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One-pot synthesis of monodispersed silica nanoparticles for diarylethene-based reversible fluorescence photoswitching in living cells†

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A small 29 nm monodispersed silica nanoparticle 1a was synthesized as a diarylethene-based reversible fluorescence photoswitch by copolymerizing silane precursors in one-pot including 3a and 4. Reversible photoswitching of nanoparticle 1a was successfully achieved in living cells to show its potential as a highly distinguishable and safe fluorescence probe for cell tracking.

Photochromic compounds (PCs) can undergo reversible photoswitching between two isomeric forms of different absorption spectra.[1] Such a property has enabled PCs to be used as key functional moieties for erasable data storage media,[2] photochromic eye glasses, and fluorescence switches.[3] Fluorescence imaging[4] is the most common technique to visualize cells, proteins, and oligonucleotides in various biological studies. Recently, to facilitate detection of the fluorescence signal emitted from a particular probe in a complex biological environment, fluorescence probes have been made switchable in response to specific stimuli.[5] Diarylethene (DAE) derivatives,[6] which are known to be one of the most efficient PCs for photoswitching, have been employed to build the reversible fluorescence switch for biological imaging.[7] Although DAE[7b] or its aggregates[7d] can be fluorescent, it may also serve as a nonfluorescent molecular switch to turn on or off the fluorescence of a neighboring fluorophore through fluorescence resonance energy transfer (FRET).[7a,e,8] As our continued effort to develop an efficient and safe reversible fluorescence switch for biological applications, here we report the synthesis of a size-controlled silica nanoparticle (NP) by copolymerizing all individual components in one-pot as silane precursors including those of DAE (FRET acceptor) and a fluorophore (FRET donor). Moreover, we show the efficient cellular uptake and reversible fluorescence photoswitching of these silica NPs—after the biocompatible surface-modification—inside two different types of living cells by alternate irradiation with UV and visible light.

Several reports have described the preparation of DAE-based reversible fluorescence photoswitch systems which were built on nanostructures.[7a,e,8a,b] Despite the many known advantages,[9] practical use of these nanometer-sized photoswitch systems, particularly for biomedical applications, can be yet limited due to their difficult preparation methods, heterogeneity, and low switching efficiency, as well as inherent toxicity and poor water-solubility. Silica NPs[10] are one of the most favored nanomaterials in biomedicine due to their relative safety and possibility to tune their size during the preparation with a low polydispersity index.[11] Further, recent advances have allowed for the incorporation of most of the small organic molecular fragments into the main silica framework by forming covalent bonds.[12] Here, our strategies to synthesize silica NPs for reversible fluorescence switching in biological systems (Fig. 1a) were the following: (1) functionalization of the FRET donor (a Cy3 derivative) and FRET acceptor (a DAE derivative), individually, with triethoxysilane (TES) groups (Fig. 1c) for their direct covalent incorporation into the silica network during the formation of NPs; (2) addition of a large excess of DAE (**3a**) in comparison to Cy3 (**4**) to achieve effective quenching of Cy3 fluorescence considering somewhat low photochromic conversion efficiency of DAE (using a carboxylic acid analogue **10**, Scheme S2, ESI†) in water under photoswitching conditions as estimated by ^{1}H NMR (Fig. S2, ESI†); (3) adoption of a synthetic method[12b] that would produce highly monodispersed silica NPs of preferably 20–25 nm in size (*i.e.*, diameter) for facile cellular uptake and to exhibit homogeneous biological effects; (4) post-modification of the amine functionalities of silica NPs into carboxylate groups for enhanced biocompatibility.

The synthesis of our photoswitchable silica NPs was carried out in a medium containing the oil-in-water emulsion (Scheme S5, ESI†).[12b] Specifically, each silane-functionalized precursor of DAE (**3a**) and Cy3 (**4**), together with vinyltriethoxysilane (VTES) as the bulk component, was added to the microemulsion made from Tween-80, and the mixture was allowed to equilibrate with stirring before polymerization. Here the most hydrophobic VTES is expected to accumulate predominantly in the interior core region

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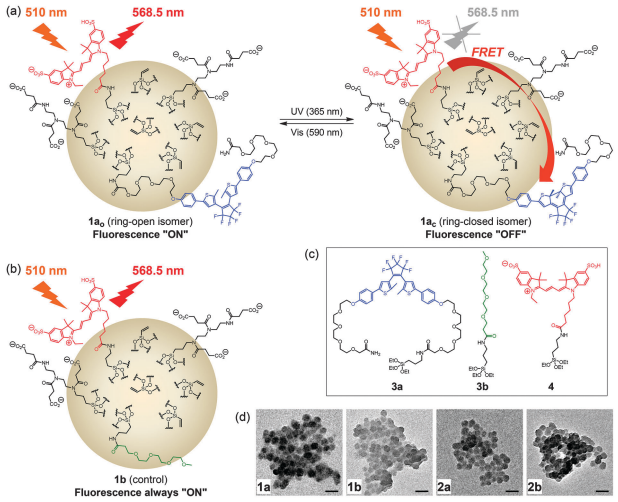

Fig. 1 (a) Silica NP **1a** for reversible fluorescence photoswitching and (b) its non-switchable control **1b**. (c) Structures of silane derivatives **3a**, **3b**, and **4** incorporated covalently into the silica network during the formation of **1a** or **1b** by copolymerization. (d) TEM images of silica NPs **1a** and **1b** and their amine-terminated precursors **2a** and **2b**, respectively (for structures, see Scheme S5, ESI†). Scale bars, 50 nm.

of the Tween-80 micelle, and **3a** and **4** with extended aromatic segments may likely as well localize in the interior. Upon initiation of the copolymerization reaction, *N*′-[3-(trimethoxysilyl)propyl]-diethylenetriamine (DETA) was added to impart amine functionalities to the NPs. It should be noted that unlike other DAE-based photoswitchable nanostructures which were prepared by attaching the premade 1 : 1 conjugate of FRET donor and FRET acceptor dyes to their surface,[7e,8b] our structure was prepared by adding individual FRET dyes as reactive silane precursors simultaneously in the copolymerization step to make the NPs. This strategy not only simplifies the preparation method by reducing synthetic steps, but also may allow for the free modulation of molar feed ratios, particularly between the FRET donor and acceptor dyes to potentially enhance the efficiency of fluorescence quenching.[7a] The amino groups on the resulting silica NP **2a** (Scheme S5, ESI†) could be converted into various functionalities by treating with amine-reactive species, and here they were reacted with succinic anhydride to enhance the biocompatibility of the NPs. The silica NP **1a** with carboxylate end groups indeed had a negatively charged surface (zeta potential value of −29 mV, Table S1, ESI†), dispersed well in aqueous media, and was highly monodispersed with *ca.* 29 nm in mean diameter as determined by transmission electron microscopy (TEM; Fig. 1d, Table S1, ESI†). Additionally, as a non-switchable control, a fluorescent silica NP **1b** with a carboxylate surface (*ca.* 23 nm by TEM, zeta potential value of −31 mV) was made in a similar manner using methoxy tetra(ethylene glycol) (mTEG) derivative **3b** instead of **3a** (Fig. 1b–d).

Next, to assess the feasibility of using our silica NP as a reversible fluorescence switch under physiological conditions, the photoswitching experiment was performed using sample solutions in phosphate buffered saline (PBS). While an absorption band centered at 586.0 nm (ring-closed isomer, Fig. S5a, ESI†) appeared clearly for the carboxylic acid derivative of DAE **10** (Scheme S2, ESI†) upon irradiation with UV light (365 nm, 2 min), only a weak broad band was exhibited around 550–650 nm for silica NP **1a** (Fig. S6a, ESI†). Indeed, this broad absorption band diminished upon irradiation with visible light (590 nm, 30 min), suggesting its correlation with the ring-closed isomer of DAE moieties. Here the absorption band of the Cy3 derivative that would normally appear around 550 nm was not explicit possibly due to the relatively low degree of Cy3 substitution on the silica NP **1a** (based on the molar feed ratio). In contrast, the reversible switching was unambiguously demonstrated in the fluorescence spectra of silica NP **1a** upon alternate irradiation with UV and visible light (Fig. 2 and Fig. S6b, ESI†). For both silica NPs **1a** and **1b**, the Cy3 emission maximum was observed at 568.5 nm (Fig. S6b and d, ESI†), which was slightly red-shifted compared with that of a free dye at 563.0 nm (Fig. S5d, ESI†). The degree of fluorescence quenching of **1a** was found to be 54% on average over five cycles of photoswitching in PBS (Table S2, ESI†). Evidently, no fluorescence quenching was observed upon irradiation with UV light under the same photoswitching conditions for neither the Cy3 dye nor the Cy3-derivatized silica NP **1b** without DAE (Fig. 2a, Fig. S5d, and S6d, ESI†). Rather, a slight drop in fluorescence intensity was consistently noticed for Cy3 and **1b** upon irradiation with visible light in repeated experiments, which appeared to be partially recovered in the subsequent irradiation with UV light. Interestingly, for all three samples—**1a**, **1b**, and Cy3—a minor degree of reduction in fluorescence intensity was noticed, with increasing number of photoswitching cycles, perhaps due to photobleaching (Fig. 2a).

Stem cells have been increasingly attracting attention as therapeutic agents, and indeed the precise tracking of these cells inside the living system is imperative for effective treatment of diseases.[13] Accordingly, we envisioned that if fluorescently labeled cells in the biological system can be reversibly switched, they can be explicitly distinguished among many other neighboring cells where detection of the fluorescence itself can be even more encumbered by the absorbance, light scattering, and autofluorescence of the surrounding media. The photoswitching potential of silica NP **1a** was investigated in two different types of living cell cultures—human epithelial cervical cancer (HeLa) cells and human adipose-derived stem (hADS) cells (Fig. 3). To determine the appropriate concentration and incubation time for effective internalization of silica NP **1a** into cells for photoswitching, we first evaluated the cytotoxicity and the cellular uptake efficiency of **1a** against each cell. Relatively high cell survival rates of 85% and

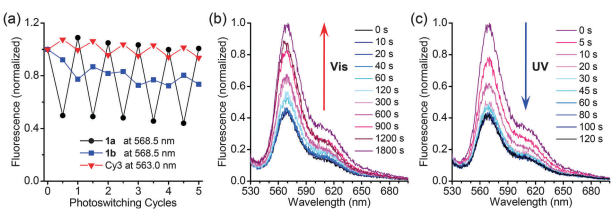

Fig. 2 Photoswitching experiments using silica NPs (100 μg mL^{-1}) **1a** and **1b**, and Cy3 (1 μM) in PBS (pH 7.4). Samples were irradiated alternately with UV (365 nm, 2 min, 2.60 mW cm^{-2}) and visible (590 nm, 30 min, 90 ± 5 mW) light. (a) Fluorescence intensity at each emission maximum (irradiation at 510 nm). Photoswitching was repeated for up to five cycles, beginning with UV light irradiation. (b) Fluorescence spectra of **1a** obtained at selected time points by brief interruption of irradiation starting from the off-state during the visible light irradiation of the third photoswitching cycle and (c) the subsequent UV light irradiation of the fourth cycle.

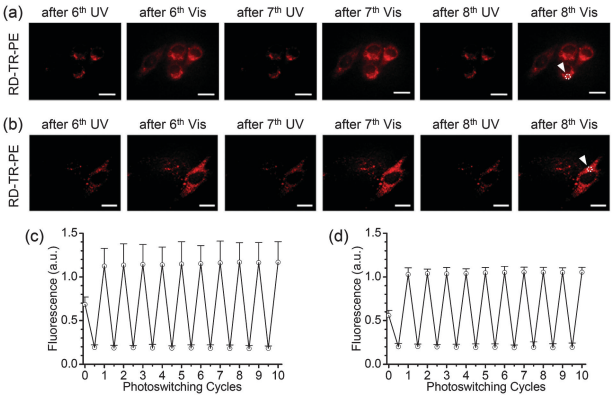

Fig. 3 Photoswitching experiments performed on living (a and c) HeLa or (b and d) hADS cells with internalized silica NP **1a**. Cells were irradiated alternately with UV (365 nm, 2 min) and visible (590 nm, 30 min) light for up to 10 cycles. The average fluorescence intensity values (mean ± SD) measured in the region of interest (dotted circles, 36 data points each) after each light application are plotted (c and d). A sequential irradiation with UV (loss of fluorescence) and visible (fluorescence recovery) light constitutes one full cycle of photoswitching (integers in the *x*-axis). RD-TR-PE: red fluorescence. Scale bars, 20 μm.

91% were achieved for HeLa and hADS cells, respectively, when cells were incubated at 50 μg mL^{-1} for 24 h (Fig. S7, ESI†). When analyzed by flow cytometry, the internalization of silica NP **1a** into either cell type became more effective (*i.e.*, higher fluorescence intensity) with increasing incubation time, and generally the uptake efficiency was somewhat higher for HeLa cells than for hADS cells (Fig. S8, ESI†). Also, similar to the previously reported anionic photoswitch with carboxylate surface groups,[7a] the internalized silica NP **1a** was mostly found in the cytosolic region and did not appear to enter the nuclei for either cell type when visualized under a microscope. Next, the photoswitching experiments were conducted for up to 10 cycles using living cells with internalized silica NP **1a** by irradiating alternately with UV (365 nm, 2 min) and visible (590 nm, 30 min) light (Fig. S9, ESI†). When Cy3 fluorescence was measured at the selected cytosolic area of each cell type upon photoswitching (Fig. 3a and b), the average quenching efficiencies of 84% and 81% were found for HeLa and hADS cells, respectively (Table S2, ESI†). These values corresponded to the fluorescence on–off contrast of 6.2 for HeLa cells and 5.4 for hADS cells, which were higher than that measured in a cuvette (on–off contrast of 2.2).[7a] Here, the morphologies of HeLa and hADS cells, which underwent 10 cycles of photoswitching, apparently remained intact. While the wavelength of light for fluorescence quenching can be shifted to a less harmful visible range by modifying the DAE structure,[6] the effect of UV light irradiation on cell viability under current photoswitching conditions was examined (Fig. S10, ESI†). Indeed, survival rates of HeLa cells with internalized **1a** that underwent up to five cycles of photoswitching were similar (within the error range) to those of the cells which were not irradiated.

In summary, we reported the one-pot multicomponent copolymerization strategy to prepare the monodispersed silica NP **1a** with a mean diameter of 29 nm for reversible fluorescence photoswitching in living cells. This silica NP was synthesized in the oil-in-water emulsion using silane precursors including those of DAE (FRET acceptor) and Cy3 (FRET donor). The carboxylate-terminated silica NP **1a** effectively internalized into living cells—both cancerous (HeLa) and stem (hADS) cells—to successfully undergo 10 cycles of photoswitching with no apparent morphological damage. Interestingly, the fluorescence on–off contrast of **1a** determined inside the living cells was much higher (5.4–6.2) than that obtained using PBS solution in a cuvette. We envision that our biocompatible silica NP-based photoswitch may find versatile utility in various applications, such as tracking therapeutic stem cells in living systems, as a highly distinguishable and relatively safe fluorescence probe.

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