Fluorescence Intermittency and Spectral Shifts of Single Bio-conjugated Nanocrystals Studied by Single Molecule Confocal Fluorescence Microscopy and Spectroscopy

HyeongGon Kang[1], Mathew M. Maye[2], Dmytro Nykypanchuk[2], Matthew Clarke[1], Peter Yim[1], Jeffrey Krogmeier,[1,3] Kimberly Briggman[1], Oleg Gang[2], and Jeeseong Hwang[1*]

[1]National Institute of Standards and Technology, Physics Laboratory, Optical Technology Division, 100 Bureau Drive, Gaithersburg, MD 20899 USA
[2]Brookhaven National Laboratory, Center for Functional Nanomaterials, Upton, NY *11973*
[3]Current Address: U.S. Genomics, 12 Gill Street, Suite 4700, Woburn, MA 01801

ABSTRACT

We have fabricated a combined measurement system capable of confocal microscopy and fluorescence spectroscopy to simultaneously evaluate multiple optical characteristics of single fluorescent nanocrystals. The single particle detection sensitivity is demonstrated by simultaneously measuring the dynamic excitation-time-dependent fluorescence intermittency and the emission spectrum of single cadmium selenide/zinc sulfide (CdSe/ZnS) nanocrystals (quantum dots, QDs). Using this system, we are currently investigating the optical characteristics of single QDs, the surface of which are conjugated with different ligands, such as trioctylphosphine oxide (TOPO), mercaptoundecanoicacid (MDA), and amine modified DNA (AMDNA). In this paper, we present the progress of our measurements of the time-dependent optical characteristics (fluorescence intermittency, photostability, and spectral diffusion) of single MDA-QDs and AMDNA-MDA-QDs in air in an effort to understand the effects of surface-conjugated biomolecules on the optical characteristics at single QD sensitivities.

Keywords: nanocrystal, quantum dot, photophysics, confocal microscopy, spectroscopy, single molecule, single particle, single quantum dot, single nanocrystal, bio-conjugation, nanosensor

1. INTRODUCTION

Colloidal semiconductor nanocrystals or quantum dots (QDs) are known to exhibit desirable optical characteristics enabling high sensitivity and high throughput multiplexed detection of multiple targets. Such optical properties include high fluorescence efficiency, photostability, broad absorption spectra and size-dependent narrow emission spectra, enabling increasingly employment of QDs in the development of next generation nano-engineered elements and devices, such as nano-optic optoelectronic assemblies, nanoscale biomedical imaging agents, and chemical and biological nanosensors[1-6]. Especially for the development of single QD-based nano-sensors, a variety of conjugation techniques to attach appropriate functional linkers and biocompatible molecules to the surface of nanocrystals, and nanocrystal conjugation to cells, have been developed, allowing for the nanocrystals to be harnessed with environment-sensing molecules on the surface to target specific sites in complex systems, or to assemble them into desired nano-structures to enhance their chemical senisitivity[3-5].

However, the optical properties of single QDs have been found to be dependent not only upon the local chemical environment in which the QDs are contained but also upon their surface functionality which may induce a change in fluorescence characteristics[7-9]. Therefore, in assessing the influence of the chemical moieties in the nanoscale surroundings of the QDs, the key challenge is to differentially measure the change in optical characteristics influenced

* jeeseong.hwang@nist.gov; phone: 301.975.4580

only by the surface-conjugated functional groups to the QDs. To this end, measurement techniques that simultaneously assess multiple time-dependent optical characteristics of single QDs in a controlled environment have been in great demand for the identification of the causes of the observed changes in optical properties. Additionally, the correlation of the optical properties of QDs with their physical and chemical states as altered by the proximity of bound or unbound organic molecules should be addressed. For instance, the exact cause of a blue shift in the emission spectra of QDs in a complex environment needs to be further elucidated. Some reports suggest a blue shift in the fluorescence of QDs may be mostly due to physical deformation (i.e. quantum confinement of excitons) of QDs when they approach or attach onto a surface[9]. Others ascribe spectral shifts to the photo-induced oxidation caused by environmental factors immediately surrounding the QDs, such as differences in hydrophobicity, hydrophilicity, pH, or electrostatic charges[10, 11]. While physical deformation of QDs would result in a definite blue shift in the emission spectra at an early stage of a time-transient measurement, the blue shift due to photo-induced oxidation would occur at a much slower rate and could be controlled by introducing an inert gas to the surrounding atmosphere of a QD. Some groups have in fact demonstrated that QD photo-oxidation can be controlled by using a nitrogen atmosphere or by varying the buffer conditions in living cells[12].

In this paper, we present the capability of our measurement technique, mainly based on combined confocal fluorescence emission microscopy and spectroscopy of single QDs, by demonstrating the differences in time-dependent optical characteristics between MDA-QDs and AMDNA-QDs at single particle sensitivities. We believe that our measurement technique will help to elucidate the photophysics of single QDs and relate their optical properties to a change in either in their local chemical environment or in their physical condition, such as nano-deformation and defects at the atomistic scale.

2. METHODS

2.1 Sample preparation

QD samples were prepared on clean borosilicate glass cover slips, 22 mm sq. No. 1.5 (Corning Inc., Corning, NY). Prior to use, the cover slips were cleaned in 1 mol·L^{-1} KOH solution for 2 hours, rinsed with ultrapure water (Barnstead International, Dubuque, IA), dried by nitrogen gas, and finally treated in an ultraviolet-ozone cleaner (Jelight Company, Inc. Irvine CA) for 40 min. Samples were prepared by spin casting approximately 20 μL of dilute quantum dot solution onto a prepared glass coverslip at the speed of 210 rad·s^{-1} for 10 s. Final concentrations of MDA and AMDNA conjugated QDs for spin casting were 8 nmol·L^{-1} and 2 nmol·L^{-1} in water, respectively. The MDA-conjugated QDs (5.8 nm ± 0.8 nm diameter, 575 nm first absorbance peak, 600 nm emission) were obtained from NN-Labs, LLC (Fayetteville, AR) and AMDNA was synthesized by Integrated DNA Technologies (Coralville, IA). The sequence of DNA in AMDNA was (5')TACTTCCAATCCAAT-$(T)_{15}$ (3') with a primary amine modification at the 3' end . AMDNA (40 μmol·L^{-1}) conjugation to MDA-QDs (1 μmol·L^{-1}) was carried out in a 10 μmol·L^{-1} solution of 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (both obtained from Sigma-Aldrich, St. Louis, MO) in 100 mmol·L^{-1} phosphate buffer at pH 7.7 overnight. Then, the mixture was quenched with 10 mmol·L^{-1} hydroxylamine solution and purified by size exclusion chromatography with a 7 min centrifuge spin at 16,000 g radial centrifugal force.

2.2 Combined confocal fluorescence microscopy and spectroscopy

A schematic of the combined confocal fluorescence microscopy and spectroscopy instrument is illustrated in Figure 1(A). The optical path of the light for confocal fluorescence detection is given as follows. A 488 nm excitation beam from a CW solid state laser (Sapphire 488-20 CDRH, Coherent, Santa Clara CA) is spatially filtered by focusing through a 50 μm diameter pinhole and subsequently passing through a collimator to expand the beam, a series of mirrors to guide the beam to the side port of an optical microscope (Zeiss Axiovert 135, Carl Zeiss MicroImaging, Inc., Thornwood, NY), a 488 nm bandpass filter to reject potential fluorescence due to optical components in the optical path, and a dichroic filter (485 DRLP, Omega Optical Inc., Brattleboro, VT) mounted at 45 degrees relative to the optical axis of an oil-immersion objective lens (100X, 1.45NA, Carl Zeiss MicroImaging, Inc., Thornwood, NY) which focused the diffraction-limited laser spot onto the upper surface of the glass cover slip. The laser power incident on the dichroic mirror was 30.0 μW ± 0.1 μW unless otherwise mentioned, and the power was measured prior to each data acquisition. For fluorescence intermittency, the fluorescence emission is collected with the same objective lens, directed through an

edge filter, a 488 nm notch filter (Kaiser Optical Systems, Inc., Ann Arbor, MI) and a 585 nm ± 35 nm band pass filter and focused onto an avalanche photodiode (APD). The APD is mounted on a 3-axis mechanical translator to optimize its position to precisely focus the fluorescence spot onto the active area (175 μm diameter) of the APD. Fluorescence emission versus time was obtained by recording the TTL output of the APD using a multichannel scaler, with an integration time of a 10 ms·bin^{-1} for each data point with a typical observation period of 10 min. Prior to the time-transient measurement, a fluorescence image was created first by raster-scanning a 5 μm × 5 μm area of the sample containing dispersed single QDs through a fixed position confocal spot using a piezoelectric stage (LP 100, Mad City Labs, Inc., Madison, WI). The 2D fluorescence intensity map (128 pixels x 128 pixels) is used to locate and record the positions of single, spatially isolated QDs. Once the position is located an individual QD (a bright spot in the image), the stage is repositioned to the center of the confocal excitation spot where it continuously excites the QD during the fluorescence measurement. Simultaneously, the fluorescence spectra from individual QDs were collected with a 5 sec integration time.

For spectral measurements, 50 % of the collected fluorescence signal was directed to a liquid nitrogen cooled, back-thinned Si charge-coupled detector (CCD, Princeton Scientific Instruments, Monmouth, NY) mounted on an SPEC300 spectrometer (Acton Research Corporation, Acton, MA), while the other 50 % was sent to the APD for intermittency measurement as described above. The incident angle of the emission signal impinging upon the notch filter was optimized for rejection of the 488 nm light by monitoring the Raman signal from hexane. Figure 1(B) shows the Raman spectra of hexane used for the wavelength calibration of the spectra on the CCD array. Figure 1B also shows the positions of three fluorescence emission peaks obtained from a mixture of QDs with emission peaks at 518 nm, 545 nm, and 603 nm. These are in good agreement with the values provided by the QD vendor (Evident Technologies Inc., Troy, NY).

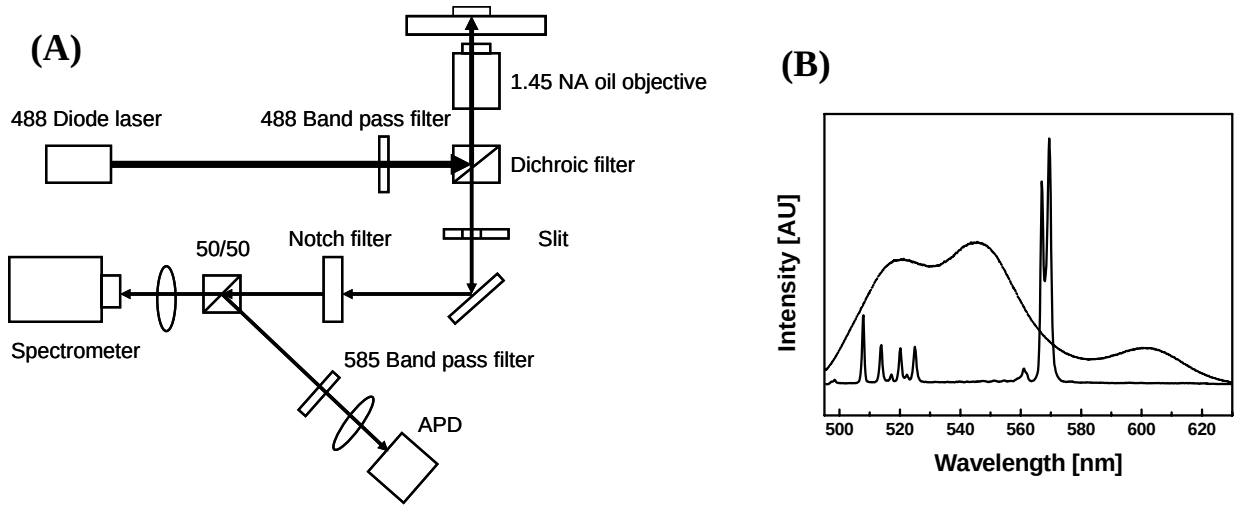

Figure 1. (A) Schematic of the combined fluorescence confocal microscopy and fluorescence emission spectroscopy system; (B) Raman spectra of hexane and fluorescence spectra of an ensemble of three different water-soluble CdSe/ZnS QDs with emission maxima at 518 nm, 545 nm, and 603 nm.

3. RESULTS and DISCUSSION

Figure 2A demonstrates a typical confocal fluorescence image of individual CdSe/ZnS QDs conjugated with MDA molecules and spin-cast on a glass coverslip. The image shows spatially resolved single or multiple QDs on a glass coverslip. The characteristics of single QDs can be confirmed by the quantized fluctuation in the time-transient fluorescence intensity (Figure 3A) which differs from clusters of multiple QDs (data not shown). This behavior is also evident in the fluorescence image (Figure 2A) where the blinking behavior can be observed as alternating bright and

dark streaks or pixels along the same scan line within the confines of a single quantum dot during the image scanning process.

Fluorescence spectra of individual CdSe/ZnS quantum dots were measured for a number of QDs. The spectral diversity of single QDs was observed even though the QDs were from the same batch solution. Additionally, the emission spectrum of an ensemble of QDs (Bulk) is much broader than the individual spectra of single QDs, due to the emission spectra being an average of many single QDs (Figure 2B). Moreover, for single QDs, the details of the fluorescence emission spectra such as peak position, intensity, and width appear to vary according to the history of light exposure prior to the measurement (Data not shown). The same behavior was also observed with 2 or 3 QDs in a cluster, while an ensemble of QDs in solution did not show the behavior due to fast diffusion of the QDs in solution in a short measurement time period

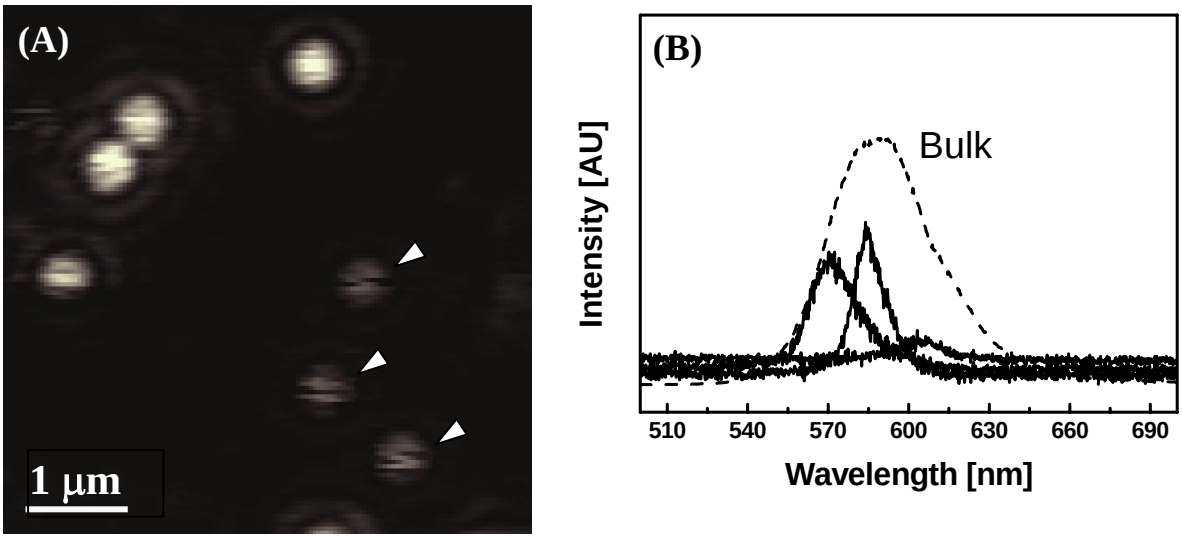

Figure 2. (A) A typical confocal fluorescence micrograph of MDA-QDs spin-cast on a glass coverslip. Four bright spots at the top left corner of the image are clusters of 2-3 single QDs, and the three dimmer spots indicated with arrows are from single MDA-QDs. (B) A Spectrum of an ensemble of QDs in solution and several single QD emission spectra are shown to demonstrate the spectral diversity of single QDs in the ensemble.

A typical fluorescence emission versus time trace of a single QD on the glass coverslip in ambient air is presented in Figure 3. Such data allows a systematic study of the excitation-time-dependent optical characteristics of single QDs. For these results, the fluorescence intensity and the emission spectra of a single MDA-QD were simultaneously obtained. The fluorescence emission in Figure 3A exhibits a characteristic signatures of single QDs in which the QD is either in a light-emitting ("on") mode or in a dark ("off") non-emitting mode also known as "blinking". In most of the MDA-QDs we studied, this intermittent behavior continued until a MDA-QD irreversibly photo-bleached after a few minutes of continuous excitation.

Although more complete analysis is needed, our initial results provide insight into understanding the photophysics of bio-conjugated QDs. First, the blinking behavior of MDA-QD at the early stages of the measurement is significantly suppressed compared to the TOPO-conjugated QDs which are not based on a mercapto- surface conjugation method[13]. The fluorescence intermittency occurs more frequently as a function of time, resulting in shorter "on" lengths of time. The switching from "on" to "off" states involves ionization of a QD by ejecting a charge carrier (electron or hole) to a trapped state (e.g. a lattice imperfection or impurity in the crystal, or to a surface impurity or defect of the glass substrate near the QD) at the QD surface or to an energy state of the surrounding environment[13]. The time-dependent shortening of light-emitting "on" lengths of time implies that the surrounding environment of an MDA-QD evolves to preferentially increase the probability for ejected charge carriers to be ejected to a "trap" state. Secondly, the fluorescence emission counts gradually decrease in time until irreversible photo-bleaching occurs. It has been proposed that the intensity decrease is more likely caused by the formation of surface defect sites in QDs resulting in

irreversible creation of photo-induced quenching states which enhance the probability of non-radiative recombination pathways[10]. However, we cannot completely exclude the contribution of a size reduction of the QD caused by the creation of a photo-induced oxide layer in the QD shell. This size reduction would also result in the decrease of photon counts due to the diminished absorption cross-section of QDs as their size become smaller than 5 nm[14]. A more thorough understanding of the relationship between fluorescence intermittency and spectral evolution would allow the variations in optical properties of individual bio-conjugated QDs to be probed.

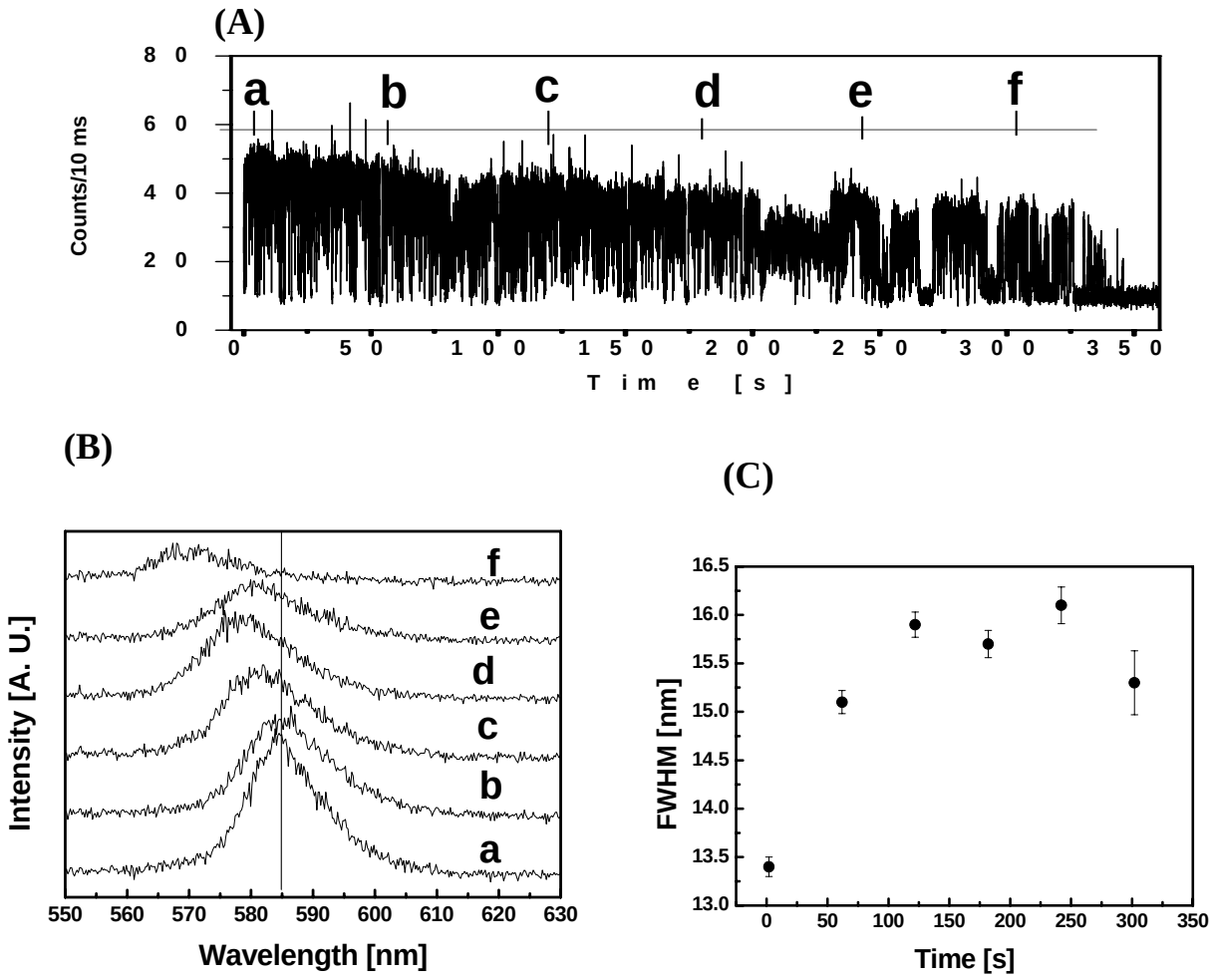

Figure 3. (A) Fluorescence intermittency from a single MDA-conjugated CdSe/ZnS QD under continuous illumination as a function of time. (B) Time-dependent spectral shift of a single QD. The time points at which each spectrum was collected are shown in (A) with indices (a) to (f). One of the single QDs marked with arrows in Fig. 2 (A) was continuously exposed to a laser spot and the data were collected with a total integration time of 5 seconds for each spectrum. (C) Full width of the

To fully explore this potential link, simultaneously measured emission spectra are presented in Figure 3B and are compared to the fluorescence intermittency data in Figure 3A. Under ambient conditions, the blue shift in the MDA-QDs emission spectrum was obvious with an estimated overall spectral shift rate of $\approx 5.710^{-2}$ nm/s with respect to the position of the initial spectrum measured at $t \approx 1$ s approximately one second after the beginning of the continuous illumination. It is noteworthy that the rate of spectral shift varies such that a slow *directional* blue shift is dominant during the early stage of illumination (Figure 3B, a, b, and c, time-traced intermediate spectra between 'b' and 'c' are not shown), but at the later stage of measurement, a faster shift was observed with occasional spectral *diffusion* (c, d, e, f).

From these results, we speculate that the fluorescence intermittency rate may be highly correlated with the increased spectral shift rate and with the spectral diffusion rate as well. To investigate a trend in the time-dependent spectral diffusion under continuous illumination, we measured the full width at half maximum (FWHM) from each emission peak of the spectra in Figure 3B by fitting them with Gaussian curves using Origin data analysis software (OriginLab Corporation, Northhampton, MA). The resulting spectral line widths versus illumination time are displayed in Figure 3C. Although the FWHM appears to decrease slightly at later times, a conclusive trend between the spectral diffusion and fast intermittency still remains unclear due to the decreased signal to noise level as the photon counts severely diminish. The relationship between fluorescence intermittency and spectral shift suggest that the decrease in photon counts during the time-transient measurement partially originates from a reduction in the quantum confinement length of a QD. This can likely be ascribed to photo-oxidation which results in a decrease in the absorption cross section of the QD. The blue shift in fluorescence spectra of QDs has been attributed to photoinduced oxidation of the QDs during the measurement[10, 11]. Van *et al* [15] reported a large blue shift (30 nm) from a single QD in air, but a much smaller blue shift in a nitrogen environment. It was suggested that the blue shift may be attributed to the shrinkage of the CdSe core by photo-induced oxidation resulting in reduced absorption cross section of the QD.

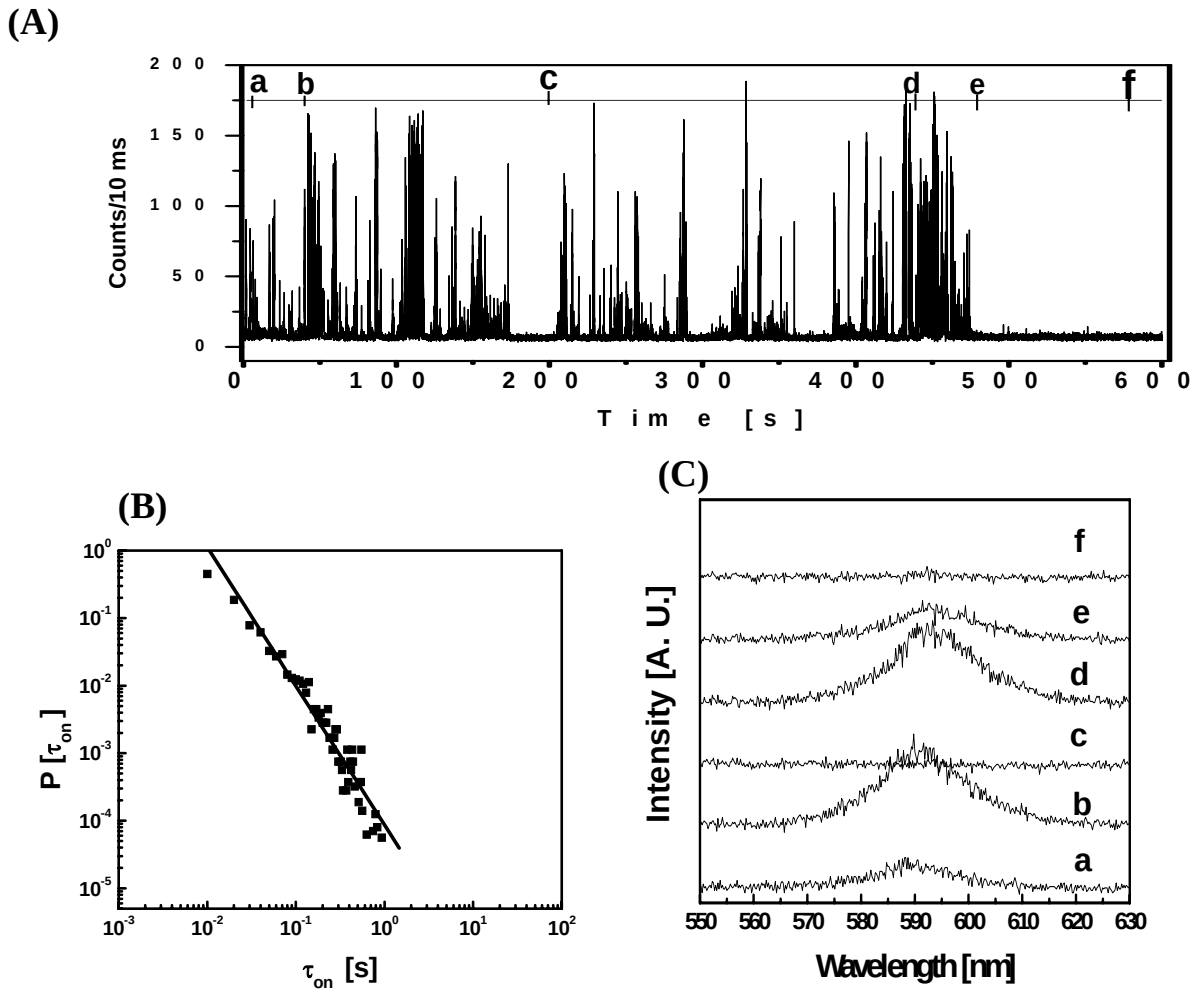

Figure 4. (A) Fluorescence intermittency from a single AMDNA-conjugated CdSe/ZnS MDA-QD under continuous illumination as a function of time. (B) Log-log weighted probability density plots of the light-emitting episodes for a single AMDNA-QD. The data was fit to a power law with an exponent of 2.06 ± 0.08. (C) Illumination-time-dependent spectral shift of a single QD. The time points for each spectrum collected are shown in (A) with indices (a) to (f).

The spectral diffusion in QDs has been attributed to Stark shifts caused by the ejected charge carrier hopping among different trap sites near the QD surface[16]. We repeated the measurements of the CdSe/ZeS MDA-QDs with AMDNA ligands conjugated to them in an effort to create a different local electrostatic environment with dissimilar trap sites to those of MDA-QDs. We anticipated that the long DNA molecules would suppress photo-oxidation of QDs by providing a "barrier" preventing oxygen molecules from reaching the QD surface therefore rendering time-invariant emission spectra. We also expected that the DNA moieties would allow for a uniform negatively charged electron donating environment resulting in the reduction of fluorescence intermittency frequency[17].

However, unexpectedly, the fluorescence intermittency dynamics of single AMDNA-MDA-QD did not exhibit the suppression of intermittency behavior in air (Figure 4A). Rather, AMDNA-QD demonstrate only short "on" lengths at all time scales with stochastic intensity fluctuations but no noticeable decrease in photon counts until the moment of irreversible photo-bleaching. For quantitative comparison of "on" time lengths between MDA and AMDNA-MDA-QDs, a log-log probability density plot of the "on" lengths of a single AMDNA-MDA-QD is shown in Figure 4B. The procedure to obtain this weighted probability density plot is described elsewhere in detail[18, 19]. The linear log-log plot of the probability density distribution confirms that the intermittency process of single AMDNA-MDA-QD is also governed by multi-exponential recovery rates, a process which can easily be described by power law kinetics as $P(\tau) \propto (1/\tau)^m$, where τ represents the length of the "on" time and m represents the power law exponent. The power law exponent of $m_{on} = 2.06 \pm 0.08$ is quite larger than 1.19 ± 0.05, of a single MDA-QD (probability density plot not shown), and 1.47 ± 0.27 of a TOPO-QD[19]. This fast intermittency may be attributed to the increase of proximal states introduced by the surface-conjugated AMDNA molecules, in which the ejected charge carriers are trapped for longer times. Stochastic and yet consistently robust photon counts also exclude the possibility of irreversible creation of photo-induced quenching states in an AMDNA-MDA-QD, which otherwise would result in gradual decrease of photon counts in time. The proximal trap states are not likely introduced by surface defects due to photo-oxidation. In fact, a blue shift in the emission spectra of only a few nanometers or less was rarely observed for the AMDNA-MDA-QDs (Figure 3C). No noticeable change in the FWHM of the spectra was observed either, implying that the spectral diffusion is also suppressed significantly.

4. CONCLUSIONS AND FUTURE PROSPECTS

In summary, we have demonstrated a combined confocal fluorescence microscopy/spectroscopy system capable of simultaneously evaluating multiple optical properties of bio-conjugated QDs. The effect of surface conjugation of the QDs with MDA and AMDNA molecules on the optical properties of single QDs were investigated including excitation-time-dependent fluorescence intermittency, and spectral characterization of the fluorescence emission. Decrease in the "on" time lengths in blinking and diminishing photon counts are concomitantly observed with a spectral blue shift and spectral diffusion only from single mercaptoundecanoic acid-CdSeZnS QDs, while DNA-conjugated QDs did not show this correlation. These results provide important information in understanding the dynamic photophysics of bio-conjugated QDs under the influence of varying chemical and physical properties using surface-conjugated ligands. We believe that our measurement and analysis techniques promise a wealth of insight in elucidating the photophysics of bio-conjugated QDs. We are currently pursuing experiments relating the optical properties of single QDs with a change in either their local chemical environment or their physical conditions such as nano-deformation and defects at the atomistic scale. The differences in optical characteristics between MDA- and AMDNA-MDA-QDs imply that the significant suppression of spectral shift and spectral drift are consequences of DNA conjugation. However, for the AMDNA-MDA-QDs, we occasionally observed emission spectra that were blue shifted by a few nanometers. This may result from a change in the amount of DNA or the conformation of DNA on the surface. To confirm the density of surface conjugated AMDNA molecules, we are making progress on implementing a quantitative DNA hybridization assay using dye-labeled complementary DNA (cDNA) oligomers specific for bonding to the sequence of the AMDNA molecules. Optical measurements under the control of an electrostatic field distribution in the vicinity of the QD surface could provide additional insight into the photo-physics of bio-conjugated QDs. To this end, experiments with adjusted parameters that affect hybridization energies and charged states of DNA backbones are being investigated. In order to control photo-oxidation of QDs, we also added a sample chamber to the system to provide control of the gaseous environment surrounding the QDs. Optical characterization of QDs in a variety of gaseous environments (Ar, N_2, etc.) are also under investigation.

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