



# In Vitro and In Vivo Uncaging and Bioluminescence Imaging by Using Photocaged Upconversion Nanoparticles\*\*

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High temporal and spatial regulation of cellular activities, biological pathways, and gene expression is critical in complex biological processes.<sup>[1]</sup> One remarkable technique that enables such control is the use of light to manipulate compounds that are photoactive (or photocaged) in various biological systems.<sup>[2]</sup> Previously, this strategy has been used to map cellular functions, monitor the expression of transgenes, and image the dynamic processes of cell–cell interactions in vitro and in vivo.<sup>[3,4]</sup> Although all of these attempts were successful in principle, there were significant limitations associated with the use of high-intensity UV or visible light in the photoactivation process. Excessive exposure to UV light can cause photoreactions in nucleic acids and result in cellular damage. Furthermore, short-wavelength UV or visible light does not penetrate into tissue very far, which limits its utility for deep-tissue imaging by photoactivation of the caged compounds. Alternatively, multiphoton photolysis with long-wavelength excitation has been used to enable deep-tissue imaging and to target gene expression<sup>[5]</sup> Despite its usefulness, the multi-

photon photolytic process typically requires a complex experimental set-up and has low conversion efficiency because of narrow absorption cross-sections. Therefore, the development of a simple approach that allows a high depth of penetration into tissue and precise control of photocaged systems as well as limiting cellular damage is highly desirable.

Recently, lanthanide-doped upconversion nanoparticles (UCNPs) have received considerable attention for applications that range from biolabeling to optical data storage.<sup>[6]</sup> These nanoparticles offer high photostability and enable deep tissue-penetration depths (up to 10 mm) by irradiation with near-infrared (NIR) light, which makes them particularly attractive for bioimaging applications.<sup>[7]</sup> Herein, we demonstrate a method for uncaging photocaged molecules in vitro and in vivo and performing bioluminescence imaging studies by combining versatile photocaged compounds with the UCNPs.

Scheme 1 illustrates the proof-of-concept design for the photolysis of caged D-luciferin by using bioconjugated UCNPs. As a commonly used bioluminescent probe, D-luciferin can recognize firefly luciferase (fLuc) reporter genes and produce bioluminescence in the presence of O<sub>2</sub>, Mg<sup>2+</sup> ions, and adenosine triphosphate (ATP). Therefore, D-luciferin provides opportunities for extensive applications in molecular imaging in vitro and in vivo.<sup>[8]</sup> In our design, Tm/Yb co-doped NaYF<sub>4</sub> core-shell nanoparticles<sup>[9]</sup> that have a reduced surface-quenching effect were chosen as the platform for the conjugation of D-luciferin. The core-shell nanoparticles were coated with thiolated silane molecules and subsequently coupled to D-luciferin that was caged with a 1-(2-nitrophenyl)ethyl group. As the absorption band of the photocaged D-luciferin overlaps with the upconverted emission band of the nanoparticle in the UV region (<sup>1</sup>I<sub>6</sub> → <sup>4</sup>F<sub>3</sub>, <sup>1</sup>D<sub>2</sub> → <sup>3</sup>H<sub>6</sub> transitions of Tm<sup>3+</sup>), excitation of the photocaged UCNPs with NIR light can trigger disassociation of D-luciferin molecules from the surface of the nanoparticle. Importantly, the uncaging process can be monitored either by tracking the luminescence intensity of D-luciferin or by using an fLuc enzyme reporter, thus providing the possibility of bioluminescence imaging studies without the need for UV or visible light.

In a typical experiment, silica-coated UCNPs with reactive terminal thiol groups were synthesized by using a water-in-oil microemulsion method (see the Supporting Information for details).<sup>[7d–f]</sup> TEM images demonstrate that the silica-modified nanoparticles have a narrow size distribution of about 50 nm (Figure S1 in the Supporting Information). Both solutions of unmodified and silica-coated UCNPs have similar emissions in the UV, visible, and NIR spectral regions

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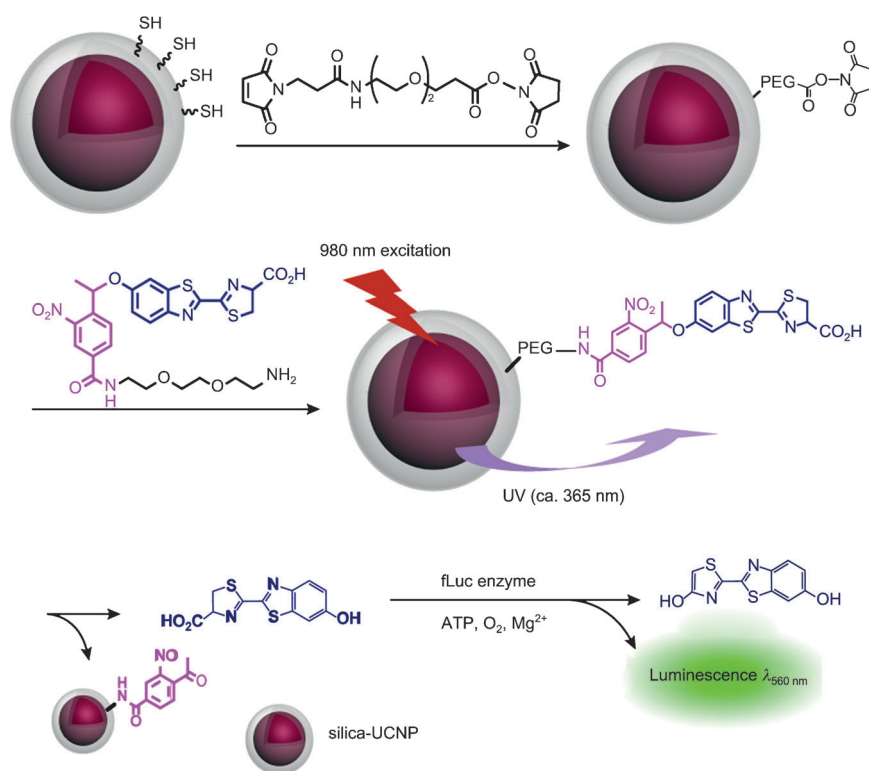
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[\*\*] This work was supported in part by NTU Start-Up Grant (SUG), Tier 1 (RG 64/10), the Singapore-Perking-Oxford Research Enterprise (SPORE), and the Singapore Ministry of Education (MOE2010-T2-083).



Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/anie.201107919>.



**Scheme 1.** Experimental design for uncaging D-luciferin and subsequent bioluminescence through the use of photocaged core-shell upconversion nanoparticles.

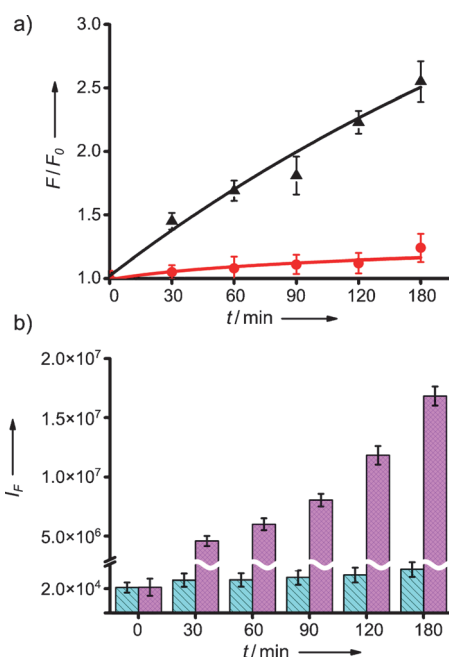
under irradiation with NIR light (Figure S1 in the Supporting Information), which demonstrates that coating the UCNPs with a silica shell did not significantly affect the upconversion properties. To ensure a high-efficiency coupling of the photocaged molecules to the particle surface, D-luciferin that was photocaged with a 1-(2-nitrophenyl)ethyl group was modified with a poly(ethylene glycol) linker that contained a terminal maleimide group, which is reactive with thiols.<sup>[10]</sup> The loading density of photocaged D-luciferin on the surface of a nanoparticle after the cross-coupling reaction was approximately 490 molecules, as determined by UV/Vis absorption spectroscopy (Figure S3 in the Supporting Information).

We examined the feasibility of uncaging D-luciferin by using UCNPs. The poly(ethylene glycol)-modified D-luciferin derivative was chosen as a control. Without irradiation with NIR light, both the caged D-luciferin derivative and the D-luciferin/nanoparticle conjugate did not exhibit D-luciferin fluorescence, which indicated that the D-luciferin molecules were effectively blocked in the photocaged systems. However, upon excitation with laser light (980 nm), the fluorescence of the solution of the D-luciferin/nanoparticle conjugate increased significantly, which can be attributed to the photo-deprotection of the 1-(2-nitrophenyl)ethyl group by upconverted UV light (Figure 1 a). Laser irradiation of the caged D-luciferin derivative that was not conjugated to the nanoparticles did not lead to a significant increase in fluorescence from D-luciferin (Figure 1 a), which indicated that the photocaged molecule itself was not sensitive to NIR light. Similar increases in fluorescence were also detected for the caged D-

luciferin derivative and the D-luciferin/nanoparticle conjugate upon exposure to UV light (Figure S4 in the Supporting Information).

The photolysis of the caged D-luciferin/nanoparticle conjugate was further confirmed by analysis of the bioluminescence mediated by the fLuc enzyme in a solution of phosphate-buffered saline (PBS, 10 mM, pH 7.2). Prior to photoactivation with NIR light, there were no detectable bioluminescence signals from either the caged D-luciferin derivative or the D-luciferin/nanoparticle conjugate in the presence of the fLuc enzyme,  $Mg^{2+}$ , and ATP. Similarly, bioluminescence signals were not detected from the caged D-luciferin derivative after excitation with NIR light for 2 h. In stark contrast, upon excitation of the D-luciferin/nanoparticle conjugate with NIR light, a significant increase in the bioluminescence signal at 560 nm was detected (Figure 1 b), which can be attributed to the light emission from the enzymatic oxidation of the photo-released D-luciferin substrate.

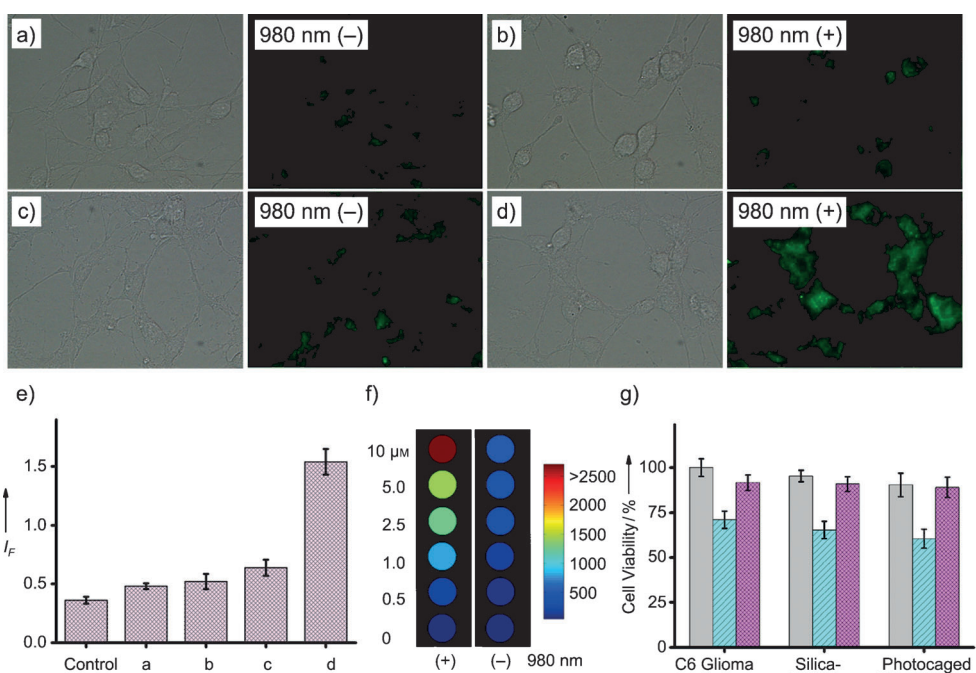
Encouraged by the results of the in vitro uncaging experiments and the bioluminescence studies, we investigated the intracellular



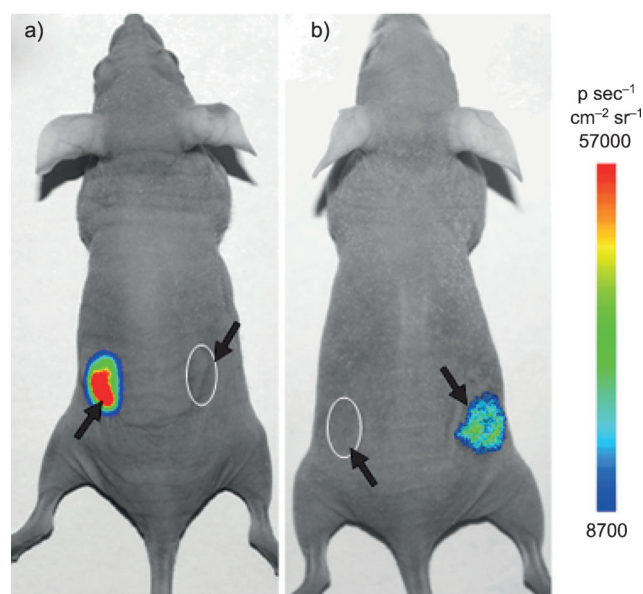
**Figure 1.** a) Time-dependent fluorescence increase from D-luciferin released from photocaged UCNPs (0.5  $\mu\text{m}$ ) under irradiation with NIR light (0–3 h). Black line = D-luciferin/UCNP conjugate; red line = photocaged D-luciferin only b) Time-dependent bioluminescence generated by adding the fLuc enzyme to uncaged D-luciferin under irradiation with NIR light (0–3 h). Purple bars = D-luciferin/UCNP conjugate; blue bars = photocaged D-luciferin only

uptake and uncaging of the D-luciferin/nanoparticle conjugate in living cells. In a typical cell imaging study, the D-luciferin/nanoparticle conjugate ([D-luciferin]:  $0.5 \mu\text{M}$ ) was incubated with C6 glioma cells in Dulbecco's Modified Eagle Medium (DMEM) at  $37^\circ\text{C}$  for 2 h. As a control, the caged D-luciferin derivative was incubated with the same type of cells under the same conditions. These two samples were then irradiated with NIR light for intracellular uncaging studies (Figures 2b and d). Before exposure to NIR light, the fluorescence signals within the cells in both of the samples were weak (Figures 2a and c), which indicated the possible quenching of the fluorescence from D-luciferin by the 1-(2-nitrophenyl)ethyl group or by the silica shell of the UCNPs.<sup>[7f–g]</sup> The NIR-activated uncaging of the D-luciferin/nanoparticle conjugate was further confirmed by bioluminescence studies in MCF-7 cells that were expressing the fLuc enzyme. Upon irradiation with NIR light, strong bioluminescent emission was detected at 560 nm (Figure 2f and Figure S9 in the Supporting Information). The emission signal from the cells that were transfected with fLuc was proportion to the number of cells used (Figure S10 in the Supporting Information). As a negative control, no bioluminescence was detected in MCF-7 cells that were incubated with the D-luciferin/nanoparticle conjugate without NIR irradiation, which clearly suggests that the use of the cell-penetrating nanoparticles enables the effective identification of fLuc enzymatic activity in living cells. More importantly, MTT assays showed that no significant cytotoxicity was observed in C6 glioma cells that were treated with the D-luciferin/nanoparticle conjugate after two hours of irradiation with NIR light. This was in stark contrast to a parallel experiment with UV irradiation, which showed significant cellular damage after a short exposure time (5 min, Figure 2g).

Another unique advantage of using photocaged silica-UCNPs is the ability to carry out bioimaging studies at substantial tissue depths. As a proof-of-concept experiment, the D-luciferin/nanoparticle conjugate and the fLuc enzyme were subcutaneously injected into living mice at a tissue depth of 2 mm, and then the bioluminescence was measured by using a modified Evolve in vivo imaging system. In contrast to the bioluminescence that was detected in the left thigh of a mouse that was directly injected with D-luciferin, there was no detectable bioluminescence signal at the point of injection in the right thigh of the mouse without irradiation with NIR light (Figure 3a). In comparison, strong bioluminescence signals were detected in the mouse that was injected with



**Figure 2.** Fluorescence imaging of C6 glioma cells incubated with photocaged D-luciferin ( $0.5 \mu\text{M}$ ) and the photocaged D-luciferin/UCNP conjugate ( $0.5 \mu\text{M}$ ) in the absence (panels a and c) and presence (panels b and d) of NIR light. Excitation filter: 460/40 nm; emission filter: 535/50 nm (exposure time to NIR light: 2 h). e) Bar graph of the fluorescence intensity of uncaged D-luciferin in cell lysate shown in panels a)–d). The cell lines without sample incubation were used as controls. f) Bioluminescence visualization of different concentrations of photocaged UCNPs in living cells (MCF-7/fLuc). g) Cell viability in the presence of silica-UCNPs or D-luciferin/UCNP conjugates that were exposed to NIR light for 2 h (purple bars), UV light for 5 min (blue bars), or no light (gray bars). As a control, cells that were not incubated with UCNPs or D-luciferin/UCNP conjugates were also exposed to UV and NIR light.



**Figure 3.** Bioluminescent images of fLuc activity in living mice that were treated with D-luciferin. a) Left: injection with D-luciferin ( $20 \mu\text{M}$ ,  $20 \mu\text{L}$ ); right: injection with photocaged nanoparticles without NIR light irradiation. b) Left: injection with photocaged nanoparticles and irradiation with UV light for 10 min; right: injection with photocaged nanoparticles and irradiation with NIR light for 1 h.



the D-luciferin/nanoparticle conjugate after irradiation with NIR light (Figure 3b). It should be noted that under UV irradiation, no notable bioluminescence was detected (Figure 3b and Figure S11 in the Supporting Information). Taken together, these data show the versatility of using photocaged UCNP for bioluminescence studies at substantial tissue depths.

In conclusion, we have reported a system for the controlled uncaging of D-luciferin and bioluminescence imaging that is based on photocaged upconversion nanoparticles. This method takes advantage of photon upconversion of NIR light to UV light to trigger the uncaging of D-luciferin from D-luciferin-conjugated UCNP. The released D-luciferin effectively conferred enhanced fluorescence and bioluminescence signals in vitro and in vivo with deep light penetration and low cellular damage. With compositional or structural tuning of the UCNP and the availability of diverse photocaged functional groups, this UCNP-based photolysis could be readily extended to other biomedical studies. These results may also offer new possibilities for non-invasively monitoring the dynamic functions of cells and selectively delivering drug molecules to targeted areas in vitro and in vivo on the basis of photocaged techniques.

Received: November 10, 2011

Revised: December 13, 2011

Published online: January 12, 2012

**Keywords:** bioluminescence · D-luciferin · nanoparticles · photochemistry · upconversion

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