Quantitative characterization of fluorophores in multi-component nanoprobes by single-molecule fluorescence

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Abstract: Multi-modal nanoparticles incorporating fluorophores are increasingly being used for medical applications. The number of fluorophores incorporated into the nanoparticles during synthesis is stochastic, leaving some nanoparticles devoid of fluorophores. Determining the number, the brightness and the photostability of the fluorophores incorporated, and the percentage of labeled nanoparticles (labeling efficiency) remains challenging. We have determined the aforementioned quantities for two synthesized multi-modal nanoparticles by exploiting the photobleaching of fluorophores at the single-molecule level using a total internal reflection fluorescence microscope. Labeling efficiency was determined by fitting the distribution of incorporated fluorophores with a statistical model and verified by independent experiments.

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1. Introduction

Multi-modal nanoparticles have been the subject of intense research in recent years for potential biomedical applications and have been approved by the US Food and Drug Administration (FDA) for use in humans [1–4]. Fluorophores are often incorporated in multimodal nanoparticles to facilitate fluorescent imaging. The number of fluorophores incorporated into nanoparticles during synthesis is statistically distributed. The statistics of fluorophore incorporation and hence the distribution of the number of incorporated fluorophores are impossible to obtain from ensemble assays. It is also challenging to characterize the incorporated fluorophores due to the inaccuracies in determining their number, brightness and photostability from ensemble assays. Typically, fluorophore content is analyzed by quantum yield (QY) analysis, which compares the relative absorbance and integrated fluorescence of the test sample to a well characterized standard sample. This technique relies on the assumption that the solvents of the two samples and the samples themselves behave similarly, which is not the case when the nanoparticle has a core structure with its own absorbance spectrum, refractive index, and light scattering properties, and the fluorophore properties are altered by incorporation. Accurate determination of the number of fluorophores can therefore be problematic even though the molar equivalence of fluorophore reacted with particles is well-defined [5]. Moreover, near infrared (NIR) fluorophores are frequently chosen for in vivo imaging because of minimal background and superior tissue penetration [6]. Choosing an appropriate NIR standard fluorophore as a comparison reference, however, is difficult because most have very low QY values that vary greatly in the literature.

In this paper, we have overcome the limitations of ensemble assays to characterize fluorophores incorporated into multi-modal nanoparticles at the single-molecule level. We present a Single Particle Observation Technique (SPOT) using a total internal reflection fluorescence microscope to directly characterize the fluorophores incorporated in individual nanoparticles by exploiting photobleaching. Photobleaching of a single fluorophore is characterized by sudden decrease of fluorescence intensity. The number of photobleaching steps indicates the number of incorporated fluorophores. By measuring the discrete decreases
Fig. 1. Single Particle Observation Technique. (a) Dual-modal magnetic resonance/optical fluorescence silica-coated iron oxide nanoparticles (SCION) were dispersed in a sample chamber. (b) When incident light hits the boundary of two media at an angle greater than a critical angle, $\theta_c$, total internal reflection occurs resulting in an evanescent field that excites fluorophores in a thin layer near the surface (~200nm). (c) Schematic of the TIRF microscope. The excitation laser (green) is refracted by the quartz prism and totally internally reflected at the quartz slide-sample interface. The fluorescence emission is collected by a microscope objective, filtered to remove the excitation light and imaged by an EMCCD camera.

in fluorescence intensity as incorporated fluorophores bleach over time, SPOT permits a direct measure of the number of incorporated fluorophores and the fluorophore labeling efficiency for multi-component nanoparticles. We demonstrate the technique by measuring the distribution of incorporated fluorophores in dual-mode magnetic resonance/optical fluorescence silica-coated iron oxide nanoparticles. SPOT analysis indicated that clusters of fluorophores rather than individual fluorophores were incorporated in these particles. The technique provides a rapid, robust, and sensitive means of determining the fluorophore labeling distribution while also providing details of encapsulated fluorophore properties, including brightness and photostability. Compared to conventional ensemble measurement techniques with inherent population averaging, SPOT (Fig. 1) is a high-throughput approach to characterize hundreds of individual nanoparticles simultaneously. Previously, single-molecule strategies related to nanomaterials have included characterizing QDs [7–9] and detecting attached targeting agents [10,11] or determining the stoichiometry of biological complexes such as membrane-bound protein assemblies [12–14]. In SPOT, photobleaching of individual fluorophores leads to a quantized decrease in emission intensity. The number of fluorophores incorporated in a nanoparticle can be determined from the number of discrete photobleaching steps, the brightness from the magnitude of the steps, and the photobleaching time from the time to the bleaching step (Fig. 2a). Fluorescence detection of individual fluorophores requires low background and high sensitivity measurements. Background fluorescence is reduced by exciting with the evanescent field generated in total internal reflection fluorescence microscopy (TIRFM), which limits the excitation to a ~200 nm layer at the surface of the slide (Fig. 1b).

2. Materials and methods

2.1. Materials

Two ~18 nm dual-reporting (magnetic resonance and optical imaging) nanoparticles were synthesized by encasing ~10 nm ultrasmall superparamagnetic iron oxide nanoparticles in a layer of silica containing one of two conjugated fluorophores as described in Bumb et al. [5]
SCION(Cy5.5) particles incorporated Cy5.5 (GE Healthcare) and SCION(Alexa555) particles contained Alexa555 (Invitrogen).

2.2. Single-molecule measurements

Single molecule measurements were performed using a home-built prism-type TIRF microscope based on an Olympus IX71 [15]. A flow cell was made with double-sided tape (SA-S-1L 0.12 mm Grace BioLabs Secure-Seal adhesive double sided tape) sandwiched between a coverslip (Gold Seal 22 mm x 40 mm No.1) and a quartz slide (SPI 25 mm x 76 mm x 1 mm). Slides and coverslips were soaked in Piranha solution (70% sulfuric acid and 30% hydrogen peroxide) overnight, sonicated in 30% detergent (Alconox) solution for 1 h, and sonicated for three cycles of 30 min each in 1M NaOH followed by ethanol and finally in acetone, using fresh solution in each cycle. After each cycle, slides and coverslips were rinsed with DI water. Proper cleaning is essential for high signal-to-noise ratio in single particle fluorescence and phase contrast images. Free fluorophore or labeled or unlabeled SCION (100 pM) were immobilized in 5% agar inside the flow cell to facilitate long observation times and limit diffusion. A quartz prism (CVI PLBC-5.0-79.5-SS) was used to obtain TIR at the interface of the quartz slide and agar in the flow cell over an area of ~150 x 150 μm². Laser powers of 3-12 mW at 532 nm (CrystaLaser GCL-025-L-0.5%) for SCION(Alexa555) and 635 nm (CrystaLaser RCL-025-640-S) for SCION(Cy5.5) were used to excite the particles. Fluorescence was collected with an Olympus water immersion 1.2 NA 60X objective (UPLSAPO60XWIR). Additional magnification of 1.6X in the Olympus IX71 was used to obtain total magnification of 96X. Excitation light was rejected using HQ550LP filter for 532 nm excitation and HQ660LP filter for 635 nm excitation (Chroma). Filtered fluorescence was detected by an EMCCD camera (Andor DV897DCS-BV). Andor iQ 1.8 software was used to control the camera and record movies. Movies of thousands of particles were recorded with 100 ms time resolution. The fluorescence intensity of each particle as a function time was extracted from recorded movies using custom written IDL programs. Statistical analysis was performed using IGOR, Origin and Microsoft Excel.

2.3. Fluorescent lifetime measurements

Lifetime measurements were performed with a PTI Easylife LS fluorescence lifetime system using 525 nm excitation. Deconvolution fitting to obtain the lifetimes was performed with the accompanying software.

2.4. Confirmation of labeling efficiency

To confirm the predicted labeling efficiency based on statistical analysis of the distribution of incorporated fluorophores, we used an Olympus IX81 microscope outfitted with a spinning disk confocal head (Yukogawa head CSU-X1) with a 100× oil immersion objective lens (N.A. 1.40) and an EMCCD camera (QuantEM 512SC, 16 μm pixel size; Photometrics). A flow cell made with a thoroughly cleaned quartz coverslip and a glass slide was used. SCION(Cy5.5) particles in PBS buffer were flowed into the flow cell. For each field of view, images of SCION(Cy5.5) were captured first in confocal fluorescence mode (640 nm excitation, laser power 50 μW, 300 ms exposure), followed by capturing a phase contrast image of the same spot with a halogen lamp (1 s exposure). This order of imaging enabled the identification of the focal plane with the fluorescent particles while minimizing photobleaching. The optimum optical set up for the phase contrast channel was determined by trial and error. The particles were most clearly visualized when the polarizer and analyzer were removed from the light path with the DPO100 DIC prism in place. It should be noted that our images are phase contrast images, not differential interference contrast images. In this configuration, the field diaphragm was adjusted to a position at which the particles appeared as black dots on a lighter background. Contrast was increased by background subtraction with a background image for each field of view from a plane 10 μm below the focal plane. The phase contrast and
fluorescence images were converted into 8-bit. The number of spots with one-to-one correspondence between the two images was determined using the ImageJ “Colocalization Threshold” plugin and the “Nucleus Counter” plugin. The number of spots with one-to-one correspondence provides the number of particles labeled with fluorophores. The average size of these labeled particles was noted and the same “Nucleus Counter” plugin was used on the phase contrast image where the parameters were set to capture particles with the same average diameter as the labeled particles. The labeling efficiency was calculated from the ratio of particles found in the fluorescent (labeled particles only) and phase contrast (both labeled and unlabeled particles) images.

Fig. 2. Number of fluorophores incorporated in nanoparticles. (a) Fluorescence intensity as a function of time for a SCION(Alexa555) particle (red) and background (grey). Bleaching of individual fluorophores results in discrete decreases in intensity (black arrows). The number of bleaching events reveals the number of fluorophores in the nanoparticle. The bleaching times are indicated by dashed lines. (b) Distribution of the number of fluorophores for SCION(Cy5.5) (red circles). Fitting to a single fluorophore incorporation model (Eq. (1)), blue line, reduced $\chi^2 = 3.3$ returned an average of $1.58 \pm 0.04$ fluorophores per particle. Fitting to a fluorophore-cluster incorporation model (see text, red line, reduced $\chi^2 = 1.6$) returned an average of $0.78 \pm 0.02$ clusters per particle, with an average of $1.7 \pm 0.1$ fluorophores per cluster determined from the free fluorophore distribution (inset). (c) Distribution of the number of fluorophores in SCION(Alexa555) (red circles). Fitting to a single fluorophore incorporation model (Eq. (1)), blue line, reduced $\chi^2 = 7.5$ returned an average of $0.49 \pm 0.02$ fluorophores per particle. Fitting to a fluorophore-cluster incorporation model (see text, red line, reduced $\chi^2 = 3.4$) returned an average of $0.25 \pm 0.02$ clusters per particle, with an average of $1.15 \pm 0.02$ fluorophores per cluster determined from the free fluorophore distribution (inset). The uncertainties in the fit parameters were obtained by bootstrap analysis [24]. The free fluorophore cluster distributions were approximated by Poisson distributions (Eq. (1)), which were corrected to account for the fact that the clusters contain at least one fluorophore.
3. Results and discussion

To demonstrate SPOT analysis, we synthesized two ~18 nm dual-reporting (magnetic resonance and optical imaging) silica coated iron oxide nanoparticles (SCION) containing Cy5.5 (SCION(Cy5.5)) or Alexa555 (SCION(Alexa555)) fluorophores. Previously, the size, surface charge, structure, and magnetic properties of these nanoparticles were characterized [5,16].

However, quantum yield analysis of the SCION particles was not feasible due to the lack of suitable reference fluorophores and confounding optical effects such as scattering and absorbance of the iron oxide core. With a home-built SPOT instrument the number and properties of fluorophores encapsulated in individual SCIONS were determined and compared to free fluorophore. Fluorescence from particles was well above background and photobleaching was clearly visible as quantized steps (Fig. 2a). SCION(Cy5.5) had a mean of 2.07 fluorophores/particle (s.d. = 1.32, n = 1324) whereas SCION(Alexa555) contained 1.31 fluorophores/particle (s.d. = 0.69, n = 2957). Note, these means correspond to the number of fluorophores per labeled particle, not the average number of fluorophores per particle over the entire sample. To understand the distribution of the number of fluorophores per nanoparticle (Figs. 2b,c), we considered a simple model in which incorporation of fluorophores in the nanoparticle during synthesis is a random, non-cooperative process. Under these assumptions, the number of fluorophores per particle will follow a Poisson distribution [17]:

\[ P(n) = \frac{\lambda^n e^{-\lambda}}{n!} \]

where, \( P(n) \) is the probability of there being \( n \) fluorophores incorporated in the particle and \( \lambda \) is the average number of incorporated fluorophores. The distributions of incorporated fluorophores were reasonably well fit by Poisson distributions for both particles (Fig. 2b, c). However, control experiments that measured the bleaching of free fluorophores in solution revealed that the free fluorophores did not all bleach in a single step as expected. Rather, a distribution of bleaching steps was observed indicative of aggregation of free fluorophores in solution (Figs. 2b, c). Aggregation of fluorophores is a well-known phenomenon of significant practical importance [18,19]. The number distributions of free fluorophore aggregates were similar to those of the labeled particles suggesting that clusters of fluorophores, rather than individual fluorophores, were incorporated into the nanoparticles. In this scenario, the distribution of the number of incorporated fluorophores would be a Poisson distribution for the number of clusters incorporated and the number of fluorophores in each cluster would be given by the measured distribution for the free fluorophore. To account for the incorporation of clusters of fluorophores rather than individuals, we fit the nanoparticle bleaching step distributions with a Poisson distribution for the number of clusters (\( c \)) incorporated:

\[ P_{\text{particle}}(c) = \frac{\lambda_c^c e^{-\lambda_c}}{c!}, \quad c = 0, 1, 2, 3... \]

and a second Poisson distribution to describe the number of fluorophores in each incorporated cluster,

\[ P_{\text{cluster}}(d) = \frac{\lambda_d^d e^{-\lambda_d}}{d!}, \quad d = 1, 2, 3... \]

Since a closed form of this expression is not readily obtained, the fitting was done via simulations. First, the distribution of free fluorophore cluster sizes (\( P_{\text{cluster}} \)) was fit with a Poisson distribution, which was assumed to be the same for the clusters incorporated into the nanoparticles. The distribution of the number of fluorophores in the nanoparticles was then simulated by assuming a Poisson distribution of incorporated fluorophore clusters, the size of...
which was drawn from the measured cluster size distribution. This process was repeated over a range of average clusters per particle ($\lambda_{\text{cluster}}$) and the chi squared deviation between the simulated and experimental distributions was computed. The $\lambda_{\text{cluster}}$ value with minimal chi squared deviation was taken as the best fit for the average number of incorporated clusters. SCION(Cy5.5) and SCION(Alexa555) bleaching step distributions were well fit by this function, resulting in lower chi squared values than the single Poisson fits (Eq. (1), Figs. 2b,c). The average probabilities of fluorophore incorporation were somewhat lower for this fit than for the single Poisson fit.

The labeling efficiency is the probability that a particle will contain one or more clusters:

$$\text{Labeling Efficiency} = P_{\text{particle}}(c \geq 1) = 1 - P_{\text{particle}}(0) = 1 - e^{-\lambda_{\text{cluster}}} \quad (4)$$

For SCION(Alexa555) and SCION(Cy5.5), the labeling efficiency obtained from the double Poisson fits were 22 ± 2% and 54 ± 2%, respectively. To experimentally confirm the calculated labeling efficiency, we directly compared the number of particles observed in fluorescence and phase contrast images of SCION(Cy5.5) (Fig. 3). Both labeled and unlabeled particles were observed by phase contrast imaging, whereas only fluorescently labeled particles were observed by confocal fluorescence imaging. Using this method, 40 ± 5% of SCION(Cy5.5) particles ($n = 382$) were labeled with at least one fluorophore, which is in reasonable agreement with the labeling efficiency of 54 ± 2% determined from the double Poisson fit, and is far less than the 79 ± 2% labeling efficiency obtained from a single Poisson fit to the data (Fig. 2b). Thus, statistical analysis of SPOT data as presented above can be used as a tool to measure the fluorophore labeling efficiency.

Using SPOT, we observed that encapsulating fluorophores in SCION increased their brightness. The distribution of intensity steps of free fluorophore (Fig. 4a) versus encapsulated fluorophore (Fig. 4b) reveals that fluorophores in SCION silica shells were brighter than free fluorophores. The weighted mean photo-intensities for encapsulated Alexa555 were 3612, 2698, and 2199 respectively at excitation powers of 12, 6, and 3 mW. This corresponded to a

![Fig. 3. Experimental confirmation of particle labeling efficiency. (a) Spinning disk confocal fluorescence image gives the number of nanoparticles with fluorophores. (b) Phase contrast image of the same field of view gives the total number of nanoparticles with and without fluorophores. Every spot on the fluorescence image has a corresponding point in the phase contrast image (examples are encircled).](image-url)
94.3%, 14.7%, and 6.6% increase over free fluorophore at the respective powers. A similar enhancement of brightness was noted by Burns et al. [20] who used fluorescence correlation spectroscopy to study silica particles containing Cy5. To further understand the origin of this brightness increase we measured the fluorescence lifetimes (Fig. 4c). The number of excited fluorophores, $F$, decay by

$$F(t) = F_0 e^{-t/\tau}$$

$$\frac{1}{\tau} = \sum k_i$$

where the fluorescence lifetime, $\tau$, is the inverse of the sum of the rates of individual decay pathways, $k_i$. In the case of free Alexa555, a single fluorescence lifetime of 228 ps was measured, whereas encapsulated fluorophores decayed with a fluorescence lifetime of 228 ps, as well as through a second pathway with a lifetime of 1.5 ns (Fig. 4c). Decrease [21] and increase [22] in emission intensity from fluorophores incorporated in silica have been reported, but were associated with a decrease of the fluorescence lifetime. Therefore, the observation of increased brightness of incorporated fluorophores with the simultaneous

Fig. 4. Photophysical properties of free and encapsulated Alexa555. (a) Distribution of fluorophore intensity drops for free fluorophore and (b) for SCION particles from individual intensity traces (Fig. 2a). (c) Fluorescence decays and fits for free fluorophore (black) and SCION(Alexa 555) particles (red). The fluorescence lifetimes were determined from the deconvolution fits. Free fluorophores had a single lifetime of 228 ± 2 ps, whereas encapsulated fluorophores had two lifetimes of 228 ± 2 ps and 1.5 ± 0.1 ns. (d) Distribution of fluorophore bleaching lifetimes obtained from individual intensity traces (Fig. 2a) of SCION(Alexa 555) particles (red circles) and free fluorophores (black circles) at 6 mW of laser power. The distributions were fit with single-exponential decays. The lifetime of encapsulated fluorophores was 89 ± 4 s (red line, reduced $\chi^2 = 2.3$), whereas the lifetime of free Alexa555 was 23 ± 2 s (black line, reduced $\chi^2 = 0.85$). (e) Bleaching time as a function of laser power for SCION(Alexa 555) particles (red) and free fluorophores (black).
appearance of an additional 1.5 ns-long decay pathway is interesting. Further studies are required to verify the origin of the second decay pathway.

We further observed that encapsulating fluorophore improved photostability as evidenced by increased photobleaching times. The distributions of photobleaching times for Alexa555 at excitation powers of 3-12 mW were well fit by exponential curves (Fig. 4d), as expected for an uncorrelated Poisson process. The average photobleaching time of SCION(Alexa555) encapsulated fluorophores was greater than three-fold longer than the bleaching time of free Alexa fluorophore (Fig. 4e). It is possible that photo-oxidation is reduced by encasing fluorophore in silica as there is a decreased amount of free oxygen. When molecular oxygen quenches a fluorophore’s dark triplet excited state, highly reactive singlet oxygen is produced that can react with and bleach the fluorophore. Thus, reducing the oxygen concentration prolongs fluorophore photobleaching time [23]. Silica is porous; hence, a non-porous coating material may further enhance the photostability of the encapsulated fluorophore.

4. Conclusion

SPOT represents an important addition to the molecular imaging and nanomedicine tool kit, as a method to analyze the molecular optical properties of fluorophores embedded into multi-modal nanoparticles. The work presented demonstrates how the brightness and the photobleaching of individual fluorophores in a nanoparticle as a function of time were recorded and analyzed. We have determined the number, the brightness, and the photostability of the fluorophores. Moreover, we have presented a statistical analysis to fit the number distribution to determine the statistics of fluorophore incorporation and to determine the percentage of labeled nanoparticles. Estimated labeling efficiency was independently confirmed using a combination of phase contrast and confocal fluorescence imaging. We observed that fluorophores encapsulated in silica had both increased brightness and improved photostability, both of which enhance the imaging properties of the nanoparticles. Our method is accurate, high throughput, and utilizes exceedingly small quantities of sample. Accurate results can be achieved with less than a femtomole of particles.

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