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Ultrabright Nanoparticles

Prevention of Self-Quenching in Fluorescent Silica Nanoparticles by Efficient Energy Transfer**

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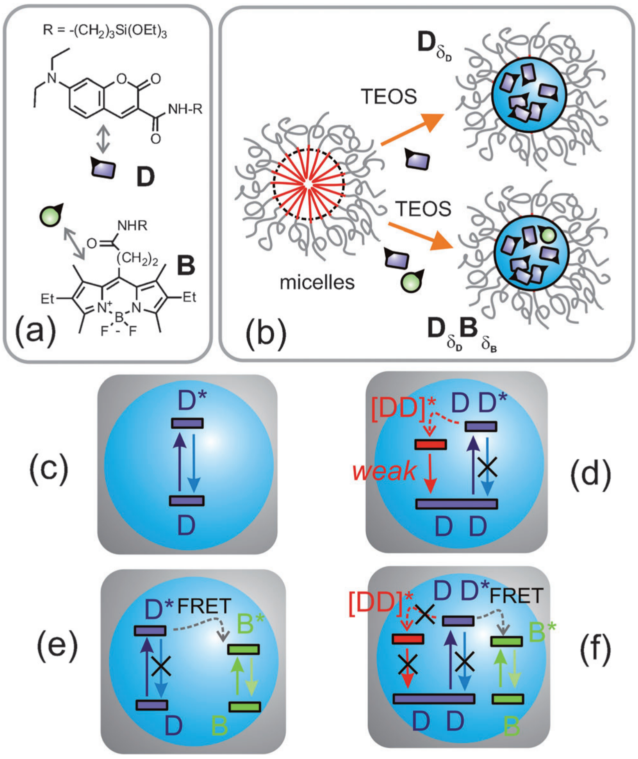
Fluorescence measurements are very sensitive, inexpensive, straightforward, and versatile. They offer submicron visualization and sub-millisecond temporal resolution.[1] For most applications, however, the sensitivity is determined by the ability of the species used as fluorescent labels or chemosensors to absorb and emit light under given excitation conditions, that is, by their brightness (defined as the product $\varepsilon\Phi$, in which ε is the molar absorption coefficient and Φ the fluorescence quantum yield).[2] In principle, therefore, the replacement of traditional molecular emitters with multifluorophore systems can strongly increase sensitivity, since the term ε of the brightness is, in the case of weak ground-state interactions, simply additive. Dye-doped silica (DDS) nanoparticles (NPs) are a particularly relevant example of potentially very bright multifluorophore systems, since the segregation of the dyes in the nanometric inert matrix typically increases also their fluorescence efficiency, Φ.[3] Nevertheless, as in the case of multilabeled biomolecules and other supramolecular multifluorophore nanostructures, bimolecular processes involving the fluorescent excited states also lead to the formation of poorly emitting species in DDS NPs. This phenomenon, known as self-quenching, dramatically jeopardizes the performance of systems with a high local dye concentration by causing a strong decrease in the fluorescence quantum yield and a broadening of the emission spectrum.[4]

Some researchers have proposed the control of the distribution of the dye molecules in the nanostructure as a strategy to avoid self-quenching.[5] This approach is quite efficient but not general, since it is applicable only to some mesoporous materials. Herein we show how Förster resonance energy transfer (FRET)[6] can be exploited to avoid intermolecular quenching and spectral broadening, and to simultaneously tune the spectral features of DDS NPs. In particular, we have investigated this approach for a family of core–shell DDS NPs that are very appealing from the point of view of applicability, since they are suitable for in vivo and in vitro imaging and as intracellular fluorescent nanosensors[7]. These NPs have a silica core with a diameter of approximately 10 nm surrounded by a poly(ethylene glycol) (PEG) shell that is about 7 nm thick.[6a,8] They are synthesized by the hydrolysis/condensation of tetraethoxysilane (TEOS) with a micellar dispersion of Pluronic F127 as a template, and they are very monodisperse and stable in water over a large pH range, including physiological pH values. Fluorescent NPs of this kind were obtained by copolymerizing the 7-*N*,*N*-diethylaminocoumarin derivative **D** at different molar ratios ($\delta_D = 0.1$, 0.2, 0.4, 0.6, 0.8 %) with respect to TEOS (Scheme 1 a,b). The resulting samples are referred to as $\mathbf{D_{0.1}}$, $\mathbf{D_{0.2}}$, $\mathbf{D_{0.4}}$, $\mathbf{D_{0.6}}$, and $\mathbf{D_{0.8}}$, respectively, to stress the different degrees of loading. After purification of the NPs, either by ultrafiltration or dialysis, no trace of the doping dye was ever detected in the waste solution. This observation confirms the quantitative inclusion and covalent bonding of the coumarin in the NPs in all cases.



Scheme 1. a) Chemical structures of the fluorescent triethoxysilanes **D** and **B**. b) Simplified representation of the synthesis of the NPs. c–f) Simplified diagrams of the electronic states of the dyes in the NPs doped only with **D** at a low (c) and high (d) loading degree (δ_D) and the corresponding NPs resulting from the co-inclusion of **B** (e,f).

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The absorption spectra of 1×10^{-7} M dispersions of the purified NPs in water are shown in Figure 1. They present the typical coumarin absorption band in the blue region and a molar absorption coefficient proportional to the doping percentage, δ_D (Table 1). The observation of only a small dependency of the maximum absorption wavelength (λ_{max}) on δ_D indicates the absence of strong interactions between the **D** molecules in the ground state in the whole investigated doping range.

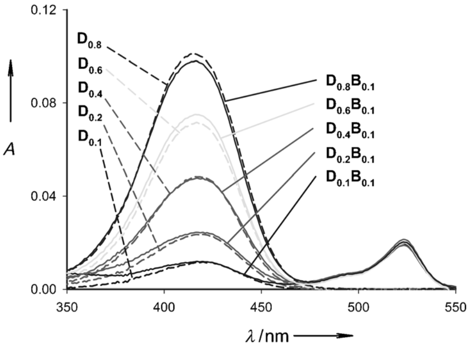

Figure 1. Absorption spectra of the NPs in water ($c = 1\times10^{-7}$ M). Dashed lines: samples containing only **D**; continuous lines: samples containing both **D** and **B**.

The fluorescence properties of the NPs, on the contrary, were strongly dependent on the δ_D value: a gradual red shift of the emission maximum together with the rise of a new band at around 550 nm were observed as the number of coumarin moieties per NP was increased. Most relevant is that, as reported for analogous systems, the fluorescence quantum yield is much lower for the more heavily loaded NPs and the emission profile much broader (Figure 2). Thus, whereas in the case of poorly doped NPs the deactivation of the **D** excited state occurs mostly radiatively (Scheme 1 c), in the heavily doped NPs the formation of weakly emitting coumarin excimers causes self-quenching and emission broadening (Scheme 1 d).[4b,9]

As mentioned above, our strategy to avoid self-quenching and tune the emission spectral features is based on energy transfer (see Scheme 1 e,f): an energy acceptor, different from coumarin, is thus colocalized in the NPs to "extract" the excitation energy in competition with the formation/deactivation of the excimer. We chose the BODIPY derivative **B** (Scheme 1 a), which presents suitable photophysical properties for use as an energy acceptor for the NPs as well as a high fluorescence quantum yield.[10] Although FRET in dual-dye DDS NPs had been reported previously, in no case was it exploited to prevent fluorescence self-quenching.

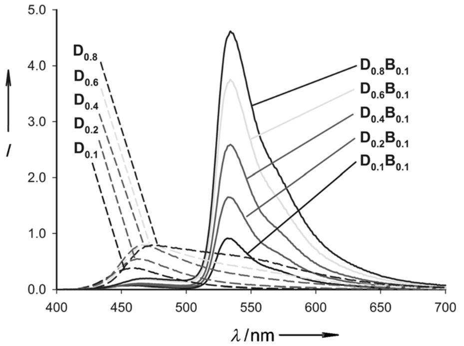

Figure 2. Fluorescence spectra of different NP samples in water ($c = 1\times10^{-7}$ M). Dashed lines: samples containing only **D**; continuous lines: samples containing both **D** and **B**.

A set of double-chromophore NPs were prepared with the same coumarin doping levels δ_D of the previously discussed set of NPs and, in all cases, a percentage of **B** (δ_B) of 0.1% (NP samples $\mathbf{D_{0.1}B_{0.1}}$, $\mathbf{D_{0.2}B_{0.1}}$, $\mathbf{D_{0.4}B_{0.1}}$, $\mathbf{D_{0.6}B_{0.1}}$, and $\mathbf{D_{0.8}B_{0.1}}$). As already observed for the single-dye NPs, both fluorophores were included quantitatively in the NPs. The presence of the BODIPY dye was confirmed by the absorption spectra of the NPs in water (Figure 1; concentration 1×10^{-7} M for all samples). The typical peak of this dye at 523 nm with a maximum of absorbance independent of δ_D was observed (Table 1). Upon excitation of this peak ($\lambda_{exc} = 500$ nm), the typical fluorescence band of BODIPY was detected (see the Supporting Information); the fluorescence quantum yields measured under these excitation conditions were very similar (Table 1). This result indicates that no electronic interaction occurs between the coumarin moieties and the BODIPY dye in either the ground or the excited state.

The same emission band characteristic of BODIPY was also observed upon excitation of the coumarin band ($\lambda_{exc} =$ 400 nm, Figure 2), and its intensity was directly proportional to δ_D. The presence of this band was accompanied in all cases by a very strong quenching of the coumarin fluorescence in the 400–500 nm range (about 10% of the residual emission, Table 1). All these findings are in agreement with very efficient FRET from **D** to **B**. Owing to its efficiency, this FRET process prevents the formation of poorly emitting coumarin excimers; this process enables the formation of ultrabright NPs with a very high molar absorption coefficient and fluorescence quantum yield. The definitive evidence of

Table 1: Photophysical data of the NPs in water.

	Absorption		Fluorescence			Absorption		Fluorescence		
	λ_{max} [nm]	ε [$\times10^5$ M^{-1} cm^{-1}]	λ_{max} [nm]	Φ		λ_{max} [nm]	ε [$\times10^5$ M^{-1} cm^{-1}]	λ_{max} [nm]	Φ	η_{FRET}
$\mathbf{D_{0.1}}$	422	2.0	458	0.79[a]	$\mathbf{D_{0.1}B_{0.1}}$	420, 523	2.1, 3.6	533	0.97,[a] 0.90[b]	0.90[c]
$\mathbf{D_{0.2}}$	418	3.6	461	0.59[a]	$\mathbf{D_{0.2}B_{0.1}}$	418, 523	4.5, 3.5	534	0.87,[a] 0.87[b]	0.91[c]
$\mathbf{D_{0.4}}$	418	7.4	464	0.46[a]	$\mathbf{D_{0.4}B_{0.1}}$	417, 523	8.8, 3.6	534	0.90,[a] 0.95[b]	0.87[c]
$\mathbf{D_{0.6}}$	417	12	466	0.38[a]	$\mathbf{D_{0.6}B_{0.1}}$	415, 523	14, 3.9	534	0.85,[a] 0.98[b]	0.86[c]
$\mathbf{D_{0.8}}$	415	16	470	0.32[a]	$\mathbf{D_{0.8}B_{0.1}}$	415, 523	18, 3.7	534	0.81,[a] 0.97[b]	0.87[c]

[a] $\lambda_{exc} = 420$ nm. [b] $\lambda_{exc} = 500$ nm. [c] The FRET efficiency (η_{FRET}) was calculated from the quenching efficiency.

the occurrence of the process is given by the excitation spectra recorded by detecting the BODIPY emission (see the Supporting Information): in all cases, the spectra coincide within the experimental error with the absorption spectrum and in particular show a contribution of the coumarin band that corresponds to an almost quantitative population of the BODIPY dye. Such a result is only possible if the energy-transfer process is fast enough to efficiently compete with all radiative and nonradiative processes involving the excited state of the coumarin moiety. The efficiency of the energy-transfer processes we observed is much higher than that observed for previously described dye-doped silica NPs,[11] most probably because the procedure used to synthesize the NPs described herein provides enhanced control of the silane condensation and thus results in a more homogeneous distribution of the dyes inside the nanostructures.[3b,9]

To test the efficiency of our approach for fluorescence imaging, we compared the signal recorded with a CCD camera (CCD = charge-coupled device) for the different samples upon excitation at (390 ± 9) nm by using either a blue emission filter ((460 ± 30) nm, Figure 3 a) or a green emission filter ((530 ± 22) nm, Figure 3 b). After integration, the total intensities acquired for all the samples were plotted (Figure 3). These data, in agreement with the spectra in Figure 2, clearly demonstrate that the presence of **B** in the NPs doped with **D** causes a shift of the emission to the green region, prevents the broadening of the fluorescence band, and most interestingly, leads to a strong increase in the signal almost in proportion to the δ_D value. We can hence conclude that our original FRET-based approach enables the prevention of self-quenching in DDS NPs and thus the construction of NPs with impressive photophysical properties: $\varepsilon \cong 10^6\,\mathrm{M^{-1}\,cm^{-1}}$, $\Phi = 0.81$, and a pseudo-Stokes shift of 129 nm.

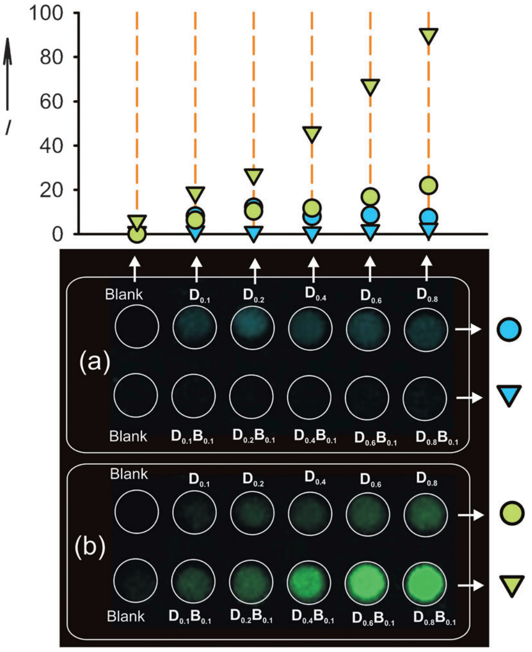

Figure 3. Fluorescence images of a set of 12 wells containing dispersions of the NPs in water ($c = 1\times10^{-7}\,\mathrm{M}$), as detected with either a blue (a, $\lambda_{em} = (460 \pm 30)$ nm) or a green (b, $\lambda_{em} = (530 \pm 22)$ nm) filter. The total intensity determined by the integration of each spot is plotted in the top graph.

In conclusion, we have demonstrated that the self-quenching of coumarin can be prevented in a family of dye-doped NPs that were recently reported to be suitable for labeling and sensing. Our approach exploits fast and very efficient FRET from the excited coumarin molecules to a suitable acceptor that upon sensitized excitation emits with almost unitary efficiency. In the described NPs, FRET is fast enough to become the predominant deactivation mechanism of the energy-donor moieties and make the formation of other weakly fluorescent bimolecular excited states inefficient. The presence of the energy acceptor shifts the maximum towards higher wavelengths and enables the emission wavelength to be tuned, spectral broadening to be avoided, and the pseudo-Stokes shift of the system to be increased. The combination of all these effects leads to a significant increase in the signal-to-noise ratio offered by these materials, and thus makes their possible application even wider and consequently more attractive.

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