Detection of single molecules in microspheres

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ABSTRACT

We have investigated the use of micron-sized liquid droplets as sample medium to detect single fluorescent molecules in solution. The use of microdroplets (5 - 15 μm diameter) offers several powerful advantages over single-molecule detection schemes involving measurements on bulk liquids where the probe volume is defined by the laser beam. In addition, cavity-quantum electrodynamical (QED) effects have been observed which influence both spontaneous emission rates and fluorescence yields of dye molecules in these microspheres.

1. INTRODUCTION

Detection of single molecules in condensed phase with high signal-to-noise ratios is important for applications which involve detection of fluorescent tags such as DNA sequencing, fluorescence immunoassay, or hydrology. However, single molecule detection in liquid phase solutions at room temperature pose problems such as solvent dependent quantum yield, finite photochemical lifetime (the number of absorption-emission cycles which occur before irreversible bleaching of fluorescence), and solvent Raman scattering and fluorescence. Our approach to minimizing these problems is to use microdroplets as the sample medium which offers several advantages over conventional detection schemes.

Single R6G molecules were first detected in the liquid phase by Shera, et al.¹ using a sheath flow technique. In this work, photon bursts from dye molecules passing through the laser beam were observed using pulsed excitation and time-gated detection. The presence of single molecules was evidenced both indirectly through a non-random autocorrelation function, and directly through peaks in a filtered data stream using a weighted quadratic sum filter. Recent extension of this technique has shown that a mixture of two different dye molecules (with different fluorescence spectra) can be analyzed in this way using two channel detection.² The primary limitation of the sheath flow technique for single molecule detection is the trade-off between probe volume size and interaction time. For a flowing stream, the interaction time is typically limited to the time it takes the molecule to transit the probe

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volume. Obviously, interaction time can be increased by reducing the flow velocity but not without bounds. At some point, the interaction time will be limited by diffusion. This problem becomes more severe as the probe volume is reduced. In addition, diffusion limits the fraction of target molecules that can be forced to transit through the optically defined probe volume.

Our experimental approach to detecting single dye molecules in solution is based on using levitated microdroplets as the sample medium and employing cw laser excitation and photon-counting detection.³ Several advantages accrue from this approach. First, the probe volume is defined by the droplet diameter which has been made as small as 35 fL in our experiments. Second, the target molecule is confined to the probe volume with no diffusive losses. Third, interaction time is an independent variable so that all molecules remain in the interaction region until photolyzed. In addition, we have recently observed a factor of 10 increase in the spontaneous emission rate of R6G due to cavity-QED effects in 4 μ m micron diameter glycerol droplets⁴ which have recently been observed to enhance the fluorescence photon yield.⁵ The most important advantage however, is that a significant reduction in the concentration detection limit over bulk measurement techniques can be achieved through "counting" of molecules, or, digital molecular detection. Depending on the fluorescence photocount statistics, extremely low concentration detection limits can be achieved with modest (< 10) signal-to-noise ratios for single molecule detection. This technique has been successfully employed in the single molecule detection of β -phycoerythrin⁶ (a protein with 34 chromophores) where a signal-to-noise ratio of ≈ 5 was obtained.

2. DIGITAL DETECTION OF SINGLE-CHROMOPHORE MOLECULES

The basis of digital molecular detection is a binary decision as to whether a target molecule is present in the probe volume or not based on the result of a fluorescence measurement. A molecule is detected if the integrated number of photocounts exceeds a certain threshold above the mean background level. The uncertainty associated with this decision is highly sensitive to the photocount statistics of the molecule of interest and is proportional to the overlap between the background and signal photocount probability distributions. In the case of multiple chromophore molecules such as β -phycoerythrin, the photocount probability distribution is nearly Gaussian. Assuming that the distribution of background counts is also Gaussian, the overlap between the signal and background distributions drops off extremely rapidly with increasing signal-to-noise ratio. Thus, even with modest signal-to-noise ratios, digital detection of multiple-chromophore molecules can be performed with very low error rates.

Most single molecule detection applications, however, involve fluorescent tracers which have only a single fluorophore. Extension of this analysis to molecules such as R6G which have only a single chromophore is non-trivial for two reasons. Obviously the first reason is the reduced fluorescence yield which will result in lower signal-to-noise ratios. The second reason is that, for a molecule such as β -phycoerythrin, the photocount probability distribution is nearly Gaussian, whereas for a molecule with a single fluorophore such as R6G, the photocount probability distribution is a decreasing exponential peaked at zero with a decay constant equal to the reciprocal of the average number of detected photocounts per molecule. The difference in the photocount probability distributions

means that the overlap between the noise and photocount probability distributions will be greater for molecules with single fluorophores. Thus, because of the different photocount probability distributions, digital molecular detection is much more efficient for molecules with multiple chromophores even when the average fluorescence yield is the same.⁷ In this paper, we report detection of single R6G molecules in micron-sized levitated glycerol droplets with signal-to-noise ratios of about 50 using an experimental apparatus which is approximately an order of magnitude more sensitive than the system described in Ref. 11. Based on this experimental value for signal-to-noise ratio for single molecule detection, we project concentration detection limits using digital molecular detection techniques as a function of total error probability and show that sub-femtomolar concentration detection limits should be achievable.

3. EXPERIMENTAL AND RESULTS

The apparatus used to measure fluorescence from levitated droplets is shown schematically in Figure 1. Our setup⁸ is similar to that previously described in Ref.s 3 and 6 although it has been modified to improve the detection efficiency by approximately a factor of 10. Briefly, droplets are levitated in a three-electrode structure similar to that used in ion trap mass spectrometers. An AC potential is applied to the ring electrode at 60 Hz to confine the particle radially, while a DC bias is applied to the two end-cap electrodes to balance against the gravitational force on the droplet. An $f/1$ collection optic mounted in the ring electrode views the fluorescence from the droplet at angle of 90° with respect to the cw Ar^+ excitation laser. The laser was polarized in the horizontal direction, and an intensity at the droplet of about 500 W/cm^2 was used. Light collected from the droplet is spectrally filtered using an interference filter centered at 575 nm with 25 nm bandwidth (Omega Optical 575DF25) and focused onto a cooled photomultiplier (Hamamatsu R943-02) operated in photon-counting mode. The anode pulses were preamplified and input into a photon counter (Stanford Research SR400) with a 1 second integration time and 2 millisecond dwell interval between count periods.

Figure 1. Schematic of levitated droplet fluorescence spectrometer. The two end-caps of the electrodynamic trap are not shown for clarity.

Droplets were produced with a piezoelectric pipet by drawing about 10 μl of R6G / glycerol solutions which have been diluted in ultrapure water (Carolina Biological Supply Co.) by a factor of between 20 and 50. Initially, the droplet diameter is about the same as the droplet generator tip orifice (40 μm) but rapid evaporation of water leaves a glycerol droplet with a nominal diameter of 10 μm . The droplet diameter was determined by measuring the distance between reflected and refracted glare-spots from He-Ne laser illumination using an eyepiece reticle with rulings that correspond to 1 μm . Uncertainty in the diameter measurement was estimated to be about 10%.

The average fluorescence yield per molecule, R_{avg} , was determined in a separate set of experiments using relatively high (10^{-10} - 10^{-9} M) concentrations and was measured to be 2400 ± 1100 photons/molecule. The fairly large standard deviation arises primarily due to the uncertainty in the measurement of the droplet diameter. With an average of 2400 photons per molecule, it is important to extract all of the fluorescence within one or two counting periods in order to maximize the signal-to-noise ratio. Therefore, the laser intensity was chosen so that most of the dye molecules are bleached within a few seconds after admitting the excitation beam while also minimizing the background signal.

Picomolar solutions of R6G in 2% glycerol/water were prepared directly in the same way as the blanks. The large container volume serves to minimize diffusion and adsorption of dye molecules to the walls of the container. Figure 2 shows fluorescence data after mean background subtraction for 3 droplets which, from a concentration of 2.8 pM, (a), (b), and (c), are calculated to contain 0, 2, and 2 molecules respectively. Trace (a) shows no distinguishable fluorescence above background indicating that no molecule is present. The number of fluorescence counts above background in trace (c) corresponds to 3 molecules and the decaying exponential time dependence of the fluorescence signal is characteristic of the presence of more than one molecule in the droplet. Trace (b) also shows a strong fluorescence signal above background whose magnitude (≈ 1800 counts) agrees well with the expected photon yield for a single R6G molecule. In addition, the time dependence of the fluorescence signal in Fig. 2(b) is much different than observed for droplets containing more than one molecule.

The exponential decay of the fluorescence signal observed for droplets containing several dye molecules arises primarily from the distribution of photochemical lifetimes. That is, if we first approximate the laser intensity within the droplet to be uniform, the absorption-emission time would be the same for every molecule. Each molecule then would bleach at different times according to the probability distribution governing the number of absorption-emission cycles before photolysis, giving rise to the exponential decay of fluorescence as shown in Figure 2(c). For a single molecule exposed to laser excitation of constant intensity during its photochemical lifetime, the fluorescence signal will be constant until photolysis occurs at which time the signal will abruptly drop to background levels. If the integration time is approximately the same as the photochemical lifetime, virtually all the photocounts are acquired in one count period - giving rise to the same type of fluorescence signal as shown in Figure 2(b).

In the case where the integration period is shorter than the photochemical lifetime, the fluorescence intensity versus time will look approximately like a step function. The fluorescence signal as a function of time was examined for a 12 μm diameter glycerol droplet (2 pM concentration) where the integration time has been

Figure 2. Fluorescence signal vs. time for 3 different droplets containing R6G at 2 picomolar concentration.

reduced to 300 ms. Here the fluorescence persists over several count periods instead of just one. In this case, the signal-to-noise ratio will be the ratio of the integrated signal divided by the square root of N times the mean background where N is the number of count periods. Although the signal-to-noise ratio is reduced by about a factor of 2, the fluorescence is still easily distinguished from the background and shows the time-dependence expected for a single molecule. The total number of fluorescence counts above background is 1650, also consistent with the photon yield expected for a single molecule. Based on the time dependence and magnitude of the signals we believe that the data represent fluorescence detection of single molecules with signal-to-noise ratios of ≈ 50 and 27 respectively.

5. CAVITY-QED EFFECTS IN LIQUID MICRODROPLETS

Cavity effects in microdroplets are well known and arise from so-called “morphology dependent resonances” (MDRs)⁹ of the droplet which occur at specific values of the size-parameter, X , defined as $X = 2\pi r / \lambda$, where r is the radius of the sphere and λ is the wavelength of light. These resonances act as high-Q cavity modes and effects such as stimulated emission¹⁰ and lasing^{11,12} as well as enhanced energy transfer¹³ have been previously reported. Quantization of the radiation field within a small volume (≤ 1 picoliter) results in a redistribution of the density of photon states, $\rho(\omega)$, so that $\rho(\omega)$ is much larger than the corresponding free-space (infinite volume) value when ω

corresponds to a cavity resonance. Conversely, $\rho(\omega)$ is also much smaller than the free-space value when ω is not resonant with the cavity. Since the spontaneous emission rate is proportional to $\rho(\omega)$, emission rate enhancement is possible when the emission frequency is resonant with the cavity. Recently, Campillo and co-workers have reported spontaneous emission rate enhancement of chelated europium ions in a stream of falling ethanol droplets.¹⁴ These workers dispersed the emission from the droplets at successive times following an excitation pulse and clearly showed that the enhanced emission has a frequency dependence which maps the droplet resonances.

Our fluorescence lifetime measurements of R6G in levitated microdroplets showed that a 10-fold increase in the spontaneous emission rate occurs for 4 μm diameter glycerol droplets. The enhancement was observed to fall rapidly with increasing droplet diameter with no enhancement observed for droplet diameters larger than 10 μm . However no inhibited emission was observed, and it was originally assumed that the absence of an inhibited rate component was due to experimental parameters in the time-correlated photon counting apparatus which emphasized the short-time behavior of the fluorescence emission. Since the number of fluorescence photons emitted per molecule should be proportional to the average rate constant for spontaneous emission, the photon yield should be sensitive to relative fraction of molecules with enhanced and inhibited emission rates as well as the magnitude of emission rate enhancement and inhibition.

Fluorescence photon yields of R6G in droplets of varying size were measured to determine whether a net gain in sensitivity could indeed be obtained as a result of cavity-QED effects. It was first suggested by Hirschfeld¹⁵ that the photon yield may be enhanced if the rate of spontaneous emission is increased which effectively reduces the relative probability of branching along decay pathways which lead to photochemical bleaching. Once an excited singlet state (S1) has been prepared through absorption of a photon, the molecule may return to the ground singlet state (S0) through stimulated or spontaneous emission of a photon. Alternative S1 removal pathways include intersystem crossing to the triplet state (T1) or absorption of another photon to a higher singlet state. These processes are presumed to give rise to photochemical bleaching and the relative probability of following one of these paths is assumed to be proportional to the rate constant for that particular process. Since the sum of all branching probabilities for removal of the excited singlet state must be unity, any change in the probability for following one path must produce a change in the probability of following another. Thus, if the spontaneous (or stimulated) emission rate can be increased, the relative probabilities per excitation cycle for other removal processes must decrease resulting in an increase in the number of absorption-emission cycles that the molecule may undergo before photobleaching occurs. For droplet diameters between 7 and 20 μm , the average fluorescence yield was observed to be independent of droplet size, while approximately a factor of 2 increase in the fluorescence yield was measured for 4 μm diameter droplets. These results clearly demonstrate that cavity-enhanced emission rates result in an increase in the fluorescence yield and suggest that the fraction of inhibited emission is small compared to the enhanced fraction. These results further imply that signal-to-noise ratios for single-molecule detection in microdroplets may be greatly enhanced by these effects.

6. SUMMARY AND CONCLUSIONS

The use of microspheres has been shown to be extremely well suited for detection of single fluorescent molecules. These microspheres have an easily controllable volume which is independent of the excitation laser and all target molecules are contained within the probe volume so that no diffusion losses occur. We have shown that single R6G molecules can be detected in 10 - 12 μm diameter droplets with a nominal signal-to-noise ratio of about 50 using cw laser excitation and a simple photon-counting measurement scheme. Cavity-QED effects associated with very small droplets (4 μm diameter) have been shown to influence the spontaneous emission rate and the integrated fluorescence yield indicating that much higher signal-to-noise ratios for single-molecule detection can be obtained by using even smaller droplets.

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