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Alternating-Color Quantum Dot Nanocomposites for Particle Tracking

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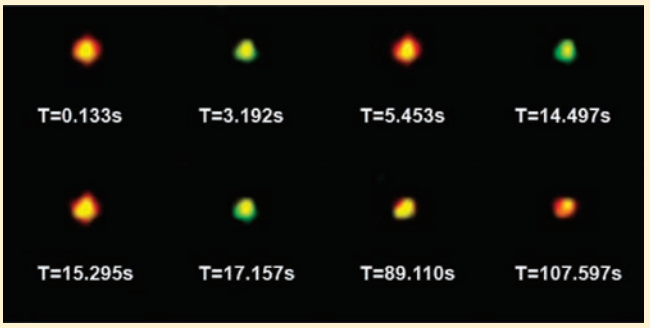
Supporting Information

ABSTRACT: Because of their extraordinary brightness and photostability, quantum dots (QDs) have tremendous potential for long-term, particle tracking in heterogeneous systems (e.g., living cells, microfluidic flow). However, one of their major limitations is blinking, an intermittent loss of fluorescence, characteristic of individual and small clusters of QDs, that interrupts particle tracking. Recently, several research groups have reported "nonblinking QDs". However, blinking is the primary method used to confirm nanoparticle aggregation status in situ, and single or small clusters of nanoparticles with continuous fluorescence emission are difficult to discern from large aggregates. Here, we describe a new class of quantum dot-based composite nanoparticles that solve these two seemingly irreconcilable problems by exhibiting near-continuous, alternating-color fluorescence, which permits aggregation status discrimination by observable color changes even during motion across the focal plane. These materials will greatly enhance particle tracking in cell biology, biophysics, and fluid mechanics.



KEYWORDS: Quantum dots, single molecule tracking, blinking, nanocomposite, molecular imaging, microfluidics

Particle tracking has made significant contributions to a wide variety of fields. For example, in fluid mechanics the local and instantaneous motion of fluids can be probed. In cell biology, this technique has been used to investigate membrane dynamics, viral infection, and gene transcription.[1−5] With their unprecedented fluorescent brightness and photostability, QDs have become a material of choice for particle tracking studies.[1,2] However, QDs are also subject to blinking, a random, intermittent loss of fluorescence intensity that disrupts particle tracking, especially in applications with rapid particle motion.[1,3]

It is generally established that QD blinking originates from enhanced nonradiative decay resulting from additional charges on the nanoparticle surface induced by surface defects.[6−12] Because QD blinking kinetics are stochastic,[1,7,8] large QD aggregates, which form as a result of colloidal instability or intracellular vesicle trapping,[13,14] do not exhibit blinking. Blinking kinetics of individual, distinct QDs in the aggregate are out of phase, yielding a continuous fluorescent signal. In contrast, small QD clusters (e.g., <5[15]) can exhibit discernible blinking responses because some individual blinking events occur simultaneously in the cluster.[16,15] Thus, blinking can be used to discern aggregation status of stationary particles. For dynamic particles, it is difficult to distinguish out-of-focus motion from blinking since both manifest as a loss of fluorescence signal.[17]

Despite this limitation, blinking is the primary in situ method used to identify QD aggregates in particle tracking.[1,17−19] Although several other techniques, such as electron microscopy and single photon counting (antibunching measurements), can also distinguish large QD aggregates from single QDs (and in fact can distinguish a single QD from a very small QD cluster), in experimental tracking studies, these methods are usually not feasible. For example, electron microscopy does not permit in situ investigation because it requires subatmospheric pressures, and single photon counting is usually performed on a fixed spot, precluding dynamic studies. Although some single photon counting devices are being developed to perform measurements at multiple spots simultaneously, the spatial resolution is very low (e.g., 20 μm diameter pixel), similar to or larger than the diameter of a biological cell or a microfluidic channel.[20] Thus, blinking has become an important determinant of particle aggregation status in particle tracking studies.

Here, we present a novel class of composite nanoparticles (CNPs) based on QDs, which address these seemingly irreconcilable issues by providing near-continuous fluorescence with an alternating fluorescent color that serves as a marker of single (or very small clusters of) CNPs. Each CNP is comprised of a few QDs with differing emission wavelengths (i.e., colors). Because blinking dynamics are stochastic, a single CNP remains nearly continuously fluorescent, while the emission wavelength alternates between those of the constituent QDs and their combinations (Figure 1). In contrast, large CNP aggregates display a nearly constant fluorescence emission color, permitting single

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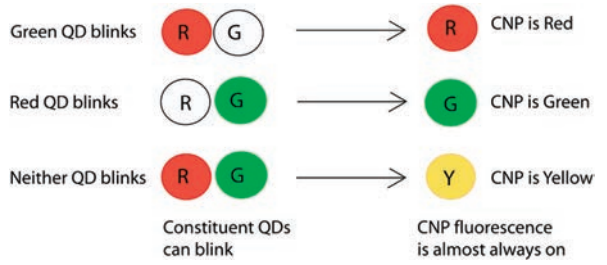

Figure 1. Schematic of alternating fluorescence emission wavelength and near-continuous fluorescence in a CNP. A CNP contains a small number of QDs with different fluorescent emission wavelengths (here red (R) and green (G) QDs). As a result of near-continuous fluorescence, CNPs can be continuously tracked, whereas their alternating color serves as an indicator of low aggregation (i.e., single or very small cluster) status.

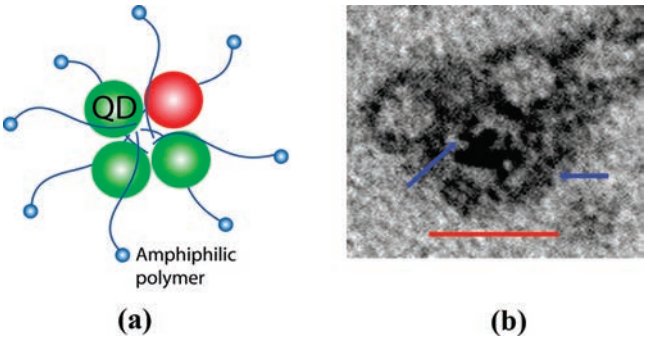

Figure 2. CNPs formed via micelle encapsulation. (a) Schematic; (b) TEM image with negative staining for a typical CNP preparation as described in the Supporting Information. QDs and the polymer micelle are indicated by left and right arrows, respectively. Scale bar (red): 25 nm.

(or very small clusters of) CNPs to be distinguished from large aggregates by their alternating-color emission. Thus, CNPs can be continuously tracked and identified as single (or very small clusters of) CNPs.

In the present work, CNPs were formed by coencapsulation of QDs with differing emission wavelengths (Invitrogen, $\lambda_{emission}$ = 545 and 605 nm for green and red QDs, respectively) in polymeric poly(styrene-b-ethylene oxide) (MW 3800-b-6500) micelles, a vehicle that we previously used for coencapsulation of QDs and superparamagnetic iron oxide nanoparticles (SPIONs).[21] Co-encapsulation was achieved using the interfacial instability process[22] in which oil-in-water emulsion droplets were transformed into micelles (~25 nm in diameter) upon evaporation of the oil phase (e.g., chloroform). Because the oil phase contained QDs with differing emission wavelengths and amphiphilic polymers, the resultant micelles consisted of polymer shells with QDs isolated in the micelle core (Figure 2a). Exact numbers of red and green QD in each micelle are difficult to determine from TEM as QDs contained in the 3D micelle overlap when projected onto a 2D image (Figure 2b, Supporting Information Supplementary Figure 1).

In the absence of interactions between encapsulated QDs and amphiphiles, the number and ratio of green to red QDs in each micelle could be controlled by the molecular structure of the polymer amphiphile employed and the quantities of polymer and QDs used. On the basis of thermodynamic considerations, the number of hydrophobic QDs in the hydrophobic micelle core should be limited by the size of QDs employed and the empty micelle core size. For example, a larger micelle core should encapsulate more QDs of the same size. Additionally, micelle size should be affected by the constituent amphiphilic molecule employed: the longer the hydrophobic/hydrophilic segment in the structure of the molecule, the larger/thicker the core/shell of the micelle. This thermodynamic analysis is supported by the work of Dubertret et al. who showed that when lipid-polyethylene glycol micelles were used to encapsulate QDs (one color only), the number of QDs encapsulated depended on the QD size employed. Larger QDs (4 nm) led to one QD per micelle, whereas smaller QDs (<3 nm) led to multiple QDs per micelle.[23] However, it should be noted that if thermodynamic equilibrium is not reached, the number of QDs encapsulated may be diminished; in this case, increasing the initial number of QDs should enhance the number encapsulated.

Overall CNP size can be controlled by choice of constituent amphiphiles. For example, poly(styrene-b-ethylene glycol) with molecular weights of 3800-b-6500 and 9500-b-18 000 Da leads to CNPs with diameters of 25 and 40 nm, respectively.[21] Smaller CNPs could be created using different amphiphiles. DSPE (distearoyl phosphatidylethanolamine)-co-polyethylene glycol (PEG) 2,000 forms ~15 nm diameter micelles with a core diameter of ~6.5 nm. As many as 10 blue QDs (diameter 2 nm) and 5 green QDs (diameter 3 nm) could theoretically be packed into each DSPE micelle.[24] Optimization of CNP size is critical; larger CNPs will experience greater steric hindrance and reduced transport, whereas smaller CNPs, which may require smaller QDs, have greater potential for Förster Resonance Energy Transfer (FRET).

CNP suitability for particle tracking applications was assessed using a fluorescent microscope ($\lambda_{excitation}$ = 488 nm), CCD camera, and long-pass filter to permit simultaneous green and red channel observation. As can clearly be seen in Figure 3a and Supporting Information Supplementary Movie 1, CNPs exhibit multiple, alternating fluorescence emission colors, including those of the constituent green and red QDs and their combination. The ratio of fluorescence in the CNP red channel to that of the green channel (R/G ratio) changed throughout the observation period (0.564−3.662 AU, or 550% difference, Figure 3b), leading to a continuous change in fluorescent color (Figure 3a and Supporting Information Supplementary Movie 1). The change in the R/G ratio was abrupt; indicating an abrupt color change between red (high R/G ratio), yellow/orange (medium R/G ratio), and green (low R/G ratio) (Figure 3a,b, Supporting Information Supplementary Movie 1). Additionally, the fluorescent colors of smaller regions within the CNP also changed continuously and abruptly (Figure 3a and Supporting Information Supplementary Movie 1). For example, in frame 41 (5.453 s), the CNP appears as a large orange core surrounded by a thin red shell (R/G ratio = 1.846), whereas in frame 110 (14.497 s) the CNP appears green (R/G ratio = 0.580). The nonuniform color distribution in the CNP indicates a heterogeneous distribution of differently colored QDs in this nanocomposite. In stark contrast, a large aggregate of CNPs (obtained from the visible precipitate of an unfiltered CNP solution after 1 week of storage) exhibited near-constant fluorescent color and R/G ratio (2.931−3.004 AU, or 2.4% difference, Figure 3a,b, Supporting Information Supplementary Movie 2). Therefore, the alternating-color feature of CNP can serve as a marker of single (or small cluster) status.

To evaluate the dynamics of fluorescence intensity, overall CNP fluorescence intensity and that of individual red and green channels were compared with the intensity of separately imaged

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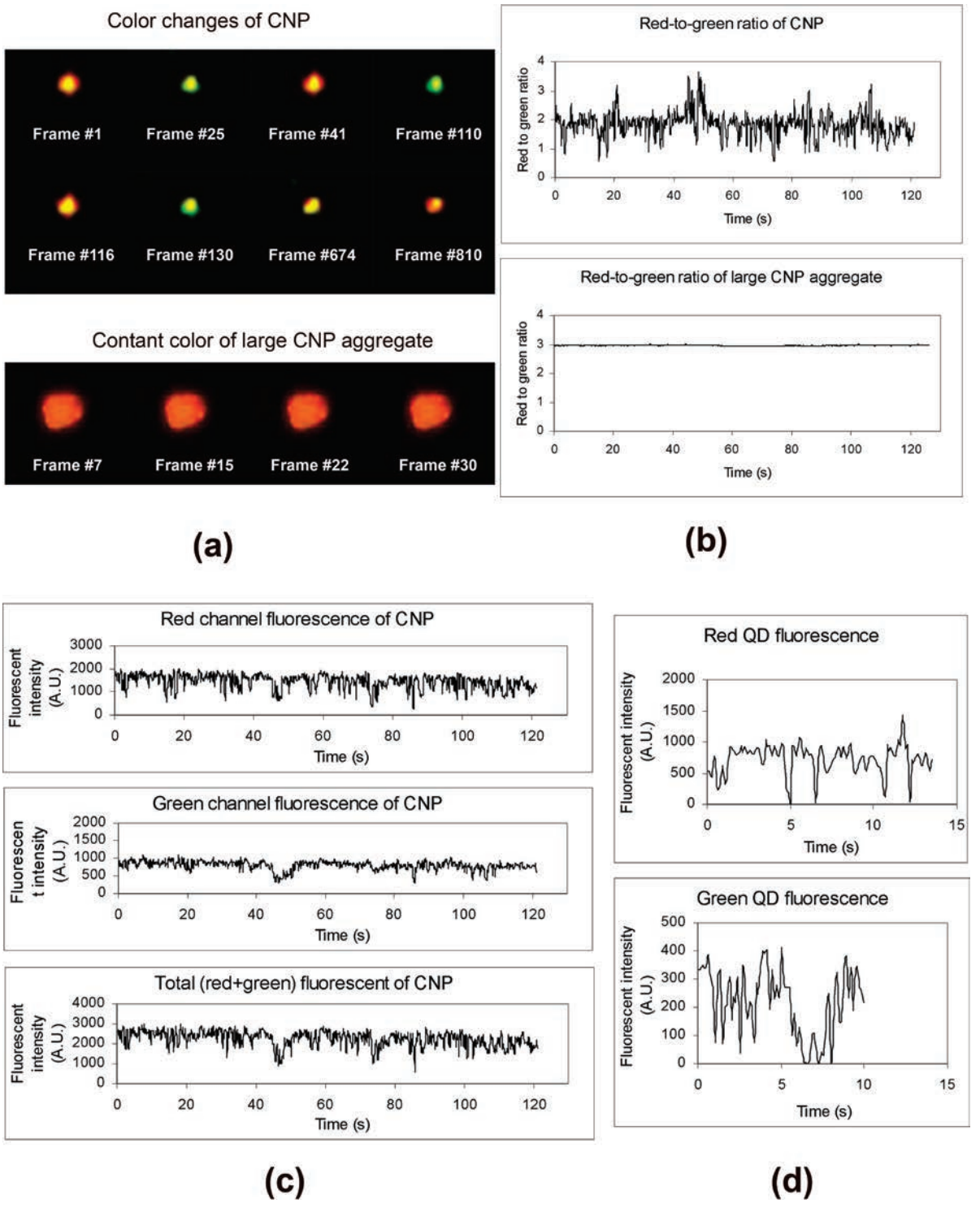

Figure 3. CNP dynamics of fluorescence (a) emission color, (b) red-to-green ratio, and (c) intensities. (d) Fluorescence intensity of a single QD. CNP fluorescence measurements were collected from the CNP on the right in Supplementary Movie 1. In (d), single QD (red or green) imaging was performed after deposition from chloroform and subsequent solvent evaporation.

single green and red QDs (Figure 3c,d). Over an observation period of 2 min, the total CNP fluorescence intensity remains high (ranging from 588.007 to 2995.998 AU), although at several time points the fluorescent intensities of individual CNP color channels were diminished as a result of constituent QD blinking (Figure 3c). Compared to individual QDs, for which fluorescence was nearly extinguished at several time points (Figure 3d, green QD, 0−408.000 AU; red QD, 13.988−1429.012 AU), CNP fluorescence was virtually continuous. Additionally, CNPs are much brighter than constituent QDs, which will significantly improve signal-to-noise ratio in tracking studies.

These measurements can also be used to estimate the number of constituent QDs in a CNP, which is important for potential multiplexing applications. If numbers of individual constituent

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QDs could be determined in situ, it would be possible to construct CNPs with known red to green particle ratios, which could then be used to track different species. From comparison of fluorescence intensity in CNP channels (Figure 3c) to that of single QDs (Figure 3d) (integrated over 10 s to compensate for blinking), we estimate that the CNP shown in Figure 3 contains four (i.e., 4.09) green QDs and two (i.e., 2.24) red QDs. We recognized, however, that given the spectral overlap and close proximity between QDs within a CNP, Förster resonance energy transfer (FRET) could occur. Thus, we calculated the FRET efficiency between green and red QDs used in this work and found that FRET efficiency is low (13.1% for 0 nm and 5.2% for 1 nm separation, respectively, Supporting Information).[25,26] Fundamentally, the low FRET efficiency observed in QD−QD pairs relative to molecular FRET donor/acceptors results from the large size of QDs. These calculations indicate that FRET does not significantly interfere with the fluorescent properties of CNPs reported here. Experimentally, CNP fluorescence imaging (Figure 3 and Supporting Information Supplementary Movie 1) clearly shows bright, continuous, and alternating-color fluorescence, confirming that FRET (if any) did not diminish the features of CNPs. Nevertheless, minimizing FRET should be an important consideration in the choice of QDs and micelle-forming amphiphiles in CNP designs.

In addition to permitting near continuous tracking and confirmation of aggregation status, we have discovered that alternating-color fluorescence emission can be used to solve another long-standing problem in QD-based particle tracking: discrimination of out-of-focus large aggregates from single (or small clusters of) nanoparticles.[17] In highly dynamic systems using conventional QDs, rapid, 3D motion out of the focal plane cannot be distinguished from blinking because both lead to disappearance of the fluorescence signal (Figure 4a). In contrast, CNPs permit facile and unambiguous confirmation of aggregation status because (1) CNPs produce alternating-color fluorescence emission and (2) the constituent QDs in a CNP move as an ensemble. Thus, a CNP aggregate completely moving out of focus manifests as a complete loss of fluorescence, which is clearly distinguishable from the alternating-color signal of a single (or small cluster of) CNP (Figure 4b).

This is a major advantage of CNP technology compared with traditional, single-color QD particle tracers. A small cluster of single-colored QDs can offer near-continuous fluorescence, and the fluctuation of cluster fluorescence intensity could be used to indicate single cluster status when the cluster is stationary. However, when the cluster moves in 3D, changes in fluorescence intensity could result from moving out-of-focus or blinking. Similarly, single photon counting cannot be used to confirm aggregation status of QDs moving across the focal plane.

As proof-of-concept of tracking ability, drop-cast CNPs were moved by manual control of the microscope stage. A typical CNP was tracked continuously for 2 min, much longer than the reported duration between blinking interruptions for any single QD-trajectory reported in the literature (Figure 5a and Supporting Information Supplementary Movie 3).[1,17,18] The CNP moved throughout the field of view while exhibiting continuous and abrupt color-changes, indicating single (or small cluster) status. CNP alternating-color fluorescence is easily distinguishable from potential fluorescent intensity and color changes that may result from particle growth (e.g., by Ostwald ripening) and aggregation. It has been previously reported that the fluorescence intensity of some large QD aggregates (e.g., QDs with poorly

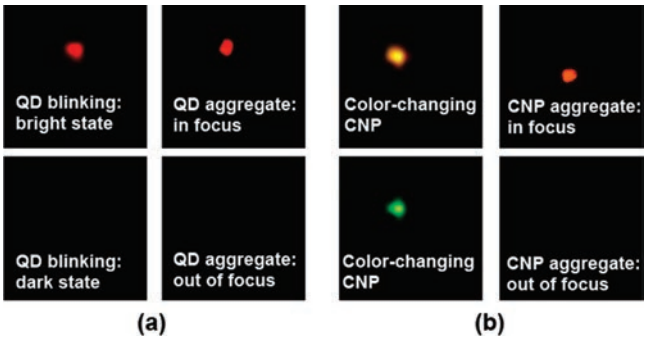

Figure 4. (a) QD blinking is not distinguishable from QD aggregates drifting outside the focal plane, whereas (b) CNP color changes are clearly distinguished from CNP aggregates exiting the focal plane.

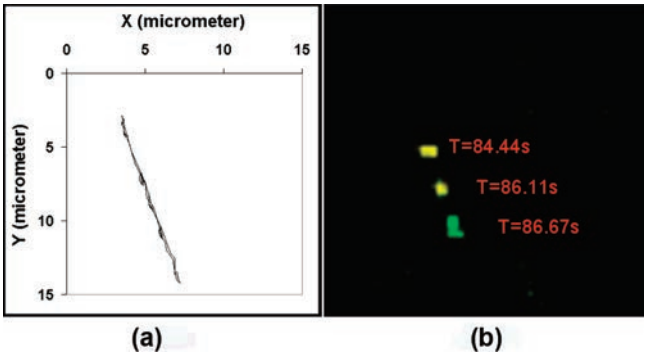

Figure 5. Dynamic CNP Tracking. (a) CNP trajectory; (b) superimposed image of the CNP at several time frames of a jump (fast and large location change).

protected surfaces) experiences a significant, but gradual, decay before reaching steady state under certain experimental conditions.[27] However, large CNP aggregates, which are composed of commercially available QDs with well-protected surfaces, emit constant fluorescence in all color channels under all experimental conditions tested (Supporting Information Supplementary Movie 2). Additionally, even if particles with poorly protected surfaces were used, because the initial decay of fluorescence would be gradual any possible alteration of fluorescence emission wavelength would also be gradual and could thus be readily distinguished from the abrupt color changes exhibited by single (or small cluster of) CNPs.

We note that there were several fast and large location changes ("jumps") in the trajectory (e.g., from 84.44 to 87.22 s). In particular, a color-changing event coincided with the jump event between 84.44 and 86.67 s (Figure 5b and Supporting Information Supplementary Movie 3). The color-changing event indicates that during this time at least one constituent QD in the CNP was blinking, which highlights the benefit of using a CNP rather than a QD for tracking. If this constituent blinking QD alone were used as the tracer particle, the trajectory after the "jump" would be lost due to the coincidence of the blinking and the jump. Alternatively, "nonblinking QDs" in which blinking is reduced or eliminated by mediators/compensators on the QD surface,[10,9] coating QDs with a thick shell,[11,6] or synthesizing QDs with a gradually changing potential energy function[12] could be used. However, these would not permit aggregation status (or lack thereof) to be confirmed, since blinking would be absent, electron microscopy and single photon counting could not be

applied in situ, and the fluorescent particle spot size can vary with camera exposure time and is subject to the diffraction limit (i.e., not the actual size of the particle).

In addition to the optical properties, CNPs have several features that make them an ideal candidate for particle tracking studies. Particle synthesis is straightforward; even without separation or optimization, ~20% of the as-synthesized CNPs show near-continuous fluorescence, alternating-color properties (with the remainder providing typical single color fluorescence). Yield could be further enhanced by fluorescence sorting (e.g., FACS); however, particles can also be used as synthesized with investigators selectively tracking those fluorescent particles with the alternating-color feature.[18] Additionally, CNPs are relatively small and are therefore not expected to interfere with most processes being tracked. Further, bioconjugation of CNPs should be facile, and can be accomplished by well-documented procedures (using amphiphilic polymers with $-COOH$ or $-NH_2$ end groups).[28] CNPs are also stable in the biological environments commonly used for particle tracking studies. After 12 h in cell culture medium (Dulbecco's modified Eagle's medium, containing 10% serum, 37 °C), CNPs were free of significant aggregation and their near-continuous-fluorescence and alternating-color properties were preserved (Supporting Information Supplementary Movie 4). Cell culture medium, blood, or cytoplasm can all potentially interact with the QD surface through oxidation/reduction reactions or molecular absorption to alter QD properties. The high tolerance for biological environments displayed by CNPs should at least partially result from protection of the QD surface by the micelle.[23]

In conclusion, we have developed a novel class of QD-based composite nanoparticles, whose near continuous, alternating-color fluorescence permits tracking of confirmed single (or small clusters of) nanoparticles and permits confirmation of aggregation status even when particles are in motion. These properties will significantly enhance dynamic particle tracking in fluids (e.g., biological environments or microfluidic flows). However, CNPs may also find applications beyond those explored here. For example, CNP technology is compatible with our recently reported method for fluorescent-magnetic nanoparticle formation.[21] Thus, CNPs could be applied in magnetic manipulation and multimodal imaging. If the exact numbers of individual constituent QDs could be determined, CNPs might also be used to create multiplexed particles that could potentially track multiple biomolecules or nanomaterials simultaneously.[29] CNPs might also serve as a platform for investigating energy transfer and electronic coupling of QDs in a controlled microenvironment. Previous researchers have noted conflicting effects of particle proximity, reporting blinking enhancement[30] as well as blinking reduction when several QDs are clustered.[15] However these studies were performed by simple drying of QD solution with little control of the clustering process. Since the structure of micelles can be easily controlled,[21,24] CNPs could be used for highly controlled QD aggregation studies, and further work to systematically study the relationship between preparation conditions, size, and intensity ratio of different color channels is planned. Thus, CNPs could serve as a platform technology leading to fundamental discoveries not only in cell biology and biophysics, but also in fluid mechanics, particularly in microfluidic settings.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Details of methods used and captions for supporting videos and images. This material is available free of charge via the Internet at http://pubs.acs.org.

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