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Exploiting Fast Exciton Diffusion in Dye-Doped Polymer Nanoparticles to Engineer Efficient Photoswitching

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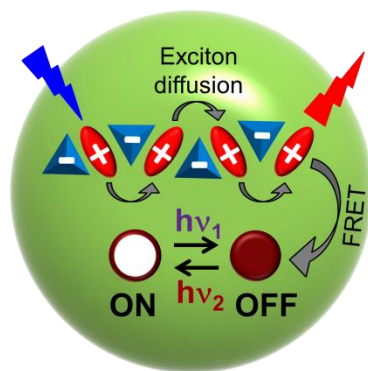
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ABSTRACT

Photoswitching of bright fluorescent nanoparticles opens new possibilities for bioimaging with superior temporal and spatial resolution. However, efficient photoswitching of nanoparticles is hard to achieve using Förster resonance energy transfer (FRET) to a photochromic dye, because the particle size is usually larger than the Förster radius. Here, we propose to exploit the exciton diffusion within the FRET donor dyes to boost photoswitching efficiency in dye-doped polymer nanoparticles. To this end, we utilized bulky hydrophobic counterions that prevent self-quenching and favor communication of octadecyl rhodamine B dyes inside a polymer matrix of poly(D,L-lactide-*co*-glycolide). Among tested counterions, only perfluorinated tetraphenylborate that favors the exciton diffusion enables high photoswitching efficiency (on/off ratio ~20). The switching improves with donor dye loading and requires only 0.1-0.3 wt% of a diphenylethene photochromic dye. Our nanoparticles were validated both in solution and at the single-particle level. The proposed concept paves the way to new efficient photoswitchable nanomaterials.

TOC GRAPHICS



KEYWORDS: fluorescent nanoparticles, PLGA polymer, photochromic dye, energy transfer, exciton diffusion, bulky hydrophobic counterions, single-particle microscopy

Photoswitchable fluorescence systems gain growing attention because of numerous potential applications, particularly for fluorescence imaging with superior resolution.¹⁻⁴ The fluorescence switching requires coupling of the photo-induced chemical transformation with the fluorescence output.⁵ This can be achieved with so-called photochromic dyes, where the light-induced chemical changes are associated with change of their optical properties.⁶ Two approaches are used in this case. The first one exploits fluorescent photochromic dyes, which switch their fluorescence properties under light illumination.⁷ Most common representatives are derivatives of spiropyran,⁸ cyanine⁹⁻¹⁰ and some fluorescent proteins.¹¹⁻¹² The second approach is to couple a fluorescent dye with a non-fluorescent photochromic dye, that operate as a FRET pair.¹³ In this case, the photo-isomerization of the photochromic dyes modulates the FRET efficiency and thus switches on/off the dye fluorescence.¹⁴⁻¹⁵ This approach is more universal, as most of photochromic dyes are non-fluorescent and their combination with fluorescent dyes enables access to any desired emission color associated with high fluorescence quantum yield. Photoswitchable FRET-based molecules work very well as the distance within a typical molecular dyad is much less than the Förster radius required for an efficient FRET. On the other hand these systems present two limitations: (1) limited brightness of organic dyes and (2) multi-step synthesis of the dyad, which makes these materials rather expensive. Both limitations could be overcome by the use of fluorescent nanoparticles (NPs). Indeed, they can be 10-100-fold as bright as organic dyes, which opens new possibilities in fluorescence bioimaging. Second, using straightforward protocols, they can be readily prepared with an appropriate photochromic dye to achieve FRET-based photoswitching. However, as the fluorescent particles are usually >5 nm large, the distance between the particle donor and the photochromic acceptor can exceed the Förster distance and thus decrease the FRET efficiency.

The most developed fluorescent NPs are quantum dots,¹⁶ dye-doped silica NPs¹⁷ and organic NPs.¹⁸⁻¹⁹ In the last decade, significant efforts have been made to make these NPs photoswitchable.²⁰ To achieve efficient switching, quantum dots were covered with multiple photochromic acceptors within the particle organic shell.²¹⁻²² However, it was found that the efficiency of switching despite optimized conditions remained modest (~2-fold). Examples in literature on dye-doped silica NPs showed better switching efficiency, compared to QDs, because in this case the photochromic acceptor could be encapsulated directly inside the particle core together with the dye donors.²³⁻²⁵ Within organic systems, photoresponsive polymer NPs

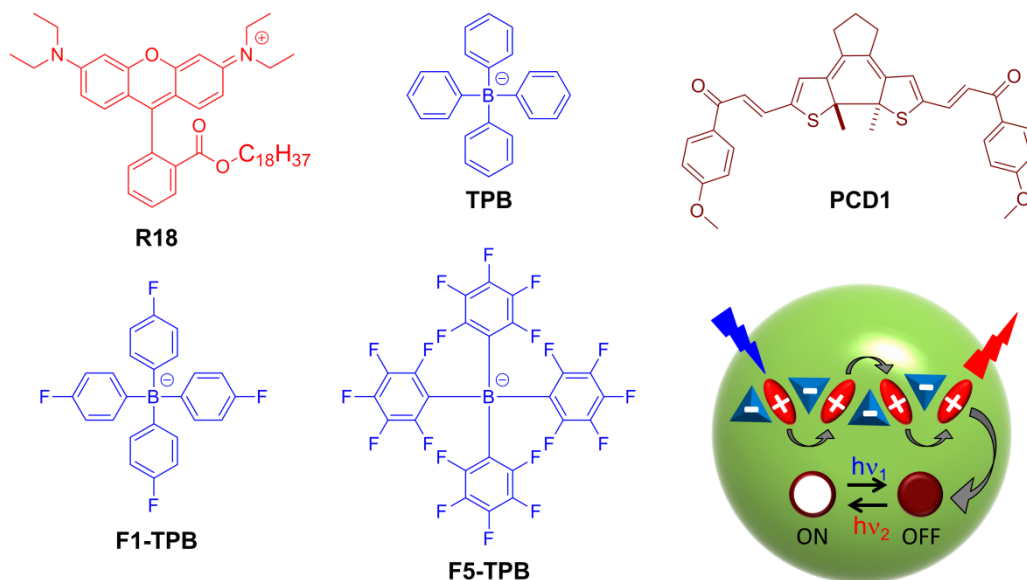
have already shown very promising results.²⁶⁻²⁷ This is notably the case of conjugated polymer NPs, which also can encapsulate photochromic dye directly inside the core.²⁸⁻³¹ In this case, the higher intensity variation (>10-fold) could be explained by fast energy transfer along the polymer conjugation, which ensures that the energy is efficiently transferred to long distances towards a single acceptor (i.e. photochromic dye).³²⁻³⁴ This phenomenon is called exciton diffusion and it is well known in sensors,³² light harvesting³⁵ and photovoltaic systems,³⁶ which rely on rapid communication within large ensemble of dyes to ensure efficient energy transfer.

Dye-doped polymer NPs are also very attractive organic systems, as they can be built from biodegradable eco-friendly materials and present exceptional brightness.³⁷⁻⁴⁰ However, only limited number of reports utilized FRET to obtain photoswitchable dye-doped polymer NPs.⁴¹⁻⁴⁴ One reason is that it is still a challenge to obtain dye-doped NPs with minimal self-quenching of the encapsulated dyes. Moreover, the problem of controlling FRET efficiency between the doped dyes and the photoswitch was rarely addressed.⁴³ In the present work, we hypothesized, that exciton diffusion, which is the reason for superquenching in the conjugated polymers, could be exploited to drive the photoswitching efficiency of dye-doped polymer NPs. To this end we selected our recently developed rhodamine doped NPs build from biodegradable polymer poly(D,L-lactide-*co*-glycolide) (PLGA).³⁸ Using hydrophobic perfluorinated counterion as an “insulator” between the cationic rhodamine dyes, we were able to prevent self-quenching at high dye loading. Unexpectedly, at high dye loading we observed for the first time a fluorescence blinking of 100-500 dyes encapsulated in one particle. This phenomenon was explained by the ultra-fast exciton diffusion, so that a single dark species (triplet or radical state of the dye) could quench the whole particle.³⁸ Remarkably, other counterions at the same dye loading did not show this behavior, so that the perfluorinated counterion was responsible for assembling rhodamine dyes in a close proximity to ensure the energy migration. Therefore, this system appeared promising for developing photoswitching NPs.

In the present work, we exploit the phenomenon of the exciton diffusion to build dye-doped polymer nanoparticles with excellent photoswitching ability. To this end, we co-encapsulated into PLGA NPs rhodamine B dye salt with perfluorinated counterion as well as a photochromic acceptor of the diarylethene family. To understand the role of the exciton diffusion, we prepared NPs with other counterions, which did not favor exciton diffusion of rhodamine dyes. It was found that with perfluorinated counterion light-driven variation of fluorescence intensity was up

to 20-fold, while for all other counterions, the switching efficiency was much lower. Thus, for the first time, we showed a direct connection between the collectiveness of the encapsulated dyes inside polymer NPs and their capacity to photoswitch. The performance of the new NPs was validated at the single particle level, where reversible photoswitching was demonstrated.

Nanoparticles studied in this work were prepared by nanoprecipitation of PLGA from acetonitrile. As we have shown earlier, diluted solutions of a polymer once precipitated in water can form NPs of 40-50 nm that are stabilized by the negative charge of carboxylate groups.^{38,45} To encapsulate a fluorophore and a photoswitch, they were simply added to the acetonitrile solution before the nanoprecipitation. As a fluorophore, we selected the octadecyl-rhodamine B (R18) bearing different counterions: inorganic perchlorate or tetraphenyl borates with different levels of fluorination (Scheme 1). As a photochromic dye, we selected the diphenylethene derivative **PCD1**, which was described earlier.⁴⁶ It is hydrophobic and thus should be efficiently encapsulated into polymer NPs, as shown for other low polar switches.⁴⁴ **PCD1** matches perfectly the optical properties of the donor dye R18, since its long-wavelength absorption band overlaps well with the emission of the donor (Fig. 1A). Moreover, the photoswitch can be selectively illuminated with 405 and 645 nm lasers, necessary for switching, without direct excitation of the R18 dye, which is important to prevent bleaching of the fluorophore during the photoswitching cycles.



Scheme 1. Chemical structure of the encapsulated fluorescent dye (R18) with different bulky counterions and the photochromic dye (PCD1). Schematic presentation of photoswitching

concept in dye-doped NPs. Small gray arrows show the exciton diffusion, while the large gray arrow presents FRET to a photochromic dye (brown).

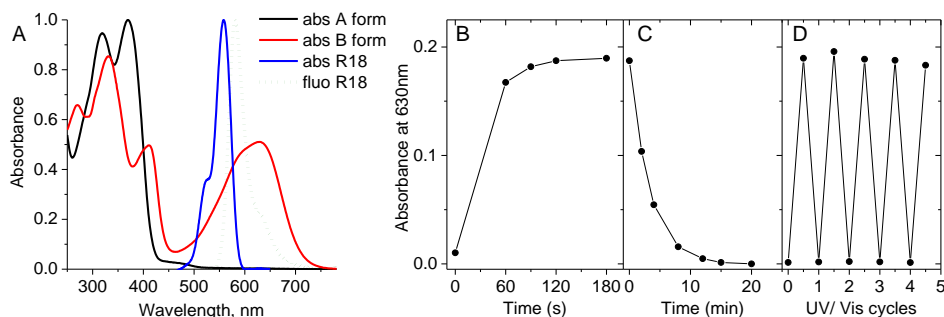


Fig. 1. Spectroscopic properties of the R18 donor and the photochromic FRET acceptor PCD1, and photoswitching behavior of the latter in dioxane. (A) Absorption spectra of PCD1 at 0.02 mg/mL in dioxane in comparison to the normalized absorption and fluorescence spectra of R18 with F5-TPB counterion in PLGA NPs (1 wt% loading). Time-dependent absorbance of PCD1 at 630 nm under illumination at 405 nm (B) and 645 nm (C). (D) Absorbance of PCD1 at 630 nm under alternate illumination at 405 nm ($\sim 1 \text{ mW/cm}^2$) and 645 nm ($\sim 200 \text{ mW/cm}^2$).

We first studied a small volume (200 μL) of the photochromic molecule PCD1 (0.02 mg/mL) in dioxane in 1 mL quartz cuvette, which was needed to irradiate the whole sample by a laser or a lamp of a fluorometer. To optimize conditions for photoswitching, the sample was illuminated at 405 nm by the lamp of the fluorometer with a light flux $\sim 1 \text{ mW/cm}^2$ and by the defocused irradiation of a 645 nm laser with a light flux $\sim 200 \text{ mW/cm}^2$. Illumination at 405 nm, resulted in the rapid growth of the absorbance at 630 nm, which reached saturation after ~ 2 min (Fig. 1B). These changes indicated the formation of the closed (colored B) form of the photoswitch. In contrast, to switch the molecule back to the open (colorless A) form, an irradiation of the sample at 645 nm for at least 20 min with much higher power was required (Fig. 1C). The observation of absorption peaks at 630 nm under alternate irradiation confirmed the reversibility of the photochromic isomerization without fatigue for at least 5 complete cycles (Fig. 1D).

To investigate the photoisomerization conditions inside PLGA polymer NPs we encapsulated 1 wt% of the photochromic dye with respect to the mass of polymer and performed the same cycles of irradiation. To obtain full photoisomerization inside PLGA, we increased the time of irradiation at 405 nm up to 10 min and at 645 nm up to 40 min. (Fig. S1).

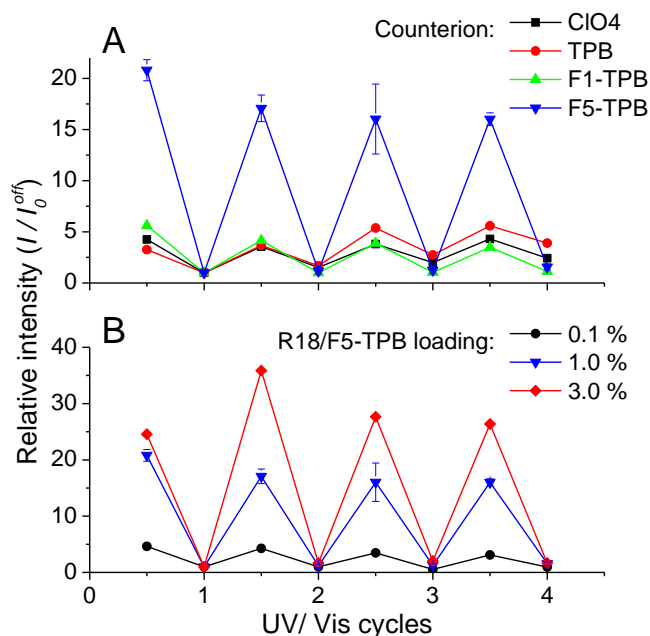


Fig. 2. Switching properties of the dye-doped PLGA NPs as a function of counterion and dye loading in PLGA NPs. (A) Variation of relative peak intensity under alternate irradiation of PLGA NPs loaded with 1 wt% of rhodamine R18 with different counterions and 0.3 wt% of photochromic dye PCD1. (B) Variation of relative peak intensity under alternate irradiation of PLGA NPs loaded with 0.1 – 3 wt% of R18/F5-TPB and 0.3 wt% of PCD1. Excitation wavelength was 520 nm.

Next, the photochromic dye PCD1 was co-encapsulated in PLGA NPs together with R18 dye bearing four different counterions: perchlorate (ClO₄), TPB, F1-TPB, F5-TPB. Dynamic light scattering (DLS) showed that the particle size remained in the range 42-47 nm (polydispersity index ~ 0.1) for most of formulations of R18 salts with or without PCD1 switch (Table S1). The only exception was NPs with R18/ClO₄ salt, which, in line with our earlier works, produced larger NPs.³⁸ The emission of the obtained NPs was further studied under illumination cycles as it was established above in model conditions. We called off state the state of NPs where the photochromic dye in closed form behaves as an energy acceptor for rhodamine B, and, on state the opposite state without FRET. To characterize the system, we determined two parameters: the *relative intensity* = I / I_0^{off} and *switching efficiency* = I_{on}/I_{off} , where I is the peak fluorescence intensity at a given condition; I_0^{off} is the peak fluorescence intensity at the off state in the first illumination cycle; I_{on} and I_{off} are the peak fluorescence intensities of the on and off states,

respectively at a given illumination cycle. First, we verified for the most promising R18 salt with F5-TPB counterion the optimal concentration of the photochromic dye inside NPs. For 1 wt% loading of R18/F5-TPB dye, the switching efficiency grew up to 0.3 wt% of the photoswitch, while for 0.5 wt% the switching was already less efficient (Fig. S2). The presence of 1 wt% of the fluorophore did not have any influence on the photoswitch fatigue (Fig. S3), whereas the presence of 0.3 wt% of photoswitch did not influence the photostability of the fluorophore (Fig. S4). Therefore, in all our further studies we used systematically 0.3 wt% of the photoswitch. We found that all NPs bearing one of the four different R18 salts were able to photo-switch their fluorescence, though the switching efficiency depended clearly on the nature of the counterion. Indeed, while for perfluorinated counterion F5-TPB, the switching efficiency was around 15, for all other counterions it was only 2-4 (Fig. 2A). The calculated Förster radius for a pair R18 and PCD1 was 2.1 and 4.9 nm for the open and closed forms of the switch, respectively. Assuming a homogeneous distribution of the PCD1 acceptors inside NPs, a volume taken by one acceptor was 240 nm^3 , which gave an average distance between the acceptors of 7.7 nm. Theoretically, the closest average distance between R18 donor and PCD1 acceptors was 4.7 nm when R18 was located in the center of tetrahedron formed by PCD1 (hexagonal arrangement of PCD1 separated by 7.7 nm). In this case, one R18 donor was surrounded with four acceptors, so that using FRET model for multiple (four) acceptors,⁴⁷ the estimated FRET efficiency was 3.1 and 84% for open and closed forms of PCD1, respectively. Thus, FRET can at least in part explain the switching behavior of our NPs. However, we should not exclude the electron transfer between R18 and the closed form of PCD1. Knowing the reduction and oxidation potentials of rhodamine B (-0.8 and 1.2 V, respectively)⁴⁸ and using the Rehm-Weller formalism,⁴⁹ the estimated reduction and oxidation potentials of this dye in the excited state were +1.38 and -0.98 V (vs SCE), respectively. For the switch, the redox data are available only for an analogue of PCD1 in the closed form with the oxidation and reduction potentials of +1.45 and -1.11 V (vs SCE), respectively.⁵⁰ The value of the oxidation potential of the model switch is close to the reduction potential of rhodamine B in the excited state, which (after neglecting small columbic effects) suggests that the electron transfer could take place from PCD1 to R18. In any case, the estimated FRET efficiency for the closed form is in line with the experimental data for all counterions, except F5-TPB (Fig. 3A). For the latter, the FRET efficiency was much higher, so that the off state in these NPs was ~12-fold less fluorescent than theoretically predicted. This is an indication

that counterion plays a particular role in the organization of R18 dyes. As we hypothesized earlier³⁸ F5-TPB could bring the donors close to each other thus favoring exciton diffusion, which could boost the FRET efficiency.

To verify whether this effect of F5-TPB is linked with the exciton diffusion process, we measured the steady-state fluorescence anisotropy of NPs with all four counterions. When the energy migrates within encapsulated fluorophores that are randomly orientated inside the polymer matrix, the fluorescence anisotropy rapidly decreases after each step of the energy hopping between dyes.⁵¹⁻⁵² As the fluorophores are confined within the rigid polymer matrix, the loss of fluorescence anisotropy in this system could be mainly attributed to the exciton diffusion process. In line with our earlier study,³⁸ NPs loaded with R18/F5-TPB salt presented much lower fluorescence anisotropy than all other salts, indicating that the most efficient exciton migration was achieved with this salt (Fig. 3B). According to our previous time-resolved anisotropy data at 1 wt% R18/F5-TPB loading, corresponding to ~100 dyes per particle, at least 40 dyes were involved in the exciton diffusion.³⁸ These results correlated well the much higher efficiency of the photoswitching observed for R18/F5-TPB NPs (Fig. 3A). The unique behavior of F5-TPB is probably related to its perfluorination, which may favor assembly of R18 dyes with short inter-fluorophore distances inside polymer matrix allowing fast exciton diffusion. The role of fluorines could be related to so-called fluorous effect (or super-hydrophobicity)⁵³ as well as to their strong electronegativity that shifts the negative charge of F5-TPB to its periphery. To further check the importance of the exciton diffusion on the photoswitching efficiency, we varied the concentration of the fluorescent donor dyes (R18/F5-TPB), while keeping the photoswitch concentration constant. In the case of non-communicating donors, the FRET efficiency, i.e. photoswitching efficiency, should be independent on the concentration of the donors.⁵⁴ However, we observed clearly that the photoswitching efficiency increased dramatically on increasing the donor dye concentration (Fig 2B, Fig. 3A). Moreover, this increase correlated perfectly with the decrease in the fluorescence anisotropy (Fig. 3B), indicating faster rates of the exciton diffusion for higher dye loading. Indeed, at higher dye loading the distance between the donor (R18) dyes should decrease, which should lead to faster energy hopping, as we showed earlier using time-resolved anisotropy and fluorescence blinking experiments.³⁸ The present data suggest that the energy transfer to the photoswitch can be drastically improved by exploiting the exciton diffusion within the donor dyes. To the best of our knowledge, it is the first time that a

connection between the photoswitching efficiency and the exciton diffusion was established for dye-doped nanoparticles.

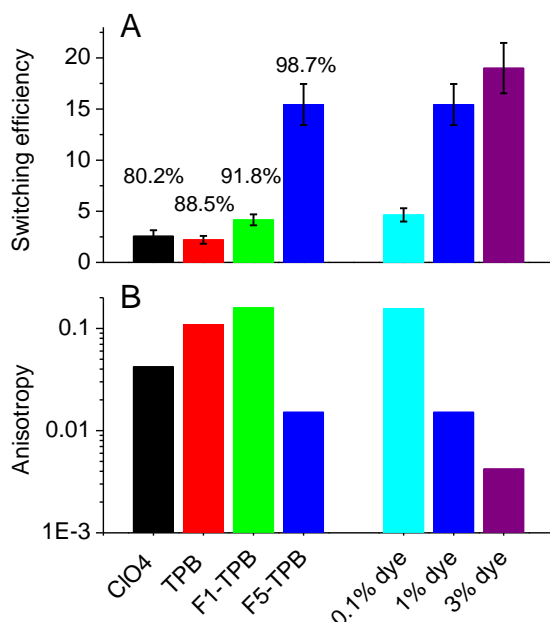


Fig. 3. Correlation between photoswitching efficiency of NPs and fluorescence anisotropy of the encapsulated dyes. (A) Average switching efficiency (I_{on}/I_{off}) within four complete on/off cycles for dye-doped NPs containing 0.3 wt% of PCD1, with different counterions and at different R18/F5-TPB dye loading. The values given in this panel present the FRET efficiency (%) for the off state calculated from I_{off} and the intensity of NPs without PCD1. (B) Fluorescence anisotropy of the encapsulated dyes for the same NPs samples but without PCD1.

Finally, we evaluated the performance of our NPs at the single-particle level using wide-field fluorescence microscopy. To this end, we immobilized fluorescent NPs containing 1 wt% R18/F5-TPB and 0.3 wt% of PCD1 at the glass surface covered with positively charged polymer PEI. Then, under the fluorescence microscope, we illuminated the observation region with alternated blue (405 nm) and red (645 nm) lasers and recorded the fluorescence image after each illumination step. We found that after illumination at 645 nm, the particles appeared bright and were well seen on the images (Fig. 4A). In contrast, after 405 nm illumination, most of the particles became dim, indicating that they underwent photo-conversion into the off state. The average intensity variation showed a clear alternation of the particle intensity (Fig. 4B). However, we should note that the value of the average intensity variation was lower than in

solution. This is in line with other studies of NPs switching showing that at the single particle level the switching appears less efficient.⁵⁵ There can be multiple factors, particularly related to the use of much higher illumination power under the microscope. The higher powers could bleach the encapsulated R18 dyes or the photoswitch and thus decrease the switching efficiency. Another reason is that at the single particle level, the signal to noise ratio is significantly lower than for NPs in solution, where a large population is simultaneously excited. Therefore, the absolute changes under the microscope could be decreased by the background noise. The other issue is the observed heterogeneity in the switching efficiency. While some particles showed switching efficiency reaching 30, for others it was below the average (Fig. S5). This heterogeneity is related to inhomogeneity in the dye and photoswitch doping, particle size, etc. Therefore, an important challenge in the future will be to improve the homogeneity in the particle switching efficiency.

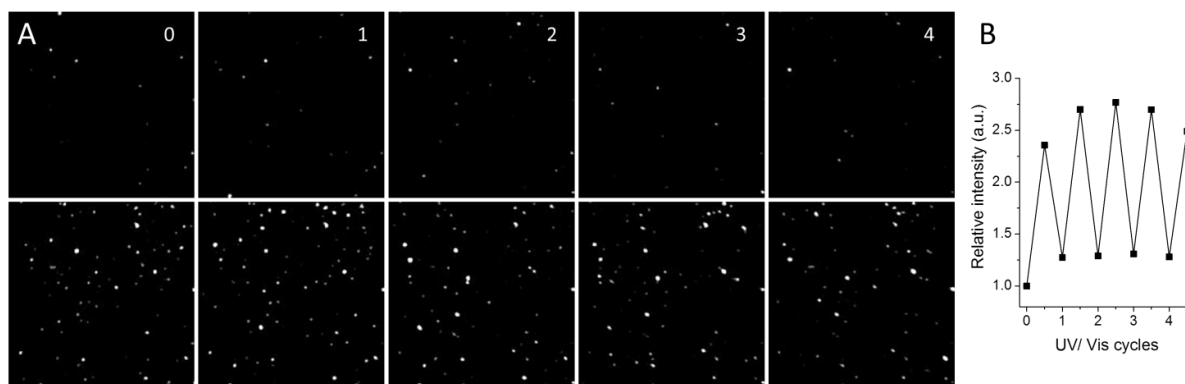


Fig. 4. Single-particle observation of photoswitching of dye-doped NPs. (A) Wide field images of NPs containing 1 wt% R18/F5-TPB and 0.3 wt% of PCD1 after illumination at 405 nm (upper panels) and 645 nm (lower panels). (B) Average relative intensity (I / I_0^{off}) of NPs in the on and off state obtained from these images. The optimal conditions for photoswitching were 60 s illumination at 645 nm with laser power 97 W/cm^2 , and 5 s illumination at 405 nm with laser power 8.7 W/cm^2 . To record fluorescence images the power of the laser at 532 nm was set to 0.2 W/cm^2 .

In the present work, we propose for the first time to utilize exciton diffusion within the encapsulated fluorophores to achieve efficient photoswitching. To this end, we utilized our recently proposed concept of hydrophobic counterion for encapsulating a cationic dye (octadecyl

rhodamine B) into NPs. This counterion serves as insulator that prevents dye self-quenching but at the same time assembles the dyes in close proximity to ensure fast dye-dye communication inside a polymer matrix. We showed that only the perfluorinated tetraphenylborate counterion favoring the exciton diffusion enables efficient photoswitching efficiency (~20) of NPs in suspensions. For other counterions, where the inter-fluorophore communication was less pronounced, the photoswitching efficiency was much lower. Wide-field fluorescence microscopy studies confirmed the photoswitching of our NPs at the single-particle level. Our study shows that exciton diffusion within fluorophores inside NPs can ensure efficient FRET to photoswitch producing highly efficient photoswitching. The proposed concept paves the way to new efficient photoswitchable nanomaterials.

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SUPPORTING INFORMATION

Detailed experimental section, and some additional optical spectroscopy and microscopy data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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