Twisted perylene dyes enable highly fluorescent and photostable nanoparticles†

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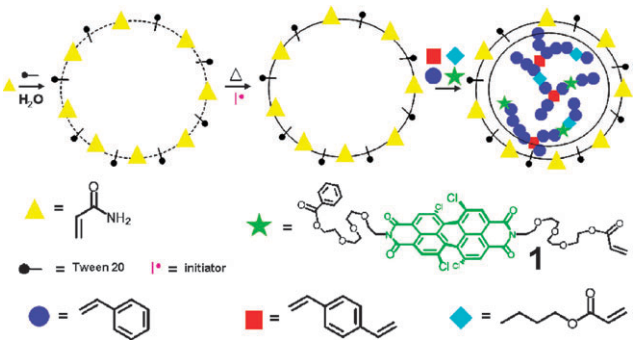
Polymeric fluorescent nanoparticles with covalently embedded perylene fluorophores were developed by facile synthesis strategy and their advanced features of extremely high fluorescence intensity, non-photoblinking and excellent photostability were experimentally confirmed at the single nanoparticle level.

Fluorescent nanoparticles (FNPs) have attracted considerable interest for their wide range of emerging applications in live cell imaging, biosensing, and optoelectronic devices.[1] Practical FNPs, especially for biological applications, require high fluorescent intensity, photostability, biocompatibility and relatively small diameter (<50 nm). Among currently available FNPs, inorganic semiconductor quantum dots possess brightness up to 20 times brighter than a single molecule, desired small diameter and improved photostability but suffer from photoblinking and cyto-toxicity.[2–4] Dye-doped polymer nanoparticles are limited by fluorophore aggregation and self-quenching as well as dye leaching.[5] Additionally, fluorescent intensity rarely scales linearly with the number of dyes imbedded in FNPs due to obstacles such as singlet-singlet annihilation, self-quenching and Stern–Volmer quenching. Thus, despite reported enhanced brightness calculated from ensemble measurements,[6] experimental data for FNPs with diameter <50 nm and fluorescence >20 times brighter than a single dye are not reported.[7,8] A relatively unexplored alternative is the use of FNPs with covalently bound fluorophores. Covalent attachment limits chromophore mobility and disperses the monomers within FNPs which potentially reduces fluorophore aggregation and therefore fluorescence self-quenching, providing a distinct advantage over doping. In this communication, we have developed photostable and biocompatible polymeric nanoparticles with covalently bound perylene dye possessing over 50 times brighter fluorescence as compared to single-dye molecules such as rhodamine 6G.

Because the tetrachloro-perylene diimide (PDI) derivative has four substituted chlorine atoms in the bay positions to twist the molecule out of plane (37°), it maintains high fluorescent quantum yield ($\Phi_{fl} \sim 0.9$) and high photostability.[9] The highly twisted structure frustrates π–π stacking, reducing self- or collisional quenching within the tight confines of a FNP, thus a strategy to fabricate bright FNP. The twisted PDI was monobenzoylated and functionalized with an acryloyl moiety to form



Scheme 1 Polymeric nanoparticle synthesis with twisted PDI monomer.

the monomer **1** which is shown in Scheme 1. Our approach to constructing polymer FNPs here is based on a modified emulsion polymerization method[10] with the twisted PDIs covalently embedded within the hydrophobic cavities of polymeric nanoparticles. Acrylamide and styrene were polymerized with minor amounts of functional monomers, including optically active perylene dye, the cross-linker divinyl benzene, and butyl acrylate for lowering the glass transition temperature of the nanoparticles. Several batches were identically prepared except for varying feed dye concentration, ranging from 0.3 to 2.4 wt%. These nanoparticles are easily suspended in water due to their hydrophilic shell, enabling feasible application in live cell experiments. Because the nanoparticle fluorescence originates from multiple dispersed dyes, one nanoparticle shines >50 times brighter than a single dye. Furthermore, no perylene dye loss was found for the aqueous nanoparticle samples after washing with organic solvent because the PDI components are covalently anchored inside the nanoparticle. This is an advantage over dye-doped nanoparticles, in which dye molecules may aggregate or leach into the environment such as a live cell.

The absorption and emission spectra for an aqueous nanoparticle sample exhibit typical spectral characteristics compared to the monomer (Fig. 1(a)) indicating minimal perylene aggregation, thus contributing to their bright fluorescence. Additionally, the FNP solutions have $\Phi_{fl} \approx 0.5$, further evidence of minimal stacking interactions. Both the absorbance and fluorescence λ_{max} are slightly red-shifted from the monomer due to differences in local environments (styrene *vs.* CH_2Cl_2). The nanoparticle emission vibronic bands are also slightly broadened due to non-homogenous broadening. FNP size was measured by both TEM and dynamic light scattering (DLS).[8] TEM, the standard measurement method, indicated an average diameter of 40 nm (Fig. 1(b)).

While various methods and calculations have been used to estimate single particle brightness from ensemble measurements,

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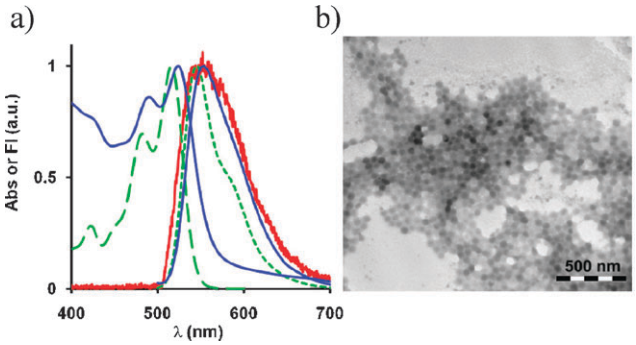

Fig. 1 (a) Normalized ensemble absorbance and fluorescence spectra of an aqueous nanoparticle colloid (solid blue) compared to the monomer in CH_2Cl_2 (green dash) and the fluorescence from a single nanoparticle (red). (b) TEM image exhibiting average particle size = 40 nm. The aggregation on TEM grids is an artifact caused by drying, as dynamic light scattering reveals no aggregation in solution.

the most accurate method to truly determine single particle brightness is by single particle experiments.[8] Thus, using a single molecule set-up as described in the ESI,† we performed wide-field and diffraction limited measurements of single particle brightness.

Fig. 2 reveals the brightness, determined by CCD wide-field imaging, of single twisted PDI monomers and FNPs prepared with varying feed dye amounts. Fig. 2(a) and (b) are shown at the same color scale with unambiguous increase in brightness of the nanoparticles over the monomer. Fig. 2(c)–(e) are at a much higher color scale and show brightness increases with increasing PDI feed concentration. Notably, many of the nanoparticles in Fig. 2(e) fluoresce >80 times brighter than a single fluorophore. Histograms generated from statistical brightness occurrence averaged over several slide samples demonstrate an unambiguous increase in brightness distribution between single nanoparticles and single PDIs. Also, with increasing PDI concentration, the FNP brightness scales approximately linearly, indicating dye incorporation is proportional to feed ratio over this concentration range (0.3–2.4 w/w%).

Why are these nanoparticles so bright compared to previous examples of dye-doped nanoparticles? First, covalent attachment allows a large number of dyes to incorporate without changing the nanoparticle structure. Second, even in the case of the highest PDI feed concentration, minimal spectral features of dye aggregation were observed. Thus, nearly all of the dyes retain their monomeric status following polymerization. Apparently, the fluorescence self-quenching is largely suppressed, likely due to frustration of π–π stacking by the twisted PDI structure and the spacing effect of other co-monomers. Third, the core–shell structure effectively encapsulates most fluorophores and reduces photobleaching by molecular oxygen.

To further probe the detailed photodynamic properties of the FNPs, fluorescence time traces of single nanoparticle from each batch were probed by diffraction-limited excitation and the fluorescence collected by avalanche photo diode (APD) as shown in Fig. 3. For comparison, a typical time trace of the monomer dye is also shown (insert). As typical for a single molecule, the monomer displays a sharp on-and-off process (photo-blinking). In sharp contrast, the fluorescence time trace for individual nanoparticles exhibit distinctively different fluorescence with a gradual decay due to multiple dye molecules within each nanoparticle. Immediately upon opening the shutter, all nanoparticles show highly bright fluorescence (the

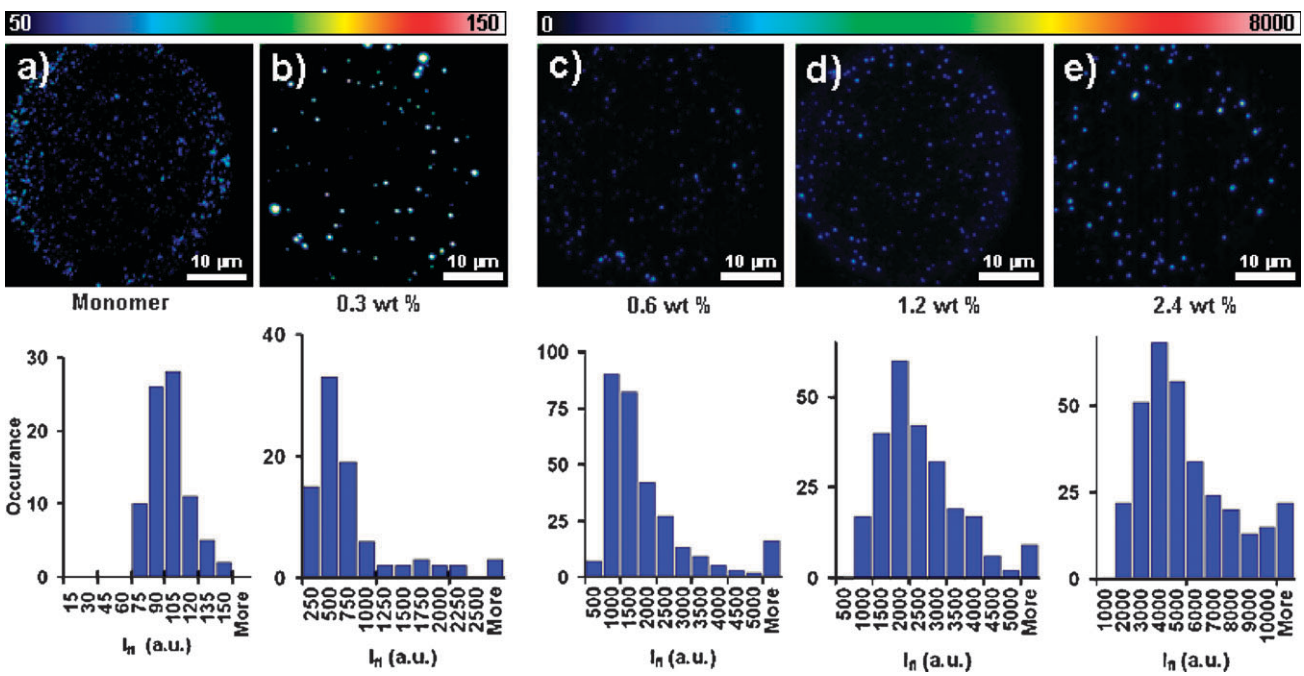

Fig. 2 (Top) Single molecule/particle wide-field CCD images (2 s integration time) for (a) monomer, (b) 0.3, (c) 0.6, (d) 1.2 and (e) 2.4 w/w% samples; (a) and (b) are at the same color scale while (c)–(e) are at a much higher scale. In sample (e), several of the particles approach 80 times brighter (I > 8000) than a single monomer ($I \approx 100$). (Bottom) Histograms summarize the statistical brightness of each batch. While a single monomer exhibits brightness of around 100, the nanoparticle samples (b)–(e) exhibit mean intensities 7, 19, 25 and 56 times brighter, respectively.

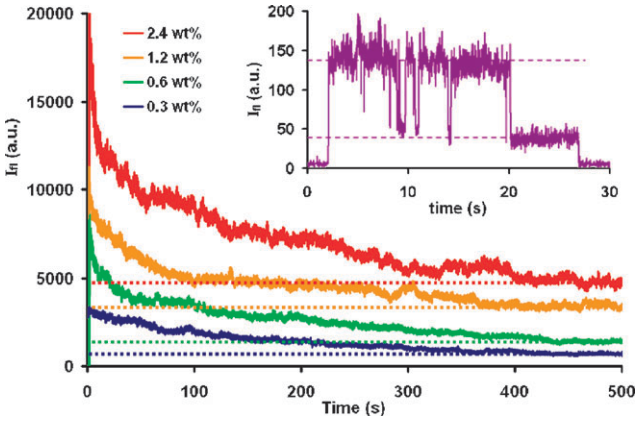

Fig. 3 Representative single particle time-traces from each batch. After photo-hardening, the nanoparticles remain quite bright, with the 2.4% sample still 50 times brighter than a single monomer after 8 min of continuous excitation. Inset: Single PDI time-trace under identical excitation power exhibits significantly lower intensity, photo-blinking, and eventual photo-bleaching.

2.4% nanoparticle is initially over 220 times brighter than a single monomer) which initially decays quickly due to photo-bleaching. Interestingly, this initial steep decay weeds out the "non-photostable" fluorophores and eventually only the most photostable fluorophores remain. Even in this photo-hardened state, the nanoparticles remain quite bright, with the 2.4% sample still emitting more than 50 times brighter fluorescence than a single monomer after 8 min of continuous laser irradiation. To the best of our knowledge, this is the brightest nanoparticle (diameter <50 nm) determined experimentally by single-particle methods.

In conclusion, we have successfully prepared polymeric nanoparticles with high fluorescent brightness and excellent photostability by covalently embedding a twisted perylene dye into core–shell type polymeric nanoparticles. The high photostability, quantum yield, and unique steric structure of the perylene monomer, coupled with high monomer concentration and core-shell encapsulation enabled by covalent attachment, are believed to mainly contribute to the remarkable brightness and improved photostability. These facile, ultrabright probes ultimately advance the capability for practical applications in live cell imaging, biosensing and in the development of optoelectronic nanodevices.

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8 See ESI† for additional information.

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