

## Photocontrolled Compound Release System Using Caged Antimicrobial Peptide

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Received March 15, 2010; E-mail: kkikuchi@mls.eng.osaka-u.ac.jp

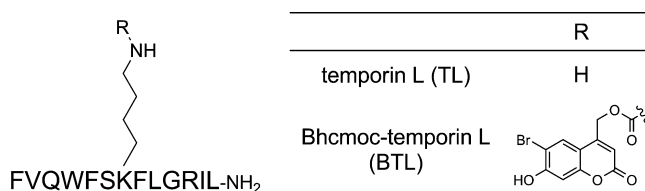
**Abstract:** A novel photocontrolled compound release system using liposomes and a caged antimicrobial peptide was developed. The caged antimicrobial peptide was activated by UV irradiation, resulting in the formation of pores on the liposome surface to release the contained fluorophores. The compound release could be observed using fluorescence measurements and time-lapse fluorescence microscopy. UV irradiation resulted in a quick release of the inclusion compounds (within 1 min in most cases) under simulated physiological conditions. The proposed system is expected to be applicable in a wide range of fields from cell biology to clinical sciences.

Caged compounds are photoactivatable probes that are biologically or functionally inert prior to their uncaging. Photoactivation of a caged compound enables the spatiotemporal regulation of various biomolecules of interest in living cells or tissues. Thus, photoactivation technologies using caged compounds have been used as powerful tools in recent biological studies. Various caged compounds such as  $\text{Ca}^{2+}$ , neurotransmitters, nucleotides, peptides, and enzymes have been reported thus far.<sup>1</sup> Caged compounds provide biologists with valuable tools for investigating biological phenomena. However, current caging/uncaging systems require the synthesis of individual caged compounds for each target biomolecule. Because such syntheses generally entail complicated and multistep reactions, the development of a new methodology is desired for enabling the widespread use of photoactivation technology that can be applicable to various biomolecules ranging from small molecules to macromolecules such as proteins and nucleic acids.

We presume that a nano- or microscale photodegradable “cage” can be used for this purpose. This photoactivation system is essentially similar to the controlled drug release systems investigated from a clinical perspective.<sup>2</sup> Among the various drug carriers, liposomes have been actively developed;<sup>3</sup> further, various types of photoinduced drug-releasing liposomes have been reported. Such functional liposomes generally consisted of designed lipids such as photoisomeric lipids<sup>4</sup> or photocaged lipids.<sup>5</sup> Very recently, photoinduced drug release systems using photocaged dendrons<sup>6</sup> or polymeric microcapsules functionalized with bacteriorhodopsin<sup>7</sup> have also been reported. Nevertheless, the development of a new and practical photocontrolled release system is still strongly required. In this paper, we report a novel photocontrolled release system that works on the basis of a strategy that is clearly different from previously known strategies.

In our proposed system, a photoresponsive drug carrier was divided into two parts: a drug carrier and a photoresponsive opener.

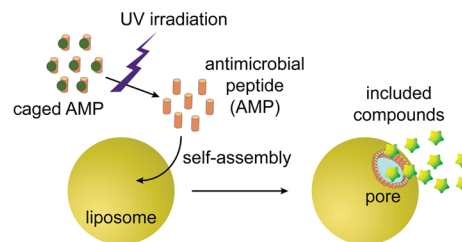
Chart 1. Structures of TL and BTL



We selected liposomes as the drug carriers because of their versatility and biocompatibility. While selecting the photoresponsive opener, we focused on the membrane-damaging properties of antimicrobial peptides (AMPs).<sup>8</sup> AMPs have attracted increasing attention as a new category of antibiotics. In natural systems, AMPs target the lipid bilayers of bacterial membranes and kill the bacteria by disrupting their membranes. AMPs also degrade liposomes whose compositions are similar to those of the bacterial membrane. Therefore, we assumed that this membrane-damaging property can be applied to construct a photocontrolled drug release system.

We designed a protected AMP derivative Bhcmoc-temporin L (BTL) (Chart 1). BTL consists of a short antimicrobial peptide temporin L (TL),<sup>8b,c</sup> which was isolated from the European red frog *Rana temporaria*, and a photoremovable 6-bromo-7-hydroxycoumarin-4-ylmethoxycarbonyl (Bhcmoc) group.<sup>9</sup> The Bhcmoc group was attached to the  $\epsilon$ -amino group of the Lys. The positively charged amino acid residues of AMPs play important roles in the bacterial membrane damaging properties of AMPs.<sup>8</sup> Thus, protective modification of the Lys in TL was expected to reduce its membrane damaging properties. It was assumed that after the protective group was removed using UV irradiation, its membrane damaging properties would be recovered. As shown in Figure 1, we expected that the combination of BTL and a liposome having the same lipid composition as the bacterial outer membranes would provide a UV-responsive drug release system.

BTL was synthesized in the following two steps: solid-phase Fmoc peptide synthesis and subsequent modification of the Bhcmoc group (Scheme S1 in the Supporting Information). All the synthetic procedures were performed on resins, and the final product was

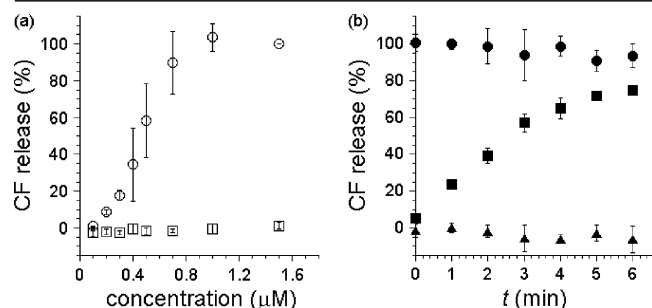


**Figure 1.** Schematic diagram of photocontrolled release system using caged antimicrobial peptide.

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**Figure 2.** CF release from LUVs induced by synthesized compounds. Lipid concentration: 2.5  $\mu\text{g/mL}$ . Error bars indicate the SD ( $n = 3$ ). (a) Correlation between CF release and concentration of compounds (circles: TL, squares: BTL) under dark conditions. (b) Correlation between CF release and UV irradiation time  $t$  in the presence of 4  $\mu\text{M}$  compounds (circles: TL, squares: BTL, triangles: none).

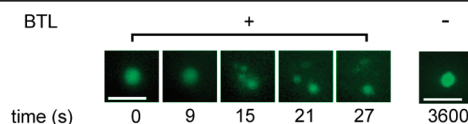
purified by reversed-phase HPLC after the cleavage from the resin and was identified by ESI-TOF MS.

The UV-light-induced conversion of BTL to TL was confirmed using HPLC analysis. Over 95% of BTL underwent conversion within 5 min of UV irradiation; simultaneously a few new peaks emerged in the HPLC analysis (Figure S1 in the Supporting Information). One of these new peaks was assigned to TL. Thus, it was confirmed that BTL was converted to TL using UV light.

Next, we examined whether or not the membrane-damaging property of TL was nullified by the modification of the Lys residue with the Bhcmoc group. Liposomes containing fluorescence dyes have been utilized as a standard tool for the evaluation of the membrane-damaging activities of AMPs and their mimics.<sup>10</sup> When the AMPs disrupt the lipid membrane of the liposomes, the dyes in the liposomes are released. We prepared large unilamellar vesicles (LUVs) and 5(6)-carboxyfluorescein (CF) as the encapsulated fluorescence dye. Since the fluorescence of CF in LUVs is partly quenched because of the high concentration effect, the collapse of the LUV membrane induces the increase in the fluorescence. A fraction of the release activity was estimated using this increase in the fluorescence. As shown in Figure 2a, addition of TL induced the release of CF with a concentration in the submicromolar range. In contrast, BTL did not induce the leakage of CF with a several micromolar concentration. These results indicate that the modification of the Bhcmoc group at the Lys of TL drastically reduced its membrane damaging property. In addition, CD spectra of TL and BTL showed that substitution at the Lys negatively affects the  $\alpha$ -helix formation in the presence of liposomes (Figure S2 in the Supporting Information).

Next, the photocontrolled release of CF from LUVs was examined by using BTL and applying UV irradiation. LUV solutions were irradiated using a UV lamp for various periods of time in the presence or absence of BTL. The lamp was then switched off for 60 s, after which the fluorescence intensities of the sample were measured. In the presence of BTL, a fraction of the CF release was dependent on the UV exposure time (Figure 2b). UV irradiation in the absence of BTL did not induce CF release. Thus, photocontrolled release was achieved by the appropriate adjustment of the BTL concentration and UV exposure time.

Finally, the applicability of the photocontrolled release system to fluorescence microscopic studies was examined. Giant unilamellar vesicles (GUVs) were selected for this purpose, because GUVs have larger diameters and the changes in their shape can be observed using optical microscopes. Prepared GUVs containing CF were loaded on a poly-L-lysine-coated glass-bottom dish and observed using a fluorescence microscopic imaging system. The fluorescence images were captured by the excitation of visible light ( $\lambda = 460\text{--}490\text{ nm}$ ). The



**Figure 3.** Fluorescence microscopic images of GUVs containing CF under UV irradiation in the presence (12.5  $\mu\text{M}$ ) or absence of BTL. Scale bar: 10  $\mu\text{m}$ .

GUVs were stable for several hours under the experimental conditions. When 12.5  $\mu\text{M}$  TL was added, the fluorescence of the GUVs completely disappeared within 40 s, although the addition of 12.5  $\mu\text{M}$  BTL did not induce the disruption of GUVs (data not shown). The GUVs were then irradiated by UV light ( $\lambda = 330\text{--}385\text{ nm}$ ) using a fluorescence microscope. In the absence of BTL, the GUVs were stable under the microscopic UV excitation. On the other hand, in the presence of BTL, leakage of the CF from the GUVs mostly with the membrane blebbing was observed within tens of seconds under the UV irradiation (Figure 3). In most cases, the leakage was completed within 1 min.

In conclusion, we developed a novel photocontrolled release system by combining liposomes and a caged antimicrobial peptide. This system was experimentally found to be effective for liposomes of different sizes. The response to UV irradiation was rapid, and the release was induced under simulated physiological conditions and also under the experimental conditions of time-lapse fluorescence microscopy. These data imply that this system could be applicable to fluorescence imaging for living cells or living animals. Because liposomes tolerate a wide variety of inclusion compounds from small molecules to macromolecules, this system could enable the development of a universal photouncaging system that might offer a breakthrough in biological studies. Further, in terms of clinical applications, this technology might be applied to other external stimuli such as specific enzyme activities in diseased tissues. This controlled release system may be useful in a wide range of research fields from cell biology to clinical medicine.

**Acknowledgment.** We thank Drs. Shigenori Kanaya and Yuichi Koga at Osaka University for the use of CD spectrometer. This work was supported in part by MEXT of Japan. S.M. thanks the Cosmetology Research Foundation.

**Supporting Information Available:** Detailed experimental procedures and supplementary results. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## References

- (1) Mayer, G.; Heckel, A. *Angew. Chem., Int. Ed.* **2006**, *45*, 4900–4921.
- (2) (a) Rapoport, N. *Prog. Polym. Sci.* **2007**, *32*, 962–990. (b) Shum, P.; Kim, J.-M.; Thompson, D. H. *Adv. Drug Delivery Rev.* **2001**, *53*, 273–284. (c) Chilkoti, A.; Dreher, M. R.; Meyer, D. E.; Raucher, D. *Adv. Drug Delivery Rev.* **2002**, *54*, 613–630. (d) Schmaljohann, D. *Adv. Drug Delivery Rev.* **2008**, *58*, 1655–1670.
- (3) Torchilin, V. P. *Nat. Rev. Drug Discov.* **2005**, *4*, 145–160.
- (4) (a) Bisby, R. H.; Mead, C.; Morgan, C. G. *Biochem. Biophys. Res. Commun.* **2000**, *276*, 169–173. (b) Liu, X.-M.; Yang, B.; Wang, Y. L.; Wang, J.-Y. *Chem. Mater.* **2005**, *17*, 2792–2795.
- (5) (a) Zhang, Z.-Y.; Smith, B. D. *Bioconjugate Chem.* **1999**, *10*, 1150–1152. (b) Watanabe, S.; Hiratsuka, R.; Kasai, Y.; Munakata, K.; Takahashi, Y.; Iwamura, M. *Tetrahedron* **2002**, *58*, 1685–1691. (c) Chandra, B.; Subramaniam, R.; Mallik, S.; Srivastava, D. K. *Org. Biomol. Chem.* **2006**, *4*, 1730–1740.
- (6) Park, C.; Lim, J.; Yun, M.; Kim, C. *Angew. Chem., Int. Ed.* **2008**, *47*, 2959–2963.
- (7) Erokhina, S.; Benassi, L.; Bianchini, P.; Diaspro, A.; Erokhin, V.; Fontana, M. P. *J. Am. Chem. Soc.* **2009**, *131*, 9800–9804.
- (8) (a) Zasloff, M. *Nature* **2002**, *415*, 389–395. (b) Mahalka, A. K.; Kinnunen, P. K. *J. Biochim. Biophys. Acta* **2009**, *1788*, 1600–1609. (c) Rinaldi, A. C.; Mangoni, M. L.; Rufo, A.; Luzi, C.; Barra, D.; Zhao, H.; Kinnunen, P. K. J.; Bozzi, A.; Giulio, A. D.; Simmaco, M. *Biochem. J.* **2002**, *368*, 91–100.
- (9) (a) Furuta, T.; Wang, S. S.; Dantzer, J. L.; Dore, T. M.; Bybee, W. J.; Callaway, E. M.; Denk, W.; Tsien, R. Y. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 1193–1200. (b) Ando, H.; Furuta, T.; Tsien, R. Y.; Okamoto, H. *Nat. Genet.* **2001**, *28*, 317–325. (c) Furuta, T.; Watanabe, T.; Tanabe, S.; Sakyo, J.; Matsuda, C. *Org. Lett.* **2007**, *9*, 4717–4720.
- (10) Tew, G. N.; Liu, D.; Chen, B.; Doerksen, R. J.; Kaplan, J.; Carroll, P. J.; Klein, M. L.; DeGrado, W. F. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 5110–5114.

JA102167M