ORGANIC LETTERS

2008
Vol. 10, No. 18
4077−4080

Preparation of Highly Photosensitizing Liposomes with Fullerene-Doped Lipid Bilayer Using Dispersion-Controllable Molecular Exchange Reactions

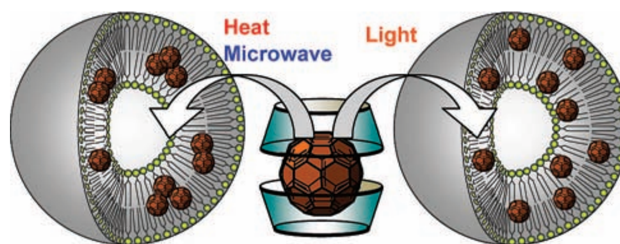
Atsushi Ikeda,*,† Tatsuya Sue,† Motofusa Akiyama,† Katsuyoshi Fujioka,† Tamami Shigematsu,† Yuki Doi,† Jun-ichi Kikuchi,† Toshifumi Konishi,‡,§ and Riichiro Nakajima||

Graduate School of Materials Science, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma 630-0192, Japan, Department of Chemistry, College of Engineering, Shibaura Institute of Technology, 307 Fukasaku, Minuma-ku, Saitama-shi, Saitama 337-8570, Japan, RIKEN (The Institute of Physical and Chemical Research), 2-1 Hirosawa, Wako, Saitama 351-0198, Japan, and Department of Chemical Engineering and Materials Science, Faculty of Engineering, Doshisha University, Kyotanabe 610-0321, Japan

aikeda@ms.naist.jp

Received July 13, 2008

ABSTRACT



Fullerene-containing liposomes with high photosensitization ability were prepared. Disaggregated fullerenes were efficiently injected into the bilayer of liposomes by a phototriggered molecular exchange reaction. These liposomes showed far higher photoreactivity than liposomes thermally produced by heating and microwave irradiation. This result indicates that control of self-aggregation of fullerene leads to a high quantum yield for the photoreaction because of the suppression of self-quenching of photoexcited fullerenes.

Liposomes have been of great interest as materials for new medical treatments related to drug delivery systems (DDSs), such as photodynamic therapy (PDT) or gene therapy.[1−3] Liposomes have the biocompatibility advantages that the immune response, inflammatory response, and biochemical synthetic cost can be regulated. In many cases, highly photosensitizers (PSs) for PDT, e.g., porphyrin derivatives, have hydrophobic properties.[4] Liposomes can be expected to act as delivery agents for these drugs. However, it is sometimes difficult to prepare lipid membrane-incorporated

† Nara Institute of Science and Technology.
‡ Shibaura Institute of Technology.
§ RIKEN (The Institute of Physical and Chemical Research).
|| Doshisha University.

(1) For reviews, see: (a) Kaneda, Y. *Adv. Drug Delivery Rev.* **2000**, *43*, 197–205. (b) Torchilin, V. P. *Nat. Rev. Drug Discovery* **2005**, *4*, 145–160.

(2) For reviews, see: (a) Pass, H. I *J. Natl. Cancer Inst.* **1993**, *85*, 443–456. (b) Dougherty, T. J.; Gomer, C.; Henderson, B. W.; Jori, G.; Kessel, D.; Korbelik, M.; Moan, J.; Peng, Q *J. Natl. Cancer Inst.* **1998**, *90*, 889–905.

(3) For a review, see: Lang, K.; Mosinger, J.; Wagnerová, D. M. *Coord. Chem. Rev.* **2004**, *248*, 321–350.

(4) For reviews, see: (a) Derycke, A. S. L.; de Witte, P. A. M. *Adv. Drug Delivery Rev.* **2004**, *56*, 17–30. (b) Ebrahim, S.; Peyman, G. A.; Lee, P. J. *Surv. Ophthalmol.* **2005**, *50*, 167–182.

10.1021/ol8015918 CCC: $40.75 © 2008 American Chemical Society
Published on Web 08/16/2008

PSs (i.e., photosensitizing liposomes) by the Bangham method because hydrophobic PSs tend to form less photoreactive self-aggregates in lipid membranes.[5,6] Recently, we prepared liposomes with target affinity and very high photosensitizing ability by using molecular exchange.[7] However, a solution to the problem of self-aggregation was not found, and there was room for further improvement of the photofunction of the liposomes.

This preparation method of liposomes includes two steps: assembly of lipid bilayer vesicles that have the molecular target-directed lipid head groups[5] and transfer of sensitizer molecules into the bilayer of the vesicles by thermally induced molecular exchange. The latter step is effected through the medium of water-soluble host molecules. This method makes it possible to reduce synthesis costs and degradation of intrinsic properties of dyes accompanying chemical modifications. We have reported that photosensitizing liposomes consisting of lipids with cationic head groups show surface affinity to DNA (DNA).[7a] Cationic, fullerene-enriched liposomes (i.e., lipid membrane with incorporated C_{60} and C_{70}; $LMIC_{60}$ and $LMIC_{70}$) have shown highly efficient DNA photocleavage compared with conventional systems (for example, water-soluble cyclodextrin-bicapped fullerenes).[7a,c] Preparation of $LMIC_x$ (x = 60 and 70) takes place by thermal molecular exchange reactions from water-soluble cyclodextrin-bicapped fullerene to liposomes. Utilization of an unstable host−guest complex improves the fullerene concentration and sensitization abilities of liposomes because of promoted fullerene transfer reactions. This host−guest molecular transfer is expected to be applicable to other sensitizer systems.

Self-aggregated sensitizers show self-quenching of their photoexcited states, and the quantum yield for photoreactions decreases dramatically.[8,9] If this situation can be improved, the sensitization ability of liposomes is expected to be still further strengthened.

In this paper, we report the preparation of extremely photoactive liposomes obtained by dispersion-controllable fullerene-exchange reactions. In the molecular exchange reaction between cyclodextrin-bicapped fullerene[10] and liposomes, optimization of fullerene dispersion was performed by using for the first time two types of "triggers" instead of heating (Scheme S1, Supporting Information):[6a] (i) microwave irradiation, and (ii) photoinduced electron transfer. Subsequently, quantitative photocleavage experiments of DNA were conducted with $LMIC_{60}$ obtained using each trigger.

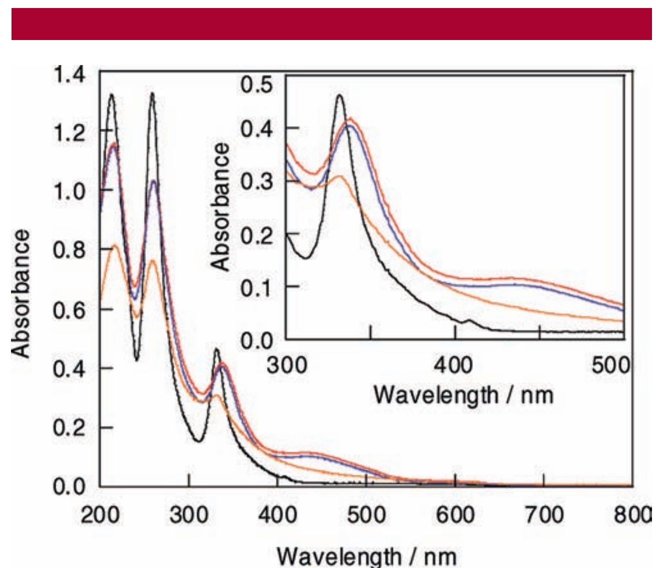
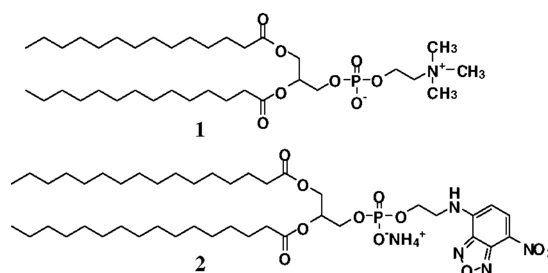


Figure 1. UV−vis absorption spectral change of the C_{60}•γ-CDx complex upon addition of **1** (a) before (black line) and (b) after heating (red line) at 80 °C for 2 h or (c) after microwave irradiation (blue line) for 7 s and (d) without **1** after heating (orange line) at 80 °C for 2 h (1 mm cell).



We first employed microwave irradiation as a trigger to accelerate the molecular exchange reaction. $LMIC_{60}$ formation by microwave irradiation was completed in only 7 s. $LMIC_{60}$ obtained by heating and by microwave irradiation gave the same absorption spectrum (Figure 1). That suggests that the transferred C_{60} made by microwave irradiation was in a microenvironment identical to that of the transferred C_{60} made by the heating method. A red shift and peak broadening of the 337 nm absorption band were observed and a new broad absorption appeared in the 400−550 nm region, implying that C_{60} was excluded from two γ-CDx cavities and C_{60} aggregated by itself (Figure 1). However, in the absence of liposomes, the application of heat to C_{60}•γ-CDx solution brought about another spectral change. The

(5) Bangham, A. D.; Standish, M. M.; Watkins, J. C. *J. Mol. Biol.* **1965**, *13*, 238–252.

(6) Several types of $LMIC_{60}$ have been also prepared using the Bangham method, but the concentrations of [C_{60}]/[lipids] were very low. (a) Hungerbühler, H.; Guldi, D. M.; Asmus, K.-D. *J. Am. Chem. Soc.* **1993**, *115*, 3386–3387. (b) Bensasson, R. V.; Bienvenue, E.; Dellinger, M.; Leach, S.; Seta, P. *J. Phys. Chem.* **1994**, *98*, 3492–3500.

(7) (a) Ikeda, A.; Sato, T.; Kitamura, K.; Nishiguchi, K.; Sasaki, Y.; Kikuchi, J.; Ogawa, T.; Yogo, K.; Takeya, T. *Org. Biomol. Chem.* **2005**, *3*, 2907–2909. (b) Ikeda, A.; Doi, Y.; Nishiguchi, K.; Kitamura, K.; Hashizume, M.; Kikuchi, J.; Yogo, K.; Ogawa, T.; Takeya, T. *Org. Biomol. Chem.* **2007**, *5*, 1158–1160. (c) Ikeda, A.; Doi, Y.; Hashizume, M.; Kikuchi, J.; Konishi, T. *J. Am. Chem. Soc.* **2007**, *129*, 4140–4141.

(8) For reviews, see:(a) Boyle, R. W.; Dolphin, D. *Photochem. Photobiol.* **1996**, *64*, 469–485. (b) Takagi, S.; Eguchi, M.; Tryk, D. A.; Inoue, H. *J. Photochem. Photobiol. C: Photochem. Rev.* **2006**, *7*, 104–126.

(9) (a) Ikeda, A.; Hatano, T.; Kawaguchi, M.; Suenaga, H.; Shinkai, S. *Chem. Commun.* **1999**, 1403–1404. (b) Hatano, T.; Ikeda, A.; Akiyama, T.; Yamada, S.; Sano, M.; Kanekiyo, Y.; Shinkai, S. *J. Chem. Soc., Perkin Trans. 2* **2000**, 909–912. (c) Ikeda, A.; Hatano, T.; Shinkai, S.; Akiyama, T.; Yamada, S. *J. Am. Chem. Soc.* **2001**, *123*, 4855–4856. (d) Ikeda, A.; Hatano, T.; Konishi, T.; Kikuchi, J.; Shinkai, S. *Tetrahedron* **2003**, *59*, 3537–3540. (e) Konishi, T.; Ikeda, A.; Asai, M.; Hatano, T.; Shinkai, S.; Fujitsuka, M.; Ito, O.; Tsuchiya, Y.; Kikuchi, J. *J. Phys. Chem. B* **2003**, *107*, 11261–11266. (f) Ikeda, A.; Ejima, A.; Nishiguchi, K.; Kikuchi, J.; Matsumoto, T.; Hatano, T.; Shinkai, S.; Goto, M. *Chem. Lett.* **2005**, *34*, 308–309.

(10) Komatsu, K.; Fujiwara, K.; Murata, Y.; Braun, T. *J. Chem. Soc., Perkin Trans. 1* **1999**, 2963–2966.

peak broadening at 337 nm was observed, but the red shift and broad absorption in the 400−550 nm region were not clearly seen (Figure 1). This observation suggests that the types of C_{60} aggregation depend on microenvironments such as a γ-CD cavity and a lipid bilayer. The complete transfer of C_{60} from two γ-CDx cavities to lipid membrane was confirmed by the disappearance of the ^{1}H NMR peaks assignable to the C_{60}•γ-CDx bicapped complex (Figure S1a,d, Supporting Information: 4.19 and 5.05 ppm). Furthermore, the ^{1}H NMR peak of self-aggregated C_{60}•γ-CDx complex was not observed (Figure S1b, Supporting Information: 5.41 ppm). The ^{1}H NMR spectrum of LMIC$_{60}$ obtained by microwave irradiation (Figure S1d, Supporting Information) is also similar to that for LMIC$_{60}$ obtained by the heating method (Figure S1c, Supporting Information).

The hydrodynamic diameters of the liposomes were measured by a dynamic light scattering spectrophotometer (DLS). The average diameters of liposomes before and after 7 s microwave irradiation (72 and 73 nm, respectively) were not significantly different (Table S1, Supporting Information). However, a longer duration of microwave irradiation (<20 s) led to an increase in the average diameters of liposomes from 72 to 151 nm because of heating by microwave irradiation over 90 °C. These findings revealed that LMIC$_{60}$ can be easily obtained by short-time microwave irradiation. However, C_{60} forms self-aggregates in bilayer as in the case of the heating method.

Second, photoinduced electron transfer was used as a trigger for the molecular exchange reaction. In the absence of β-nicotinamide adenine dinucleotide (NADH), UV−vis absorption spectra of C_{60}•γ-CDx and lipid 1 show no significant change during photoirradiation, indicating that the exchange reaction of C_{60} occurred to an insignificant extent (Figure S2, Supporting Information). This finding shows that (i) γ-CDx can maintain the inclusion phenomena toward a photogenerated singlet excited state ($^1C_{60}$*) and a triplet state ($^3C_{60}$*) and (ii) local evolution of heat accompanying internal conversion of photoexcited C_{60} does not cause destruction of C_{60}•γ-CDx complex. In the presence of NADH as reductant, the absorption band at 334 nm became slightly less intense with photoirradiation because of the disappearance of the absorption of NADH as a result of its decomposition after photoinduced electron transfer (Figure 2b). The spectrum of the magenta solution indicated that no C_{60} derivatives (e.g., $C_{60}O$) were generated. Figure S1e (Supporting Information) shows that the ^{1}H NMR signal of self-aggregated C_{60}•γ-CDx complex (5.41 ppm) did not appear and the peaks assignable to the C_{60}•γ-CDx complex (4.19 and 5.05 ppm) disappeared. These findings strongly imply that NADH reduced $^3C_{60}$* to $C_{60}^{\bullet -}$,[11] $C_{60}^{\bullet -}$ was released from two γ-CDx cavities, and then $C_{60}^{\bullet -}$ was immediately returned to neutral state by dissolved O_2 (Scheme S2, Supporting Information).[12b] To confirm the photoinduced C_{60} transfer into lipid membranes, we measured fluorescence quenching of **2** by C_{60} transferred into lipid membrane incorporating fluorochromes (LMIF). The LMIF was pre-

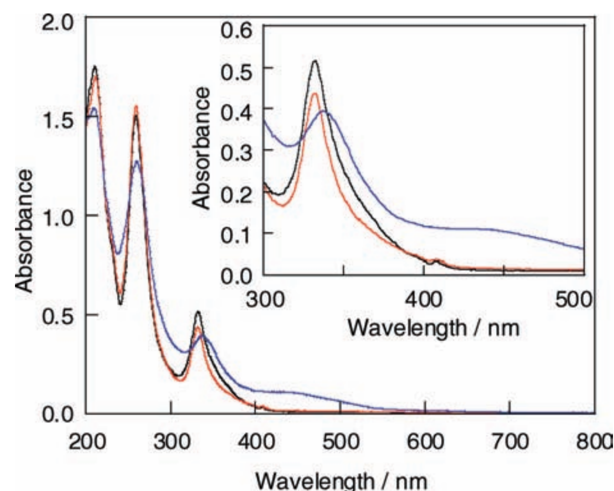


Figure 2. UV−vis absorption spectral change of the C_{60}•γ-CDx complex upon addition of **1** and NADH (a) before (black line) and (b) after photoirradiation for 6 h (red line) and (c) after photoirradiation for 6 h and heating at 80 °C for 2 h (blue line) (1 mm cell).

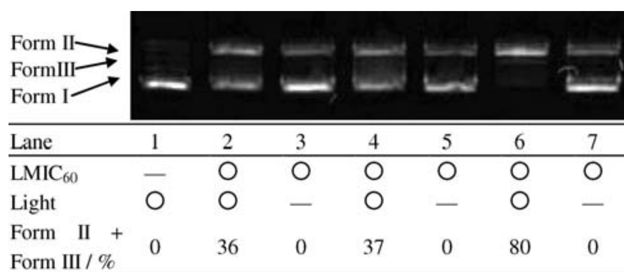
pared using lipids containing 0.5 mol% of **2**. Quenching of photoexcited **2** by photochemically transferred C_{60} was clearly observed as in the case of LMIF−C_{60} system obtained by the heating method (Figure S3b, Supporting Information). Before the phototriggered molecular exchange reaction, no fluorescence quenching of **2** was observed because C_{60} exists as a γ-CDx complex in water. On the other hand, after photoirradiation for 6 h, the exchange reaction of C_{60} results in about 50% fluorescence quenching. The implication is that C_{60} was transferred from two γ-CDx cavities to lipid membranes containing **2** to yield vesicle-incorporated C_{60}. To elucidate the details of the fluorescence quenching, a reference sample was prepared by addition of **2** into LMIC$_{60}$ following the photoinduced introduction of C_{60}. The sample also showed ca. 50% fluorescence quenching of photoexcited **2** (Figure S3b, Supporting Information). That observation indicates that the quenching is not based on photodecomposition of **2**. In the absence of lipids, the C_{60}•γ-CDx complex decomposed, and C_{60} formed a self-aggregated structure by photoirradiation in the presence of NADH (Figure S4, Supporting Information).

When the LMIC$_{60}$ that was obtained by light illumination was held at 80 °C for 1 h, the UV−vis absorption spectrum became similar to that of LMIC$_{60}$ obtained by the heating or microwave methods (Figure 2c). Consequently, C_{60} in LMIC$_{60}$ that was obtained by light illumination existed in an isolated (i.e., disaggregated) state (Scheme S3, Supporting Information).

Finally, we evaluated photoreactivities of a series of LMIC$_{60}$'s formed with each trigger. Supercoiled plasmid

(11) Fukuzumi, S.; Suenobu, T.; Patz, M.; Hirasaka, T.; Itoh, S.; Fujitsuka, M.; Ito, O. *J. Am. Chem. Soc.* **1998**, *120*, 8060–8068.

(12) NADH was added at relatively high concentration in following papers ([NADH] = 10 mM). (a) Yamakoshi, Y.; Sueyoshi, S.; Fukuhara, K.; Miyata, N.; Masumizu, T.; Kohno, M *J. Am. Chem. Soc.* **1999**, *120*, 12363–12364. (b) Yamakoshi, Y.; Umezawa, N.; Ryu, A.; Arakane, K.; Miyata, N.; Goda, Y.; Masumizu, T.; Nagano, T. *J. Am. Chem. Soc.* **2003**, *125*, 12803–12809.



Lane	1	2	3	4	5	6	7
$LMIC_{60}$	—	○	○	○	○	○	○
Light	○	○	—	○	—	○	—
Form II + Form III / %	0	36	0	37	0	80	0

Figure 3. Agarose gel electrophoretic patterns of DNA nicked by $LMIC_{60}$ prepared by heating, microwave irradiation, and photoirradiation. Reaction samples contained 1.9 mg L^{-1} of ColE1 supercoiled plasmid. Lane 1: distilled water containing no chemicals. Lanes 2−7: 273 μM of lipids and 27 μM of C_{60}. Lanes 1, 2, 4, and 6: incubated under visible-light irradiation at a distance of 10 cm using a 500 W Xe-arc lamp (UI-502Q; Ushio, Inc.) at 25 °C for 80 min under aerobic conditions. Lanes 3, 5, and 7: incubated in the dark for 80 min under aerobic conditions. Lanes 2 and 3: $LMIC_{60}$ prepared by heating. Lanes 4 and 5: $LMIC_{60}$ prepared by microwave irradiation. Lanes 6 and 7: $LMIC_{60}$ prepared by photoirradiation. After addition of loading buffer (Wako Pure Chemical Industries Ltd.), electrophoresis was performed using a 0.9% agarose gel. The gel was stained with SYBR Gold (1:10000 dilution of stock supplied by Molecular Probes Inc., Eugene, OR) and viewed on a UV transilluminator. This experiment was performed at least three times; the reported values of photocleavage efficiency are averages of the separate runs.

DNA, ColE1 was not cleaved under visible light irradiation in the absence of $LMIC_{60}$ (Figure 3, lane 1) or in the dark in the presence of $LMIC_{60}$ (Figure 3, lanes 3, 5, and 7).

Under visible-light irradiation (λ_{ex} >350 nm) for 80 min, $LMIC_{60}$ showed distinct DNA-cleaving activity (lanes 2, 4, and 6). In lanes 2 and 4, about 36 and 37% of supercoiled DNA (form I) was converted to nicked DNA (form II) and linear DNA (form III) because of photoirradiation of $LMIC_{60}$ prepared by the heating (lane 2) and microwave (lane 4) methods. Furthermore, in lane 6, $LMIC_{60}$ prepared with photoirradiation showed markedly higher photocleaving performance (80%). Figures S5−S8 (Supporting Information) show photocleavage of DNA by $LMIC_{60}$'s as a function of irradiation time. This reaction curve also shows that quantitative DNA conversion can be achieved by sufficient illumination of $LMIC_{60}$ prepared by photoirradiation. We suggest two possible explanations for the difference in photoactivities of $LMIC_{60}$ by focusing on the three methods of production. (i) Trace amounts of NADH[12] remained in the $LMIC_{60}$ solution prepared by photoirradiation. Photocleavage of DNA is efficiently promoted by $O_2^{\bullet-}$, which was generated by photoinduced electron transfer from NADH to $^3C_{60}^*$ and then from $C_{60}^{\bullet-}$ to $^1O_2$.[13] (ii) The difference arises from the isolation of C_{60} in $LMIC_{60}$ prepared by photoirradiation. In the isolated C_{60} system, the self-quenching of photoexcited C_{60}'s ($^{1,3}C_{60}^*$) is remarkably suppressed, and therefore, the long-lived photoactivated C_{60}s are able to generate.[9d,e] If explanation (i) is correct, the photocleaving activity of $LMIC_{60}$ prepared by heating should improve with addition of NADH (0.13 mM in the solution of $LMIC_{60}$; final concentration 35 μM). However, the photocleaving activity was not influenced by the addition of NADH (28 and 29% without and with NADH, respectively; under visible-light irradiation for 1 h; Figure S9, lanes 2 and 4, Supporting Information).[12] To test explanation (ii), we compared the photoactivity of isolated C_{60} and aggregated C_{60} in $LMIC_{60}$. Two types of $LMIC_{60}$ were prepared, using the photoirradiation method (giving isolated C_{60}) and heating after photoirradiation (giving aggregated C_{60}). $LMIC_{60}$ prepared by heating after photoirradiation showed inferior photocleaving performance (29%; Figure S9, lane 4, Supporting Information) to that of $LMIC_{60}$ prepared without heating (80%; Figure 3, lane 6). These findings strongly support explanation (ii).

In summary, this study revealed that efficient implantation of fullerenes in the liposome bilayer was triggered by microwave irradiation and photoinduced electron transfer. With the former method, $LMIC_{60}$ was prepared in a short period of time, while the latter method produced extremely highly photosensitizing liposomes with isolated C_{60}. The isolation of C_{60} resulted in dramatic improvements in the performance of liposomes. The DNA photocleavage ability of the improved $LMIC_{60}$ is twice that of $LMIC_{60}$ obtained by thermal triggers. Three photochemical strategies exploited the full potential of photosensitizing liposomes. (i) A surface affinity for the target molecule (DNA), (ii) sufficient optical density in the visible region (high C_{60} concentration), and (iii) efficient photoinduced reactions derived from the isolation of sensitizer (C_{60}). The photosensitizing molecular assembly fulfilled all of the necessary conditions for photochemistry in solution or tissue. These findings have important implications for various applications in biological, medicinal, and materials chemistry because miscellaneous sensitizer-incorporated liposomes can be prepared easily using the phototriggered molecular exchange reactions. Applications of these systems using other sensitizers are being studied in our laboratories.

(13) After photoirradiation for 6 h, no NADH was detected in the ^1H NMR spectrum.

Acknowledgment. This work was supported by Grants-in-Aid for Scientific Research (B) 20310133 from the Japan Society for the Promotion of Science (JSPS).

Supporting Information Available: Detailed descriptions of experimental procedures and Supporting Figures (S1−S9), Schemes (S1−S3), and Table S1. This material is available free of charge via the Internet at http://pubs.acs.org.

OL8015918