magnification investigation.

2.2 Single Molecule Experimental Study Details

Observations of single molecule fluorescence were made with a scanning confocal Picoquant MicroTime 200 microscope with time-correlated single-photon counting capabilities. The excitation laser was reflected by a dichroic mirror to a high numerical aperture (NA) oil objective (100 $\times$, NA 1.3) and focused to a diffraction limited spot (~ 300 nm) on the sample surface. The fluorescence from the FMN was collected by a single-photon counting avalanche photodiode (SPAD) (SPCM-AQR-14, Perkin Elmer Inc) through the dichroic beam splitter and a band-pass (550-600 nm, Chroma) filter. Single molecule

fluorescence images were recorded by raster scanning the sample through the excitation light focus by means of a linearized piezo scanner. The samples were excited with a typical power density of 200 W/cm² from a 470 nm pulsed laser (20-MHz repetition rate, 80-ps FWHM). Intensity-time trajectories and intensity-time decays were obtained by positioning the excitation beam above the individual FMN molecules. The arrival time of each photon (100-ns resolution) as well as the fluorescence delay time relative to the laser pulse (37-ps resolution) were recorded for each detection channel and stored for later analysis. We have added a spectrograph and an electron-multiplied CCD to the Microtime 200 to obtain spectra from each individual molecule. For spectroscopy of individual molecules, a 150 mm spectrograph (Princeton Instruments Acton) has been employed. The spectrograph consists of high-reflectance mirrors (used for collimation and focusing), and a 150 G/mm dispersion grating with 500 nm blaze wavelength; this grating provides efficient imaging from 450-750 nm. The detector for the spectrograph is a high quantum efficient (>90% visible range) electron-multiplied CCD (Princeton Instruments Photon Max 512).

2.3 FDTD Computational Study Details

Three-dimensional FDTD simulations are performed using the *FDTD Solutions* package from Lumerical Solutions, Inc. (Vancouver, Canada)¹³⁻¹⁶ *FDTD Solutions* was implemented using the parallel option on a Dell Precision PWS690 Workstation with the following components: Dual Quad-Core Intel Xeon E5320 processors at 1.86 GHz, and 8 GB RAM. Additional post-processing of the FDTD data were performed using *MATLAB* (version 7.0) from Mathworks (Natick, MA), and OriginPro 7 from Originlab Corporation (Northampton, MA). For the fluorophore based calculations it is assumed that excitation of the fluorophore has already occurred and the fluorophore is now emitting dipole radiation. Hence the fluorophore is modeled as a time-windowed, oscillating point dipole source for the electric field, with frequency content spanning the spectral range (300-700 nm) of interest and polarization perpendicular to the metal nanoparticle surface. After testing for convergence, we employed a grid size of 1 nm for all our calculations. The typical durations of our simulations were 400 fs.

The FDTD calculations were done for a fluorophore near an aluminum nanoparticle or a silver nanoparticle. Further details of our computational setup have been reported elsewhere.¹³⁻¹⁶ In our calculations, dipole polarizations along the *x*-axis are considered, where the *x* orientation of the dipole is perpendicular to the metal nanoparticle surface. We used 1.33 for the refractive index of water. The FDTD program implements a realistic frequency-dependent, lossy dielectric model for aluminum and silver.¹³⁻¹⁵ We calculate the total radiated power enhancement as P_{rad}/P_0 , where P_{rad} is the integral of the Poynting vector over a surface enclosing the fluorophore and metal nanoparticle, and P_0 is the result of this integral with only the fluorophore present.^{13-15,23} This enhancement can be equated with inferences on radiative decay rate changes according to $\gamma_{rad}/\gamma_{rad}^0$, where γ_{rad} is the radiative decay rate of the dipole in proximity of the metal nanoparticle and γ_{rad}^0 is the radiative decay rate of an isolated dipole (in water):^{13-15,23}

$$\frac{\gamma_{rad}}{\gamma_{rad}^0} = \frac{P_{rad}}{P_0} \quad (5)$$

Eq. (5) implies that an enhancement in the total radiated power is indicative of a corresponding increase in the relative radiative decay rate of the system and vice-versa. An increase in the radiative rate will usually result in an increased quantum yield, unless the non-radiative processes overwhelm this effort.

3.0 Results and Discussion

Figure 2 (a) shows the high resolution FE-SEM image of SIFs and reveals the formation of silver nanoparticles of various sizes ranging from $\sim 20 - 500$ nm with multitudes of shapes. Figure 2 (b) shows the characteristic extinction spectra of SIFs with the plasmon resonance showing a peak at ~ 450 nm and the characteristic dip in the plasmon resonance occurring at ~ 310 nm. The FE-SEM image of a 10 nm thick aluminum film is presented in Figure 2 (c). This image reveals that the aluminum forms nanoparticles of various sizes and shapes when evaporated on the quartz substrate, with an average particle size of approximately 80 nm. Figure 2 (d) shows the extinction spectra of the 10 nm thick aluminum film with the plasmon resonance being very broad across all wavelengths measured and with no clear spectral peak region. These results are in agreement with our previously reported observations.²⁵ One of the most important conclusions we can draw from Figure 2 is that both the SIFs as well as the aluminum substrate show significant surface roughness on the 'nm' scale. It is well documented that surface roughness promotes coupling of incident light (plane waves) to surface plasmons to create localized regions of enhanced field especially in the near-fields around the particles.²⁵ Fluorophores located in these enhanced field areas experience a much higher excitation field than if it were isolated in a dielectric and directly excited only by the incident light. This leads to higher excitation rates of the fluorophore, which leads to greater excitation-emission cycles in a given time period.

Figure 3 (a) shows the fluorescence emission spectra of NADH on a quartz substrate as well as on a 10 nm thick aluminum film. We observe an emission enhancement of ~ 5 -fold on the aluminum film when compared with the quartz (control). It can be seen that the emission peak of both the spectra are located at approximately 440 nm thus indicating minimal change in spectral properties of NADH by the interaction with the aluminum film. Figure 3 (b) shows the fluorescence emission spectra of FAD on a glass substrate as well as SIFs substrates. It can be seen that the interaction of SIFs with FAD leads to a significant emission enhancement of over 14-fold. The intensity decays of the agarose film containing FAD on glass and SIFs substrates are shown in Figure 3 (c). IRF is the instrument response function. The solid lines indicate the best fit to the experimental decay curves. It can be clearly seen from the figure that the intensity decay of FAD on the SIFs surface is faster than on the glass substrate. The intensity-decay of the FAD on the glass substrate was fitted with a double exponential with two lifetime components of 5.41 ns (31%) and 1.63 ns (69%) with the total amplitude-weighted lifetime being 2.82 ns. The intensity decay of FAD on the SIFs substrate was also fitted with a double-exponential with two lifetimes of 4.5 ns (19%) and 0.43 ns (81%). Hence the amplitude-weighted lifetime of FAD on SIFs was calculated to be 1.23 ns. Therefore the intensity decays in Figure 3 (c) show that the lifetime of FAD was decreased by over 2-fold on the SIFs when compared to the glass control substrate. This shortening of the FAD lifetime on the SIFs substrate suggests that the enhanced emission intensity observed on SIFs cannot be from the FAD molecules alone but rather due to the radiation from the plasmon-fluorophore complex²⁵⁻²⁶ that results when excited fluorophores interact with the silver nanoparticles in their immediate proximity (near-field). This helps support to our radiating plasmon model where we believe the enhanced fluorescence emission observed in MEF experiments is due to radiation from the entire excited state fluorophore-metal nanoparticle complex acting as a singular radiating entity.²⁵⁻²⁸ We have coined the term called "plasmophore" to describe this entity.²⁶ Later on in this study, we will present FDTD calculations which will further promote our understanding of the "plasmophore" radiation theory. Additionally, we would like to point out that precise agreement between the increases in emission intensity (14-fold) and decrease in lifetime (2-fold) cannot be expected. This is because time-domain measurements often result in over-weighting of the lifetime by the longer lifetime components in cases involving a heterogeneous decay, especially when the decay of the short components overlaps IRF.

Figure 4 (a) shows the fluorescence emission spectra of FMN on a glass substrate as well as SIFs substrates. It can be seen that the interaction of SIFs with FMN leads to a significant emission enhancement of over 11-fold. The intensity decays of the FMN agarose film on glass and SIFs substrates are shown in Figure 4 (b). The solid lines are the best fit to the experimental decay curves. It is seen from the figure that the intensity decay of FMN on the SIFs surface is faster than on the glass substrate. The intensity-decay of the FMN on the glass substrate was fitted with a double exponential with two lifetime components of 5.4 ns (35%) and 1.49 ns (65%) with the total amplitude-weighted lifetime being 2.87 ns. The intensity decay of FMN on the SIFs substrate was also fitted with a double-exponential with two lifetime components of 3.89 ns (18%) and 0.64 ns (82%). The amplitude-weighted lifetime of FMN on SIFs was calculated to be 1.21 ns. Therefore the intensity decays in Figure 4 (b) show that like FAD, the lifetime of FMN was decreased by over 2-fold on the SIFs when compared to the glass control substrate. In Figure 4 (c) we present the photostability of FMN on SIFs and glass substrates. Using the same incident excitation power, we observed significantly more fluorescence of from the SIFs substrates when compared to the glass sample (data not shown). In the case of Figure 4 (c) the incident excitation power on the FMN film on SIFs was attenuated to give the similar initial emission intensity as the glass case. It is clearly seen in Figure 4 (c) that FMN is more photostable on the SIFs substrate when compared to the glass substrate when the incident excitation yields similar initial emission intensities. Our results confirm that an increase in emission intensity coupled with a decrease in lifetime of the FMN emission on SIFs substrates causes a significant increase in its photostability, which in turn is expected to increase its detectability as the excited state molecule is able to go through a larger number of excitation-emission cycles in a given period of time without photobleaching.

We now present theoretical results which helps explain our experimental observations and also can shed more light into the MEF process. In Figure 5 (a), we present the calculated normalized scattering cross section of an 80 nm [scattering cross sections normalized by geometric area, $\pi(d/2)^2$ – where d is the diameter of the metallic nanoparticle] aluminum and silver nanoparticle in water respectively. For the case of the aluminum nanoparticle, we see that the scattering cross-section of the particles is several fold greater than its physical cross section. This is an important aspect of plasmonic nanostructures and makes them efficient substrates for radiating plasmon-coupled emission (MEF). The scattering cross section shows three notable peaks. The primary peak is the dipolar scattering peak located at approximately $\lambda = 330$ nm. In addition we also observe higher order peaks at approximately $\lambda = 214$ nm and 180 nm. Note that surface plasmon resonances of the aluminum nanoparticle is blue shifted by approximately $\lambda = 150$ nm when compared to similar sized silver nanoparticles, which has its plasmon resonance typically in the $\lambda = 470$ nm region. Of course these trends are readily inferred by consideration of the different metallic dielectric constants for the two metals. For the silver nanoparticle, we also observe that the normalized scattering cross section is several fold greater than its physical cross section, which makes it an efficient substrate for MEF. The location of the scattering cross section peaks is an indication that silver will a better substrate for MEF applications in the visible region whereas aluminum will be optimally suited for applications in the UV-blue region of the spectra. Hence we also present in Figure 5 (b) and (c), FDTD calculations of the effect of the radiated power enhancement as a function of wavelength when a dipole is in close proximity of an aluminum and a silver nanoparticle. Understanding the results of Figure 5 (b) and (c) will add more information that will help us unlock the mystery of the MEF mechanism. These set of FDTD computations were performed by calculating the total radiated power as inferred by integrating the flux normal to the six sides of a closed box containing the fluorophore-nanoparticle system, and then dividing it by the corresponding power radiated by an isolated fluorophore (in water). An enhancement or quenching in the total radiated power by a system is related to relative changes in the radiative decay rate of the system in

comparison to an isolated dipole as described by Eq. 5.^{13-15,23} Two different fluorophore-metal surface distances, 5 nm and 10 nm are considered and in all cases the dipole is oriented perpendicular to the surface of the metal. We deliberately chose the perpendicular orientation of the fluorophore because we shown in our previous reports that this orientation gives us the maximum enhancement in the radiated power and hence we chose to present the “best case” scenario.¹³⁻¹⁵ Figure 5 (b) shows the radiated power enhancements when a fluorophore (oscillating dipole) is placed near an 80 nm aluminum nanoparticle in water. We see that the enhancement in the radiated power peaks at approximately $\lambda = 440$ nm for both the dipole-aluminum separation distances. The maximum enhancement of approx. 13-fold occurs for the 5 nm separation and for the 10 nm separation the maximum enhancement is approximately 8-fold. The enhancement observed with this particular dipole orientation is similar to our previous reports,¹³⁻¹⁵ and can be attributed to the fluorophore’s dipole inducing a dipole in the aluminum nanoparticle in a configuration that allows the dipoles to align along the axis head to tail, leading to a much larger effective radiating dipole than in the case of an isolated fluorophore (constructive interference). We have previously shown that for fluorophores oriented parallel to the metal surface, significant quenching of the emission is observed and hence do not present calculations with this “worst case” scenario.¹³⁻¹⁵ We recognize that considering a complete ensemble of molecular orientations (both parallel and perpendicular) would provide a more “realistic” prediction for enhancement. However, we note that a greatly enhanced emission of the perpendicular fluorophores when combined with quenched emission from the parallel fluorophores, will still result in an increase of the total emission. In Figure 5 (c) we present the radiated power enhancement of a dipole placed 5 and 10 nm away from the surface of an 80 nm diameter silver nanoparticle immersed in water. For both separation distances we see the dipolar enhancement peak located at approximately $\lambda = 490$ nm, with corresponding maximum enhancement factors of approximately 40-fold for the 5 nm separation distance and 20-fold for the 10 nm separation distance. An interesting observation is the appearance of the additional blue-shifted enhancement peaks at approximately $\lambda = 400$ nm. We believe these secondary peaks are due to higher order enhancement modes and become more prominent as the fluorophore comes in closer proximity to the metal – and is consistent with our previous reports.¹³⁻¹⁵

The results of Figure 5 (b) and (c) deals with the power radiated from a fluorophore next to metal nanoparticles – which essentially deals with propagating radiation that travels to the far-field. It is also important to investigate the near-fields created around metal nanoparticles due to the interaction with excited-state dipoles in their immediate proximity. Figure 6 presents the effect of an excited-state fluorophore emitting at 530 nm on the near-fields around an 80 nm silver and aluminum nanoparticle with the dipole-metal spacing of 5 nm. We choose 530 nm as the emission wavelength as this is where the emission maximum of FAD occurs. For these FDTD calculations, the dipole is in water and is oscillating at a fixed frequency corresponding to 530 nm throughout the entire simulation time, and a time average of the square of the electric field vector over the last period of evolution was constructed. The dipole has its electric field oriented along the x -axis and we present the near-field intensity image ($E^2 = E_x^2 + E_y^2 + E_z^2$). Figure 6 (a) shows the near-field around an isolated dipole. It is interesting to note that the isolated dipole has near-fields along both the x and y axes. We have verified in our previous reports that the intensity of Figure 6 (a) is very similar to the near-field of a Hertz dipole.¹³⁻¹⁵ Figure 6 (b) shows the enhanced near-fields around an 80 nm silver nanoparticle that occurs due to the interaction with the dipole. We observed that the intense-near field enhancements are not located on the side of dipole but rather located on the side of the metal opposite the dipole. We also found that the intense near-field enhancements extend tens of nanometers from the edge of the particles into the free space as observed by the extent of the red areas in Figure 6 (b). These results suggests that it is not the dipole itself but rather the dipole coupled to the metal that acts as a unified

radiating entity that leads to enhanced emission intensities observed. This supports the radiating plasmon model that we have previously reported.²⁶⁻²⁸ Figure 6 (c) shows the enhanced near-fields around an 80 nm aluminum nanoparticle that occurs due to the interaction with the dipole. A comparison of Figure 6 (c) with Figure 6 (b) clearly shows the near-fields generated around the silver nanoparticle are stronger than that of aluminum (with both figures on the same color scale). This is seen by the yellow-orange areas in Figure 6 (c) and the intense red areas in Figure 6 (b). These results can be explained by the plasmon resonance properties of silver being in the visible range when compared to aluminum and hence the coupling with the visible dipole is stronger in silver than in aluminum. In both these cases, the enhanced fields are on the side of the metal that is distal to the dipole.

For all SMD studies it is important to reduce the observed volume around the fluorophore as this helps reduce background emission and increases signal-noise ratio. Small observation volumes also provide the opportunity to analyze higher concentration of fluorophores. SMD and FCS are currently performed using probe concentrations in the nanomolar (nM) range.¹ The low concentrations are necessary to increase the possibility of having a single molecule with the observation volume (for solution studies) or two molecules separated by distances greater than the diffraction limit (for surface based studies). The binding constants of cofactors with their associated proteins such as NADH for dehydrogenases are typically in the micromolar (μM) range – 1000-fold greater concentration than what is currently possible to observe using SMD. Hence protein-bound NADH will co-exist with μM concentrations of free NADH. Under these conditions there will be hundreds of NADH molecules in the typical diffraction limited volume, which will prevent studies of single binding reactions. Metal particles can be used to decrease the effective observed volume by localization of the fields near the surface of the particles due to their interaction with incident radiation. This gives us the opportunity to monitor binding reactions at higher and importantly biologically relevant concentration range (μM). Additionally, the near-field information can provide insight into the nature of metal enhanced fluorescence (MEF) that is interesting from the perspective of applications involving molecular spectroscopy.^{13-15,26-28} Figure 7 (a-b) shows the effect of the $\lambda = 340$ nm plane wave excitation on the near-fields around an 80 nm silver and aluminum nanoparticle respectively. The excitation wavelength of $\lambda = 340$ nm was chosen for this set of calculations because it is used for exciting NADH fluorescence. All the near-field calculations shown are performed along a single plane, that is, the x - y plane running through the center of the metal nanoparticles. In the calculations the incident plane wave is travelling in water with the propagation vector along the z -axis (out of the plane of paper) and the electric field is oriented along the x -axis. We present the near-field intensity enhancement image which is created by dividing the near-field intensity ($E^2 = E_x^2 + E_y^2 + E_z^2$) around the metal nanoparticle as a result of its interaction with the incident plane waves by the field intensity along the planar wavefront that exists along a plane wave when no metal particle lies in its propagation path. Comparing Figure 7 (a) to 7 (b), we see that aluminum nanoparticles interact with the $\lambda = 340$ nm plane wave far more strongly than silver nanoparticles. This is not surprising because the plasmon resonance properties of aluminum lie in the UV range and that of silver is red-shifted to the visible regime as reported by us earlier.¹³⁻¹⁵ Figure 7 (b) also shows the near-fields are localized in a small region around the metal particle. It is this intense localization of the fields around the metal particle that can lead to the small observation volumes that can potentially be exploited for SMD studies. So for the case of the $\lambda = 340$ nm excitation, the pronounced excitation enhancements are around the aluminum nanoparticle and are in the range of approximately 10-30 fold that extends up to approximately 20 nm away from the surface of the metal nanoparticle. Figure 7 (c-d) are the near-field intensity enhancement images that show the effect of the $\lambda = 440$ nm plane wave excitation on the near-fields around an 80 nm silver and aluminum nanoparticle respectively. The excitation wavelength of $\lambda = 440$ nm was chosen for this set of calculations because it was used for excitation of FAD/FMN fluorescence.

Comparing Figures 7 (c) with Figure 7 (d) we see the silver nanoparticle interacting more strongly with the $\lambda = 440$ nm plane wave and the enhancements range between 30-100 fold at distances up to 20 nm from the surface of silver nanoparticle. For the aluminum nanoparticle we observe more modest near field excitation enhancements in the range of 20-30 fold also at distances up to 20 nm from the surface of the nanoparticle. This discrepancy can be explained by virtue of silver having its resonance properties in the visible range and aluminum in the UV. The calculations of Figure 7 are important because it helps us better understand the MEF process.²⁶⁻²⁸ MEF has essentially two components. The first is on the excitation side where the increased fields around the metal nanoparticles leads to significantly higher excitation rates for a cofactor molecule (or any other fluorophore) localized in this region when compared to the same molecule in a dielectric which is excited only by the incident light. This will lead to greater excitation-emission cycles of the fluorophore in a given time period. Secondly, on the emission side the coupling of the excited-state fluorophore with the plasmons of the metal nanostructure leads to the formation of a “plasmophore” that also contributes to the to increased emission intensities.

In Figures 8-9 we present the first single molecule MEF studies on cofactors. Figure 8 (a) and (b) shows representative single-molecule images of FMN on glass and SIFs collected using confocal optics on a stage scanning microscope. Well-separated bright spots represent fluorescence emission from the individual FMN molecules. The significant difference in the peak intensities of the two images shown are clear proof that SIFs are highly effective in enhancing the intensity of the intrinsic emission of single FMN molecules. We have observed that the density of spots observed on glass (data not shown) increases with higher FMN concentrations, thus further providing evidence that we are observing single FMN molecules. To explore the changes in the underlying photophysics of single FMN molecules on glass and SIFs surfaces, the fluorescence intensity of individual FMN molecules as a function of time was recorded while under continuous excitation. While observing the individual FMN molecules under continuous illumination on the glass substrate, the cofactor molecules display severe blinking. An example of such blinking is shown in the intensity time trace of Figure 8 (c). We can clearly see that the intensity fluctuates dramatically, and eventually the emission stops when the molecule undergoes complete photodestruction. Figure 8 (d) shows the intensity time trace of an FMN molecule on SIFs. Here we not only observe a significantly higher count rate but also a significant decrease in blinking. We have only provided the time trace of a single FMN molecule on both glass and SIFs but this phenomenon was observed across a set of 50 molecules on each substrate that was investigated for this study. Blinking occurs when an excited state molecule transitions back and forth between the singlet state (on-state) and triplet state (off-state). We know that in the case of MEF, the enhancement in the emission intensity occurs concurrently with a decrease in the lifetime of the emission.^{1,27-28} As a result of the shortened lifetime, the fluorophore does not remain in the excited state long enough for the inter-system crossing into the triplet state to occur and thus we observed reduced blinking. Another important factor to note here is that both the blinking and photobleaching events of the FMN molecules on either glass or SIFs are always a single step quantum event. This is further proof that we are only observing single FMN molecules.

In Figure 8 (e) and (f) we present the fluorescence intensity decays of single FMN molecules on glass and SIFs. The lifetimes of single FMN molecules were measured using the time-correlated single-photon counting (TCSPC) method and were recovered by non-linear least-squares (NLLS). The average lifetime of FMN on glass is about 4.4 nsec and the lifetime is dramatically shortened on the SIFs surface to approximately 0.32 nsec. It is interesting to see that the decrease in lifetime of FMN on SIFs at the single molecule level is much greater than that at the ensemble level. This is because for the ensemble case we study a very large number of molecules with a wide range of dipolar orientations with respect to the glass or

SIFs substrate and with also a wide variety of nano-environments around the cofactor molecules. This heterogeneous group of molecules leads to an average lifetime value. In the single molecule case, we are studying only a single cofactor molecule with a fixed nano-environment and at the fixed orientation with respect to either the glass or SIFs. Hence we recognize that for SMD studies it is important to provide a histogram of the intensities and lifetimes for a large number of molecules to get a sense of the different orientation and nano-environments around multiple molecules. We will provide histogram data and discuss this topic further in Figure 9. Figure 8 (g) and (h) presents the representative spectra of individual FMN molecules on glass and SiFs surfaces respectively. Individual FMN molecules both on glass and SiFs surfaces displayed spectra with unique properties that are not always identical to the bulk phase spectra. These spectral changes could be a result of a number of mechanisms such as different local nano-environments of the cofactor molecules. We believe this is the first observation of the spectra of individual cofactor molecules on SIFs substrates. Figures 8 (g) and (h) show that there is a significant spectral overlap between the emission spectra of FMN molecules from glass and SIFs thus further confirming that the emission spectra collected were indeed from the FMN molecules and not corrupted by any noise in the system (such as silver nanoparticle luminescence).

In Figures 9 (a) and (b) we present the intensity histograms of over 50 FMN molecules on glass or SIFs surfaces. Examination of intensity histograms shows that on average the FMN molecules were more than 20-fold brighter on the SIFs than on glass. These results further confirm that silver nanostructures can increase the brightness of FMN molecules on SIFs when compared to glass. We also measured the lifetimes of over 50 FMN molecules, on glass and SIFs surfaces to determine the range of lifetimes present in each substrate. These results are presented in Figures 9 (c) and (d). The mean of the lifetime distribution on the SIFs is ~ 0.32 nsec, and that on glass is 4.4.nsec with the histograms showing that on average the lifetimes are about 12-fold shorter on the SIFs than on glass. An important observation is that the cofactor molecules with the shorter lifetimes had higher intensities, which suggests that the radiative decay rate of the FMN molecules is much larger on the SIFs than on glass. The results of Figure 9 (a-d) show that silver nanostructures can increase the brightness and reduce the lifetime of FMN molecules. This also indicates that FMN molecules can potentially have higher photostability on SiFs when compared to glass.

4.0 Conclusions

The focus of this study was to determine whether metal nanostructures can be employed to enhance the intrinsic emission of enzyme cofactors. We see in ensemble studies for the cofactors FAD and FMN, silver nanostructures increased the emission intensity over 10-fold for both FAD and FMN. There was also concurrently a 2-fold reduction in the lifetime of FAD and FMN on SIFs. The results of these “proof of concept” studies are in agreement with our previous observation of MEF of various fluorophores. Similarly for the case of NADH, aluminum was the metal of choice for enhancing its intrinsic emission. This is because absorption and emission of NADH lies in the UV and blue region respectively where the plasmon resonance properties of aluminum are ideal. Our experimental observations were corroborated by theoretical FDTD calculations which show that silver nanoparticles enhance the radiated power of fluorophores in the visible region, having dipolar peak enhancements at approximately $\lambda = 490$ nm. The silver nanoparticles also computationally showed strong enhancements in the near-fields when a dipole radiating at $\lambda = 530$ nm is in close proximity to it. On the other hand, the near-fields created by the interaction of an aluminum nanoparticle next to a dipole radiating at $\lambda = 530$ nm was not as strong as that of silver. Additionally, the silver nanoparticles also computationally showed strong interactions with an incident plane wave at $\lambda = 440$ nm leading to intense near-fields localized around the nanoparticle. These set of FDTD results showed that silver is the ideal

metal to enhance the intrinsic emission of FAD and FMN. Similarly FDTD calculations on aluminum showed that the radiated power enhancements are maximized at approximately $\lambda = 440$ nm. Additionally, we show that aluminum nanoparticles also create intense near-fields as a result of their interaction with incident plane waves at $\lambda = 340$ nm. These enhanced near-fields are not seen for the case of the silver nanoparticles at this wavelength. Hence our FDTD results indicate that aluminum is the ideal metal to generate the MEF effect for the intrinsic emission of NADH. We then present for the first time results of experimental SMD studies where we show that SIFs surfaces lead to significant enhances in the intrinsic emission intensity of single FMN molecules. Our SMD studies also clearly show that SIFs significantly reduce blinking from single FMN molecules. These set of theoretical and experimental results proves that by exploiting the appropriate plasmonic properties of metallic nanostructures, it is possible to perform SMD studies on single cofactors without the need of extrinsic labeling. Our results indicate that by exploiting the intrinsic emission of cofactors using metals, there is great potential to design assays to obtain direct measurements of the catalytic events by a single enzyme molecule and thus obtain a wide range of information about enzyme function, biomolecule association reactions and other biochemical interactions.

Acknowledgments

This work was supported by the National Institutes of Health (NIH) - Grant Nos. RC1GM091081, and EB006521, HG005090.

References

1. Lakowicz, JR. Principles of Fluorescence Spectroscopy. 3rd Ed. Springer; New York: 2006.
2. Rigler, R.; Edman, L.; Földes-Papp, Z.; Wennmalm, S. Fluorescence Correlation Spectroscopy in Single-Molecule Analysis: Enzymatic Catalysis at the Single Molecule Level. In: Rigler, R.; Orrit, M.; Basché, T., editors. Single Molecule Spectroscopy - Nobel Conference Lectures. Springer; New York: 2001. p. 177
3. Visser, AJWG.; van den Berg, PAW.; Hink, MA.; Petushkov, VN. Fluorescence Correlation Spectroscopy of Flavins and Flavoproteins. In: Rigler, R.; Elson, ES., editors. Fluorescence Correlation Spectroscopy – Theory and Applications. Springer; New York: 2002. p. 9
4. van den Berg, PAW.; Visser, AJWG. Tracking Molecular Dynamics of Flavoproteins with Time-Resolved Fluorescence Spectroscopy. In: Valeur, B.; Brochon, B-V., editors. New Trends in Fluorescence Spectroscopy – Applications to Chemical and Life Sciences. Springer; New York: 2001. p. 457
5. van den Berg PAW, Widengren J, Hink MA, Rigler R, Visser AJWG. Spectrochim. Acta A. 2001; 57:2135.
6. Li H-W, Yeung ES. J. Photochem. Photobiol. A: Chem. 2005; 172:73.
7. Voet, D.; Voet, JG.; Pratt, CW. Fundamentals of Biochemistry. John Wiley & Sons; New York: 2002.
8. Visser AJWG. Photochem. Photobiol. 1984; 40(6):703. [PubMed: 6522458]
9. Chosrowjan J, Taniguchi S, Mataga N, Tanaka F, Visser AJWG. Chem. Phys. Letts. 2003; 378:354.
10. Visser, AJWG.; Muller, F. Time-resolved fluorescence on flavins and flavoproteins, Methods in Enzymology. Academic Press; 2002. 1980
11. Mataga N, Chosrowjan H, Taniguchi S, Tanaka F, Kido N, Kitamura M. J. Phys. Chem. B. 2002; 106:8917.
12. van den Berg PAW, van Hoek A, Walentas CD, Perham RN, Visser AJWG. Biophys. J. 1998; 74:2046. [PubMed: 9545063]
13. Chowdhury MH, Ray K, Johnson MJ, Gray SK, Pond J, Lakowicz JR. J. Phys. Chem. C. 2010; 114:7448.

14. Chowdhury MH, Ray K, Gray SK, Pond J, Lakowicz JR. *Anal. Chem.* 2009; 81:1397. [PubMed: 19159327]
15. Chowdhury MH, Pond J, Gray SK, Lakowicz JR. *J. Phys. Chem. C.* 2008; 112:11236.
16. Chowdhury MH, Gray SK, Pond J, Geddes CD, Aslan K, Lakowicz JR. *J. Opt. Soc. Am. B.* 2007; 24:2259. [PubMed: 19777118]
17. Taflove, A.; Hagness, SC. *Computational Electrodynamics: The Finite-Difference Time-Domain Method.* 3rd Ed. Artech House; Norwood: 2005.
18. Sullivan, DM. *Electromagnetic Simulation Using the FDTD Method.* IEEE Press; Piscataway: 2000.
19. Taflove A, Brodwin ME. *IEEE Trans. Microwave Theory and Techniques.* 1975; 23:623.
20. Yee KS. *IEEE Trans. Antennas Propagat.* 1966; AP-14:302.
21. Guiffaut G, Mahdjoubi K. *IEEE Ant. And Prop. Mag.* 2001; 43:94.
22. Berenger JP. *J. Comput. Phys.* 1994; 114:185.
23. Kaminski F, Sandoghdar V, Agio M. *J. Comp. and Theor. Nanoscience.* 2007; 4:635.
24. Lakowicz JR, Shen Y, D'Auria S, Malicka J, Fang J, Gryczynski Z, Gryczynski I. *Anal. Biochem.* 2002; 301:261. [PubMed: 11814297]
25. Ray K, Chowdhury MH, Lakowicz JR. *Anal. Chem.* 2007; 79:6480. [PubMed: 17685553]
26. Lakowicz JR, Ray K, Chowdhury M, Szmazinski H, Fu Y, Zhang J, Nowaczyk K. *The Analyst.* 2008; 133:1308–1346. [PubMed: 18810279]
27. Lakowicz JR. *Anal. Biochem.* 2001; 298:1–24. [PubMed: 11673890]
28. Lakowicz JR. *Anal. Biochem.* 2005; 337:171–194. [PubMed: 15691498]

Figure 1.

(a) Normalized absorption and emission spectra in water of NADH; (b) Normalized absorption and emission spectra in water of FAD; (c) Normalized absorption and emission spectra in water of FMN.

Figure 2.

(a) FE-SEM image of SIFs; (b) Extinction spectra of SIFs; (c) SEM image of 10 nm thick Al film; (d) Extinction spectra of 10 nm thick Al film.

Figure 3.

(a) Fluorescence emission spectra of NADH on 10 nm Al film and quartz; (b) Fluorescence emission spectra of FAD on SIFs and glass; (c) Intensity decay of FAD on glass and SIFs substrate showing a shorter lifetime of the FAD on SIFs than on glass. IRF is the instrument response function.

Figure 4.

(a) Fluorescence emission spectra of FMN on SIFs and glass; (b) Intensity decay of FMN on glass and on a SIFs substrate showing a shorter lifetime of the FMN on SIFs than the glass. IRF is the instrument response function; (c) Photostability of FMN on glass and SIFs substrates.

Figure 5.

FDTD calculations showing: (a) the normalized scattering cross-section of an 80 nm aluminum and silver nanoparticle respectively; (b) the radiative power enhancement of a dipole next to an 80 nm aluminum nanoparticle in water with dipole-metal distances of 5 nm and 10 nm; (b) the radiative power enhancement of a dipole next to an 80 nm silver nanoparticle in water with dipole-metal distances of 5 nm and 10 nm. In both figures the dipole is oriented perpendicular to the metal surface.

Figure 6.

(a) Near-field intensities arising from an isolated point dipole radiating at $\lambda = 530$ nm; (b) Near-field enhancement around an 80 nm diameter silver nanoparticle due to the interaction with a 530 nm emitting dipole located 5 nm from the surface; (c) Near-field enhancement around an 80 nm diameter aluminum nanoparticle due to the interaction with a 530 nm emitting dipole located 5 nm from the surface. Images are on a log scale (base 10), and the dipole is oscillating along the x -axis, which corresponds to the perpendicular orientation described in the text.

Figure 7.

(a) FDTD calculations of the near-field intensity enhancement image in water of the fields created around a 80 nm silver nanoparticle by its interaction with the plane wave of wavelength $\lambda = 340$ nm; (b): Near-field intensity enhancement image in water of the fields created around a 80 nm aluminum nanoparticle by its interaction with the plane wave of wavelength $\lambda = 340$ nm; (c) Near-field intensity enhancement image in water of the fields created around an 80 nm silver nanoparticle by its interaction with the plane wave of wavelength $\lambda = 440$ nm; (d) Near-field intensity enhancement image in water of the fields created around an 80 nm aluminum nanoparticle by its interaction with the plane wave of wavelength $\lambda = 440$ nm. For all the cases the plane wave is oriented along the x -axis and propagating along the z -axis (out of the plane of paper). Note all images are in the log scale (base 10).

Figure 8.

Scanning confocal images of FMN molecules on (a) Glass and (b) SIFs. Scale bar shows the intensity counts in 1-ms bin; Intensity-time trajectories of individual FMN molecules on (c) SIFs and (d) Glass; Fluorescence intensity decays of individual FMN molecules on (e) glass and (f) SIFs surfaces; Single-molecule fluorescence spectra of individual FMN molecules: (g) on glass and; (h) SIFs surfaces.

Figure 9. Fluorescence intensity histograms of FMN on (a) glass and (b) SIFs. Average fluorescence lifetime histogram of FMN on (c) glass and (d) SIFs surface.