

Photoactivable bioluminescent probes for imaging luciferase activity†

Qing Shao,^a Tingting Jiang,^a Gang Ren,^b Zhen Cheng^{*b} and Bengang Xing^{*a}

Received (in Cambridge, UK) 28th April 2009, Accepted 1st June 2009

First published as an Advance Article on the web 9th June 2009

DOI: 10.1039/b908346d

A set of stable and efficient photoactivable bioluminescent probes for imaging luciferase activity has been developed, which displayed robust bioluminescent signals upon brief UV illumination in buffer, cells and living animals.

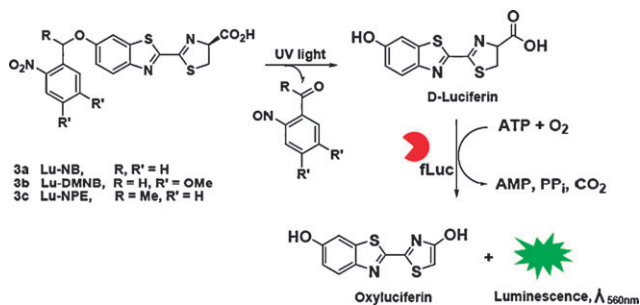
The firefly luciferase (fLuc) has been widely utilized for optical reporter gene imaging in biochemical assays, cell culture and living animals.¹ fLuc can catalyze the oxidation of its substrate D-luciferin in the presence of O₂, ATP, and Mg²⁺ to generate the bioluminescence that can be imaged by a CCD camera.¹ The luciferase based bioluminescence imaging technique displays low background and high sensitivity compared to fluorescence imaging modality, since fLuc nonexpression cells and tissues do not produce significant bioluminescence during normal cellular processes. Therefore, it has been extensively applied for noninvasive imaging of gene expression, studying *in vivo* cell trafficking² and detecting enzyme activities such as for β -galactosidase, caspases, monoamine oxidase and β -lactamase.³ Normally, cellular structures exhibit complex spatiotemporal organization. The precise tracking of the dynamic properties of cellular functions and repetitive monitoring of reporter genes at a desired time and/or location in intact cells, tissues or living animals will be crucial for many biomedical applications including cell trafficking, gene therapy studies and transgenic model engineering.⁴ The most notable and promising strategy for this purpose is the photolysis of photoactivable or “caged” molecules, by which the activation process can be readily modulated by a beam of light with high spatial and temporal precision.⁴ In line with this direction, several photocaged fluorophores have been reported for fluorescent labeling of proteins,⁵ monitoring the gene silencing and real-time imaging dynamics of cell–cell coupling *in vivo*.⁶ However, simple and specific photoactivable luciferase probes for efficient imaging of bioluminescence reporter gene in living animals are unavailable, since most of the current “caged” luminescence substrates are less stable, unable to be applied in the living animals or only limited to *in vitro* fluorescent detection.⁷

Here we present one set of stable and novel photo-releasable luciferin derivatives and report the first-time study of imaging luciferase activity using photocage technology in living mice.

^a Division of Chemistry & Biological Chemistry, School of Physical & Mathematical Sciences, Nanyang Technological University, Singapore. E-mail: bengang@ntu.edu.sg; Fax: 65 67911961; Tel: 65 63168758

^b Molecular Imaging Program at Stanford (MIPS), Department of Radiology, Stanford University Medical Center, California, 94305, USA. E-mail: zcheng@stanford.edu

† Electronic supplementary information (ESI) available: Chemical synthesis and characterization, photolysis, quantum yield determination, luciferase activity studies. See DOI: 10.1039/b908346d

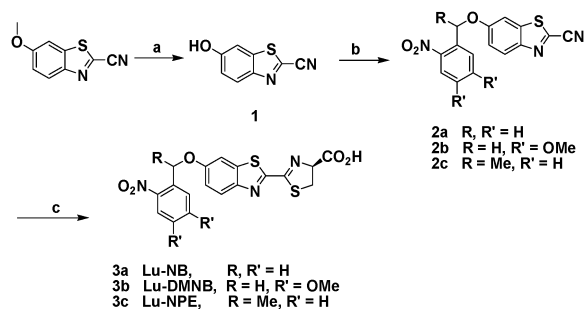


Scheme 1 The structure of the “caged” D-luciferin derivatives and proposed photolysis reaction for detection of fLuc.

These compounds were designed by masking the 6-hydroxy group with different cage groups (3a–c, Scheme 1), and all of them showed rapid uncaging and significant bioluminescence enhancement upon UV irradiation. Their inherent fluorescent properties were studied and their bioluminescent properties were further investigated by using fLuc as reporter enzyme in buffer, cell and living animals.

As shown in Scheme 2, the chemical synthesis of caged bioluminescent derivatives was started from alkylation of 2-cyano-6-hydroxybenzothiazole under basic conditions with different cages: 2-nitrobenzyl (NB, 3a), 4,5-dimethoxy-2-nitrobenzyl (DMNB, 3b) and 1-(2-nitrophenyl)ethyl (NPE, 3c). Then the alkylated transmediates were directly condensed with D-cysteine, followed by ring cyclization to provide compounds 3a–c with reaction yields of 83.4, 87.6 and 75.8%, respectively (see ESI†). Care needs to be taken to minimize the free luciferin as trace amounts of luciferin contamination would result in a significant bioluminescent background.

After obtaining the precursors, the photochemical properties of the “caged” luciferin derivatives were evaluated. These compounds did not show fluorescence and bioluminescence signals before photolysis, indicating the substrates were



Scheme 2 Synthesis of the “caged” D-luciferin derivatives: Reagents and conditions: (a) pyridine hydrochloride, 180 °C; (b) K₂CO₃/acetone, 2-nitrobenzyl bromide; (c) D-cysteine, MeOH–DCM, room temperature.

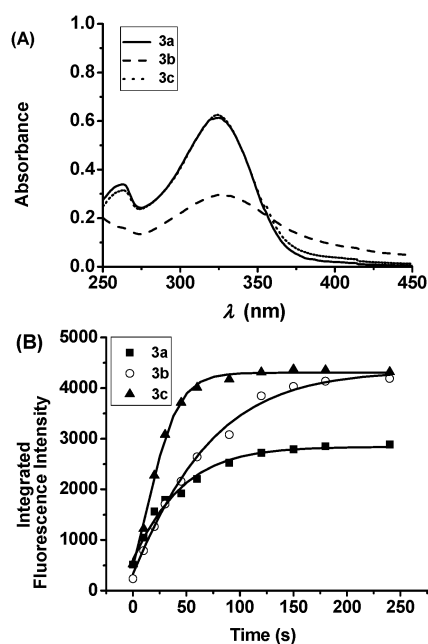


Fig. 1 (A) Absorption spectra of “caged” compounds (50 μM) in PBS buffer (pH 7.4); (B) time course of integrated fluorescent intensity of “caged” compounds (1 μM) upon UV illumination at 365 nm: $\lambda_{\text{exc}} = 350 \text{ nm}$, λ_{em} integrated from 425 to 670 nm.

efficiently blocked by the three different cages. UV illumination (365 nm) acted to remove the caging groups and progressively generated fluorescent products (Fig. 1). The excitation and emission spectra of photolyzed products showed no difference from those of D-luciferin, suggesting the release of luciferin from photoconversion of “caged” luciferin derivatives 3a–c. The uncaging products were further confirmed by HPLC. The quantum yield and efficiency of photolytic release of different cages were determined accordingly (see ESI[†]). Among the different “caged” substrates, NPE-luciferin (3c) appeared to be the best one affording the greatest fluorescence activation upon shortest time of UV illumination, which was consistent with the general observation made in reported “caged” systems.⁸

Similar uncaging properties were also estimated based on the measurement of fLuc activity in PBS buffer and cell lysate. As shown in Fig. 2, before photoactivation, there was almost no luminescence observed in all “caged” derivatives in the presence of fLuc, ATP and PBS buffer. However, upon UV illumination, the photoactivation switched on the luminescent activity and produced robust bioluminescent emission enhancements at 560 nm (Fig. 2A). Similar results were also obtained when the “caged” luciferin derivatives were treated with fLuc transfected C6 glioma cell lysates. As shown in Fig. 2B, significant luminescent emissions for all three different derivatives were observed after UV illumination. The bioluminescent signal from fLuc transfected cells was proportionally dependent on the cell numbers. All three derivatives showed similar spectral features. In comparison with NB (3a) and DMNB “caged” luciferin (3b), substrate NPE-luciferin (3c) exhibited a higher uncaging luminescent property which was in accordance with the results in buffer and in fluorescent uncaging measurements. As a control, the

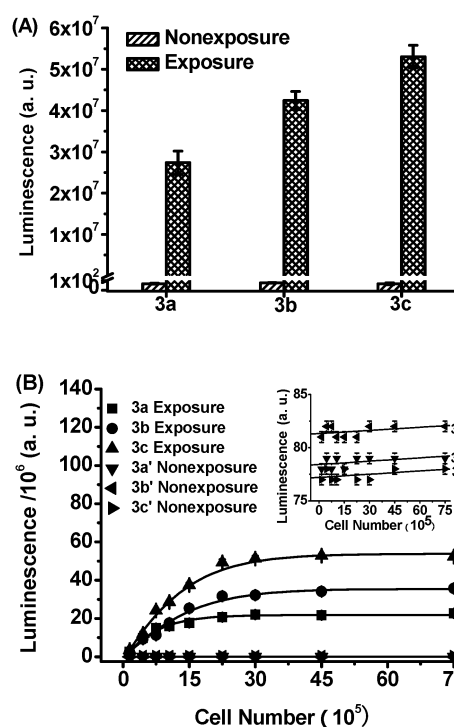


Fig. 2 (A) Bioluminescence change of “caged compounds (12.5 μM) before and after UV excitation in the presence of 75 $\mu\text{g ml}^{-1}$ fLuc, 1 mM ATP and 2.5 mM MgCl_2 . (B) Dependence of bioluminescence emission on cell number. Irradiation time is 1 min. The inset curve is the magnification of non-exposure data.

substrates were treated with the cell lysates without UV irradiation. No luminescence was observed, demonstrating the “caged” luciferin derivatives were stable in the cell lysates and able to identify the fLuc activity only after photoactivation.

To test the cell-membrane permeability of D-luciferin and the three “caged” luciferin derivatives and further determine their photolytic effectiveness, we examined their imaging properties in living cells. In a typical experiment, 25 μM of D-luciferin or the three “caged” substrates were incubated with C6 glioma cells for 1 h. There were no significant fluorescence signals observed in the cells loaded with D-luciferin, indicating that luciferin itself crossed the cell membranes with low efficiency (Fig. 3A and D). Before photoactivation, the three “caged” luciferin derivatives showed very weak fluorescence signals in the cells due to quenching of the single excited state by a photoinduced electron-transfer (PET) process (Fig. 3E and ESI[†]).⁷ However, after 60 s continuous low dose of UV irradiation, the “caged” luciferin derivatives were photolyzed and imaging experiments revealed the bright fluorescence signals inside the cytoplasm of cells. Among the different derivatives, NPE-caged luciferin (3c) demonstrated the highest fluorescence activation under the comparable conditions (Fig. 3C and F). There was no obvious cytotoxicity observed in the process of fluorescence measurements, demonstrating that the rapid fluorescence enhancement upon brief UV irradiation did not induce significant cellular damage (see ESI[†]). The cellular imaging studies indicated clearly the good cell membrane permeation and photocleavable activities of these “caged” substrates in living cells.

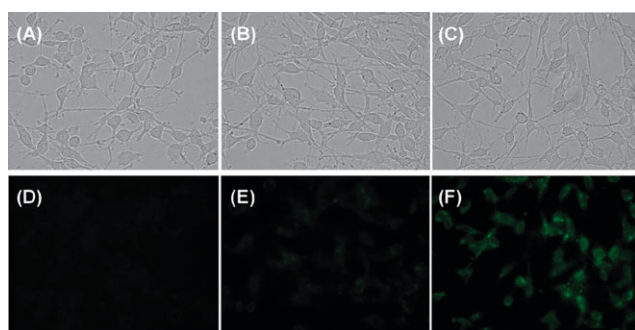


Fig. 3 Fluorescence imaging of C6 glioma cells loaded with (A), (D); D-luciferin only (25 μ M); (B), (E); NPE-luciferin (**3c**) only (25 μ M) but no UV excitation; (C), (F); NPE-luciferin (**3c**) (25 μ M) and followed by 1 min UV excitation. Excitation filter: 460/40 nm; emission filter: 535/50 nm.

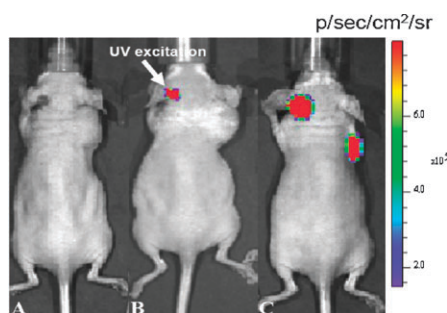


Fig. 4 Imaging of fLuc activity in living mice ($n = 4$). The tumors were implanted in mice by injection of C6-fLuc cells in the left ear and right shoulder. (A) 3 mg of **3c** injection without UV excitation; (B) 3 mg of **3c** injection and 4 min UV excitation of left ear tumor only; (C). 3 mg of D-luciferin injection.

Finally, we also applied the “caged” luciferins for *in vivo* imaging measurements. All experiments with live animals were approved by the Stanford University Administrative Panel on Laboratory Animal Care. In a typical experiment, we injected 1×10^5 of C6 cells stably transfected with fLuc into the left ear and 8×10^5 cells into the right shoulder of a nude mouse. After ten days tumor implantation, the mice were intraperitoneally injected with 3 mg of NPE-caged luciferin (**3c**) for bioluminescent imaging. As shown in Fig. 4, there was no emission signal observed in the mouse without UV excitation, indicating the enzyme activity was blocked by the “caged” substrate. A strong emission from the left ear was detected in the mouse upon substrate **3c** injection and UV illumination of the left ear C6-fLuc tumor (Fig. 4B). The emission peaked at 20 min post-injection and then gradually decreased over 1 h, whereas no bioluminescent signal was observed in the right shoulder tumor because no UV excitation was applied. In comparison, the same amount of D-luciferin was injected into a mouse and significant emission was observed in both left ear and right shoulder tumors (Fig. 4C), indicating that the fLuc activities of both tumors were similar and the observed contrast was not due to different activity in each tumor. These results confirmed that

uncaging luciferin by photolysis might work as an efficient bioluminescent probe for tracking the dynamics of the luciferase expression in living animals.

In conclusion, this work presents the design and evaluation of new photoactivable bioluminescent substrates for imaging the luciferase activity in buffers, cells and living animals. These stable, less toxic and cell permeable “caged” luciferin derivatives display rapid photorelease of D-luciferin and confer robust fluorescent and bioluminescent signals with minimum background after brief UV irradiation. We expect that these novel “caged” firefly luciferase substrates will offer new opportunities for real-time monitoring the dynamics of the cellular functions *in vitro* and *in vivo*.

The authors gratefully acknowledge URC (RG56/06), A*Star BMRC (07/1/22/19/534) and SEP (RG139/06) grants in Nanyang Technological University, Singapore. NCI Small Animal Imaging Resource Program (SAIRP) grant R24 CA93862, and NCI *in vivo* Cellular Molecular Imaging Center (ICMIC) grant P50 CA114747, USA. We also thank Prof. Ken-Shiung Chen, in the School of Biological Sciences, NTU, for his luminometer facility.

Notes and references

- (a) C. H. Contag, *Mol. Microbiol.*, 1995, **18**, 593; (b) O. Shimomura, *Bioluminescence: Chemical Principles and Methods*, World Scientific, NJ, 2006, pp. 1–29; (c) F. McCapra, *Methods Enzymol.*, 2000, **305**, 3.
- (a) C. H. Contag and M. H. Bachmann, *Annu. Rev. Biomed. Eng.*, 2002, **4**, 235; (b) A. M. Leoning, A. M. Wu and S. S. Gambhir, *Nat. Methods*, 2007, **4**, 641; (c) P. Ray and S. S. Gambhir, *Methods Mol. Biol.*, 2007, **411**, 131.
- (a) T. S. Wehrman, G. Von Degenfeld, P. O. Krutzik, G. P. Nolan and H. M. Blau, *Nat. Methods*, 2006, **3**, 295; (b) K. Shah, C. H. Tung, X. O. Breakefield and R. Weissleder, *Mol. Ther.*, 2005, **11**, 928; (c) W. H. Zhou, M. P. Valley, J. Shultz, E. M. Hawkins, L. Bernad, T. Good, D. Good, T. L. Riss, D. H. Klaubert and K. V. Wood, *J. Am. Chem. Soc.*, 2006, **128**, 3122; (d) H. Q. Yao, M. K. So and J. H. Rao, *Angew. Chem., Int. Ed.*, 2007, **46**, 7031; (e) B. G. Xing, J. H. Rao and R. R. Liu, *Mini-Rev. Med. Chem.*, 2008, **8**, 455.
- (a) D. M. Rothman, M. D. Shults and B. Imperiali, *Trends Cell Biol.*, 2005, **15**, 202; (b) D. S. Laurence, *Curr. Opin. Chem. Biol.*, 2005, **9**, 570.
- (a) C. Orange, A. Specht, D. Puliti, E. Sakr, T. Furuta, B. Winsor and M. Goeldner, *Chem. Commun.*, 2008, 1217; (b) M. E. Hahn and T. M. Muir, *Trends Biochem. Sci.*, 2005, **30**, 26; (c) T. Kawakami, H. Cheng, S. Hashiro, Y. Nomura, S. Tsukiji, T. Furuta and T. Nagamune, *ChemBioChem*, 2008, **9**, 1583; (d) J. Ottl, D. Gabriel and G. Marriott, *Bioconjugate Chem.*, 1998, **2**, 143.
- (a) Y. M. Guo, S. W. Chen, P. Shetty, G. H. Zheng, R. L. Lin and W. H. Li, *Nat. Methods*, 2008, **5**, 835; (b) S. Shah, S. Rangarajan and S. H. Friedman, *Angew. Chem., Int. Ed.*, 2005, **44**, 1328; (c) X. J. Tang, S. Maegawa, E. S. Weinberg and I. Dmochowski, *J. Am. Chem. Soc.*, 2007, **129**, 11000; (d) I. A. Shestopalov, S. Sinha and J. K. Chen, *Nat. Chem. Biol.*, 2007, **3**, 650; (e) G. Han, T. Mokari, C. Ajo-Franklin and B. E. Cohen, *J. Am. Chem. Soc.*, 2008, **130**, 15811.
- (a) J. W. Yang and D. B. Thomason, *Biotechniques*, 1993, **15**, 848; (b) R. S. Givens and L. W. Kueper, *Chem. Rev.*, 1993, **66**, 55.
- (a) T. Kobayashi, Y. Urano, M. Kamiya, T. Ueno, H. Kojima and T. K. Nagano, *J. Am. Chem. Soc.*, 2007, **129**, 6696; (b) Y. R. Zhao, Q. Zheng, K. Dakin, K. Xu, M. L. Martinez and W. H. Li, *J. Am. Chem. Soc.*, 2004, **126**, 4653.