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Photoreversible cellular imaging using photochrome-conjugated fullerene silica nanoparticles†

Jinyoung Jeong,[a] Eunju Yun,[a] Yohan Choi,[a] Hye-youn Jung,[a] Sang J. Chung,[a] Nam Woong Song[b] and Bong Hyun Chung*[a]

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Photochromic compound-conjugated fluorescent fullerene-silica nanoparticles prepared by the reverse-microemulsion method was utilized for photoswitchable cellular imaging by repeatable irradiation of ultraviolet and visible light.

Fluorescent nanoparticles have been extensively investigated for biomedical applications such as bioimaging because of their unique properties, which include strong photoluminescence, tunable colour based on size, and tolerance for cell penetration.[1,2] Semiconducting quantum dots, fluorescent dye-encapsulated silica nanoparticles, and fluorescent carbogenic nanoparticles are widely used for cell imaging, monitoring of target molecules in tissue, and detection of biological interactions *in vivo*.[3,4] However, there remain several challenges, including high background autofluorescence from biomolecules, photo-blinking, and high cytotoxicity.[5] Recently, photoswitchable nanoparticles have been studied as a means to overcome these drawbacks in bioimaging applications. In particular, polymer nanoparticles containing fluorescent dyes and spiropyran were used for dual-colour imaging of complex biological systems based on fluorescence resonance energy transfer (FRET).[6–8] Although the FRET-based imaging system enables precise, specific and multiplex imaging of target molecules, there are considerable drawbacks, such as the need to design acceptor and donor molecules, to maintain the proper distance between molecules, and to make further modifications for cell penetration.

Photochromism occurs when a chemical species has two forms that are reversibly transformed by irradiation at different wavelengths. This phototransformation phenomenon can be affected by a variety of physicochemical properties, including the refractive index, dielectric constant, and geometric structure. Dithienylethene is a promising photochromic compound because of its thermal irreversibility and resistance to fatigue.[9–11] It can be reversibly switched between two conformations: a closed-ring state upon UV irradiation and an open-ring form upon visible light irradiation, by either breaking or forming a chemical bond.[12] On the basis of these characteristics, we designed a photoswitchable nanoparticle by combining a dithienylethene derivatised photochromic compound (PC) and a highly biocompatible, photoluminescent fullerene-silica nanoparticle (FSNP), which were previously developed and utilised as photoreversible bioimaging agents with low autofluorescence in a variety of cells.[13]

In this study, we synthesised an amine-functionalised dithienylethene that can directly conjugate to fullerene *via* nucleophilic addition by the following procedure (see Fig. S1, ESI†). The photochrome-conjugated fullerene silica nanoparticles (PC-FSNPs) were prepared by addition of ammonium hydroxide (NH_4OH) and the PC solution to a reverse microemulsion containing the C_{60} fullerene and silica precursor (TEOS) (Fig. 1). Because the amine group of the PC was very attracted to the C_{60} fullerene, it was necessary to add the NH_4OH to the microemulsion with the PC to induce simultaneous cross-linking of the silica and fullerene *via* hydrolysis of TEOS and addition of the amine to C_{60}.

The size and shape of the as-prepared PC-FSNP was measured by scanning electron microscopy (SEM). As seen in Fig. 2, the PC-FSNP was highly monodisperse with an average diameter of 85.22 ± 5.85 nm. It was slightly larger than the previously prepared FSNP (61.5 ± 6.0 nm), indicating that the conjugation of the PC into the nanoparticle may affect its size.

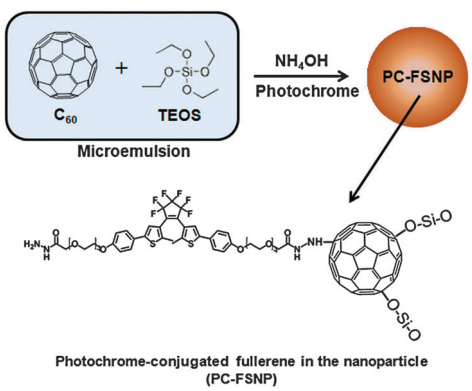

Fig. 1 Schematic illustration of synthesis of photochrome-conjugated fullerene-silica nanoparticles *via* the reverse microemulsion method.

[a] *BioNanotechnology Research Center, Korea Research Institute of Bioscience and Biotechnology, Gwahangno, Yuseong, Daejeon 305-806, Republic of Korea. E-mail: chungbh@kribb.re.kr; Fax: +82 42 879 8594; Tel: +82 42 860 4442*
[b] *Cell Dynamics Research Center at GIST and Nanobio Fusion Research Center, Korea Research Institute of Standards and Science, Daejon 305-340, South Korea*

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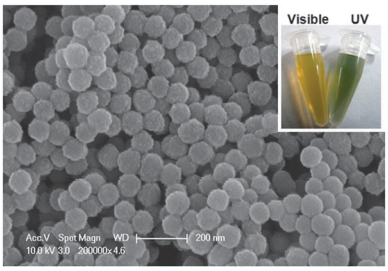

Fig. 2 FE-SEM image of as-prepared PC-FSNP. In the inset, the photograph shows the colour change of the PC-FSNP aqueous solution under UV and visible light irradiation.

The nanoparticles were light yellow under white light due to the structure-broken fullerene; however, they clearly became greenish-yellow after UV irradiation (Fig. 2, inset), due to the mix with blue colour of the closed-ring state of PC. On the contrary, the nanoparticle prepared without C_{60} did not change the colour upon either UV or visible light illumination (data not shown), indicating that PC did not interact with the silica precursor.

To characterise the chemical composition of the PC-FSNP, Fourier-transform infrared (FTIR) spectroscopy was conducted. In the FTIR spectrum of the PC-FSNP, the Si–O signal from 1100 cm^{-1} to 1300 cm^{-1} was too strong to indicate whether the covalent C–N bond between C_{60} and amine-functionalised dithienylethene was present. Therefore, the PC-FSNP was treated with sodium hydroxide to eliminate silica, and an FTIR spectrum was obtained. Fig. S2 (ESI†) shows the C–N absorption in the range of 1250 cm^{-1} to 1350 cm^{-1}, indicating the formation of an aromatic nitrogen–carbon bond. It demonstrates that amine-terminated PC was covalently conjugated to C_{60} *via* nucleophilic attraction.[14] It is consistent with the result obtained from the IR spectrum of the conjugate of PC and C_{60} which shows the aromatic carbon–nitrogen bond at 1261 cm^{-1} (inset in Fig. S2, ESI†). In addition, the C–F vibration peaks corresponding to the PC at 951.6 cm^{-1}, 1056.4 cm^{-1}, and 1383.5 cm^{-1} were also observed. Thermogravimetric analysis (TGA) of the particles shows a weight loss at 512 °C, corresponding to the elimination of the silanol (Si–OH), that was about 20% less for the PC-FSNP than the FSNP control, which implies the PC was conjugated to the nanoparticles (see Fig. S3, ESI†). This conclusion assumes that the PC competes with silica to conjugate to the C_{60} during the particle formation. Taking the above results into account, we believe that the nanoparticles were formed by the covalent immobilisation of the PC and silica onto C_{60} *via* nucleophilic substitution and silanisation, respectively.

The optical properties of PC-FSNP in aqueous solution were also investigated under both UV and visible light irradiation. As seen in Fig. 3a (inset), the PC had an absorption peak at 600 nm under visible light that increased after UV (365 nm) irradiation as a result of the transformation to the closed-ring state. The absorbance recovered upon irradiating with visible light (512 nm). Although the change in the absorption of the PC-FSNP was relatively small, the spectra were similar to those of the pure PC under sequential UV and visible light irradiation, whereas the FSNP control had no change in the absorption spectra (data not shown). Because the absorption

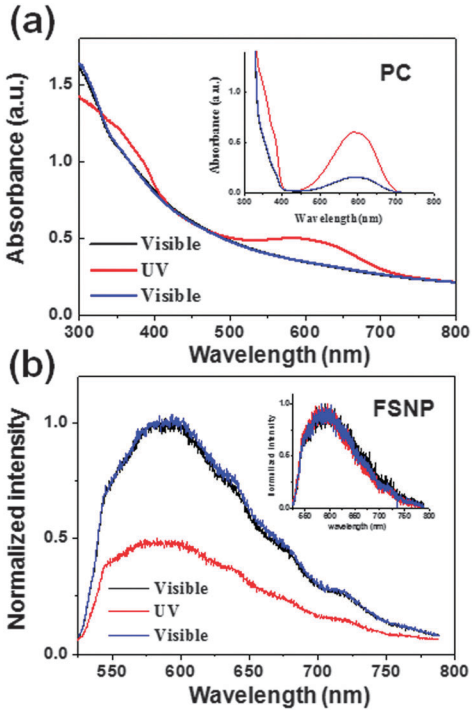

Fig. 3 Optical properties of PC-FSNP in aqueous solution under UV and visible light irradiation. (a) UV/visible absorption spectra of PC-FSNP (inset: UV/visible spectra of photochrome in ethanol) and (b) Photoluminescence spectra of PC-FSNP (inset: photoluminescence spectra of FSNP).

region of the PC matched the photoluminescence (PL) wavelength (~600 nm) of the FSNP, we expected that the PL of the PC-FSNP was influenced by the change in absorption due to the phototransformation of the PC. To confirm this hypothesis, we measured the PL of the PC-FSNP and FSNP control using an Ar ion laser (excitation at 488 nm). The resulting PL spectrum of the PC-FSNP under visible light was similar to that of the FSNP, indicating that the conjugation of the PC did not affect the luminescence of the FSNP, which originates from the C_{60}–O–Si bond. However, the PL intensity decreased about 52% after UV light irradiation but recovered after visible light irradiation. The dependency of the PL on the wavelength used was assumed to be caused by the emissive energy of the FSNP being transferred to the PC, as it strongly absorbs visible light at 600 nm after UV irradiation. In other words, the PL of the PC-FSNP was quenched by the intermolecular energy transfer between the C_{60}–O–Si and the PC in the nanoparticle after UV irradiation.[15] After the ring structure of dithienylethene was transformed back by visible light irradiation, the PL also recovered to its original intensity, in clear contrast to the FSNP, which had no change in PL whether irradiated with either UV or visible light (Fig. 3b, inset). The quenching effect can be influenced by various factors, such as the molar ratio of donor and acceptor molecules in the system, the distance between the two molecules, and the surrounding elements. Although it is complicated to distinguish the exact effect the different wavelengths used had on the quenching efficiency of PC-FSNP, it is roughly determined by the number of PC molecules in a single nanoparticle, calculated from weight loss analysis in TGA, and the distance from the dithienylethene

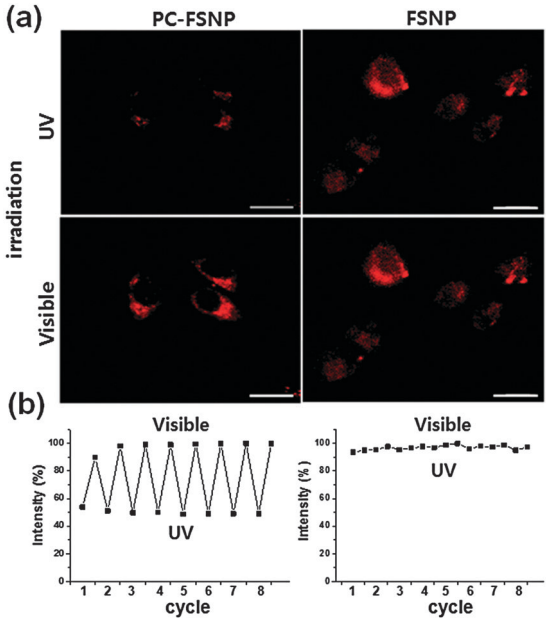

Fig. 4 (a) Fluorescence images of PC-FSNP-containing HeLa cells. The images were taken under a combination of 490 nm excitation/617 nm emission filter after UV and visible light irradiation for 5 min each. (b) Comparison of fluorescence intensity of PC-FSNP and FSNP under repeatable UV and visible light irradiation.

moiety to the C_{60} through the tetraethyleneglycol linker, which is close enough for energy transfer to occur (<5 nm). Compared to the quenching efficiency of FRET and other systems, PC-FSNP is a reasonable system for photoreversible analysis or imaging owing to its rigid structure surrounding the photo-transformation moiety, its high photostability, and the biocompatibility originating from the FSNP.

In light of these advantages, we evaluated the photoreversible cellular imaging of PC-FSNP uptake in cells with sequential UV and visible light irradiation. Before the cellular imaging, we investigated the time-dependent quenching effects of PC-FSNP to determine an adequate irradiation time. The relative fluorescence intensity of the PC-FSNP in an aqueous solution was measured every minute after either UV or visible light irradiation. As seen in Fig. S4 (ESI†), the intensities after UV irradiation gradually decreased by almost 50%, while the intensities after visible irradiation increased within 5 min. After 5 min, there were no further changes in either the quenching or recovery of the PL of the PC-FSNP. Therefore, the irradiation time for the photoreversible effect of the PC-FSNP was fixed at 5 min.

For cellular imaging, the HeLa cells were incubated with PC-FSNP and FSNP (50 μg ml^{-1} for each) for cellular uptake by endocytosis. Next, the cells were washed with the media and PBS after one-hour incubation. The fluorescent images of the nanoparticle-containing cells were obtained by fluorescence microscopy with an emission filter of 617 nm under excitation at 488 nm. As seen in Fig. 4a, both the PC-FSNP- and FSNP-containing cells showed bright red emission in the cytosol region of the cells after visible light irradiation, indicating that both the PC-FSNPs and FSNPs were significantly internalised into the cells. However, there was a distinguishable difference in the red fluorescence of the PC-FSNP and the FSNP after UV irradiation. The intensity from the PC-FSNP decreased considerably compared to that of the FSNP, which had no major changes in fluorescence under either UV or visible light. Furthermore, the intensity was reversibly changed by repeatedly irradiating with UV and visible light up to 8 times (Fig. 4b). The average intensity difference between UV and visible light irradiation was 48.1 ± 1.7%, which is similar to the quenching efficiency of PC-FSNP (52%) in an aqueous solution. It is noteworthy that the photoreversible reaction of the PC-FSNP occurred consistently in both the pristine solution and the intracellular environment.

In conclusion, we prepared photoreversibly switchable photoluminescent nanoparticles through the conjugation of an amine-functionalised photochrome, C_{60}, and silica by the reverse microemulsion method. The PL of the nanoparticles was reversibly and repeatedly switched on and off upon UV and visible light irradiation through the intermolecular energy transfer between the photo-induced transformation of PC and C_{60}–O–Si, the PL species. The quenching efficiency (about 50%) of PC-FSNP by UV irradiation in both a pristine solution and an intracellular environment was comparable to that of other photoreversible switching systems. This nanosystem consisted of a photoswitchable molecule and a rigid, photostable and biocompatible photoluminescent moiety could be used for photoreversible analysis during cellular imaging and detection of target molecules in a complex biological system with a high signal-to-noise ratio.

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