Supramolecular Nanocarrier of Anionic Dendrimer Porphyrins with Cationic Block Copolymers Modified with Polyethylene Glycol to Enhance Intracellular Photodynamic Efficacy

ARTICLE *in* ANGEWANDTE CHEMIE INTERNATIONAL EDITION · FEBRUARY 2005

Impact Factor: 11.26 · DOI: 10.1002/anie.200461603 · Source: PubMed

11 AUTHORS, INCLUDING:

Woo-Dong Jang
Yonsei University
82 PUBLICATIONS **2,660** CITATIONS
SEE PROFILE

Atsushi Harada
Osaka Prefecture University
131 PUBLICATIONS **9,802** CITATIONS
SEE PROFILE

Donglin Jiang
Japan Advanced Institute of Science and T…
113 PUBLICATIONS **4,920** CITATIONS
SEE PROFILE

Kazunori Kataoka
The University of Tokyo
688 PUBLICATIONS **37,549** CITATIONS
SEE PROFILE

Drug Delivery

Supramolecular Nanocarrier of Anionic Dendrimer Porphyrins with Cationic Block Copolymers Modified with Polyethylene Glycol to Enhance Intracellular Photodynamic Efficacy**

*Woo-Dong Jang, Nobuhiro Nishiyama, Guo-Dong Zhang, Atsushi Harada, Dong-Lin Jiang, Satoko Kawauchi, Yuji Morimoto, Makoto Kikuchi, Hiroyuki Koyama, Takuzo Aida, and Kazunori Kataoka**

A great number of challenges have been overcome to create efficient photosensitizers (PSs) for photodynamic therapy (PDT) which have a high photocytotoxicity and selectivity for

[*] Dr. W.-D. Jang, Dr. N. Nishiyama, Dr. G.-D. Zhang, Dr. A. Harada, Prof. Dr. K. Kataoka
Department of Materials Science and Engineering
Graduate School of Engineering, The University of Tokyo
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656 (Japan)
Fax: (+81) 3-5841-7139
E-mail: kataoka@bmw.t.u-tokyo.ac.jp

Dr. D.-L. Jiang, Prof. Dr. T. Aida
Department of Chemistry and Biotechnology
Graduate School of Engineering, The University of Tokyo
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656 (Japan)

Dr. W.-D. Jang, Dr. G.-D. Zhang, Dr. A. Harada, Prof. Dr. K. Kataoka
CREST
Japan Science and Technology Corporation (Japan)

Dr. N. Nishiyama, Dr. H. Koyama
Department of Clinical Vascular Regeneration
Graduate School of Medicine, The University of Tokyo
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655 (Japan)

Dr. S. Kawauchi, Dr. Y. Morimoto, Prof. Dr. M. Kikuchi
Department of Medical Engineering
National Defense Medical College
3-2 Namiki, Tokorozawa, Saitama, 359-8513 (Japan)

[**] This work was supported by Core Research for Evolutional Science and Technology (CREST), JST.

 DOI: 10.1002/anie.200461603

the diseased tissue.[1] To obtain high quantum yields and effective energy absorption, PSs generally need to have large π-conjugation domains such as a porphyrin structure. Therefore, most conventional PSs easily form aggregates, which produce a self-quenching effect of the excited state, in aqueous medium as a result of their π–π interactions and hydrophobic characteristics. These issues can be overcome, as we reported previously, by segregating PSs into the focal core of dendrimers (dendrimer porphyrins (DPs), Figure 1b).[2] DPs are attractive for biomedical purposes because of their predictable structures, that is, their monodisperse molecular weight and tunable three-dimensional structures, and their flexibility for a high density of tailored functional groups on the periphery.[3] Indeed, third-generation DPs with 32 cationic or anionic peripheral groups exhibit a high solubility in aqueous medium and have a high quantum yield for the generation of singlet oxygen, which leads to an appreciable photocytotoxicity.[2] These advantageous features of DPs are facilitated to an even greater extent by their inclusion into stealth nanocarriers, thus improving their longevity in blood circulation and results in their gradual accumulation in solid tumors through the enhanced permeation and retention (EPR) effect.[4] Furthermore, as demonstrated here, the inclusion of DPs into a novel type of nanocarrier, that is, polymeric micelles, has led to an unprecedented increase in the photocytotoxicity without compromising either the photophysical properties of DPs in regard to their efficient photochemical reactions or the physicochemical properties of the carriers necessary for tumor-selective delivery.

A novel polymeric micelle system[5] for PDT is based on an electrostatic assembly of an anionic DP,[6] which consists of zinc porphyrin at the focal core with a third generation of poly(benzyl ether) dendritic frameworks having 32 negative charges on the periphery, and poly(ethylene glycol)–poly(L-lysine) block copolymer (PEG-*b*-PLL) in aqueous media (polyion complex (PIC) micelles; Figure 1b).[7] The DP-incorporated micelles (DP/m), prepared with a stoichiometric ratio of negatively charged DP and positively charged PEG-*b*-PLL, were approximately 64 nm in diameter with an extremely narrow size distribution in physiological saline solution (Figure 2a). Our previous study using static light scattering (SLS) measurements demonstrated that an individual DP/m contains an average of 38 DP molecules and the micelles have a remarkable stability against salt concentrations,[7] which indicates the clear stabilization effect by the 32 negative charges of DP in the micellar structure. The dependency of the formation of DP/m on the pH value was investigated by dynamic and static light scattering (DLS and SLS) measurements. Figure 2b shows the pH-dependent changes in the translational diffusion coefficient (D_T) and normalized $(Kc/\Delta R(0))^{-1}$ (normalized to the micelle at pH 7.4) of DP/m, where D_T is related to the hydrodynamic size based on the Stokes–Einstein equation, and the normalized $(Kc/\Delta R(0))^{-1}$ value is related to the changes in the average apparent molecular weight of the micelles. Both the hydrodynamic size and normalized $(Kc/\Delta R(0))^{-1}$ value basically remained unchanged in the pH range from 6.4 to 8.5 (Figure 2b). However, the diameter of the micelles gradually

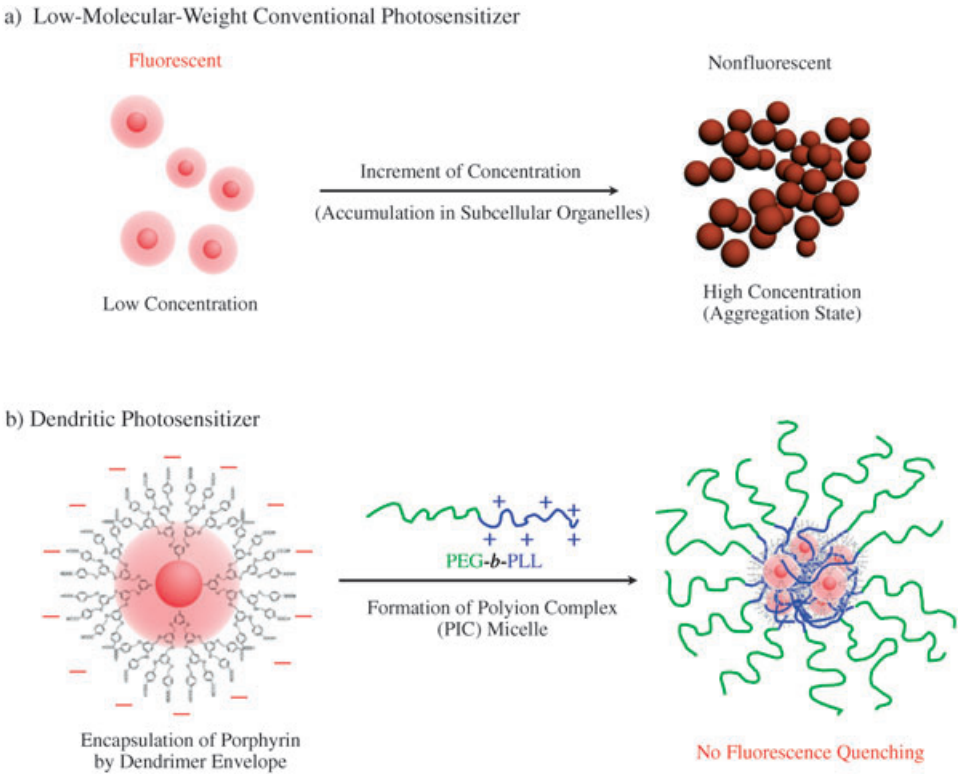

Figure 1. a) Conventional PS aggregate at a high concentration which results in quenching of PSs. b) Formation of polyion complex (PIC) micelles through electrostatic assembly of anionic dendrimer porphyrins (DPs) and PEG-*b*-PLL copolymers. The dendrimer envelope of DP can sterically prevent aggregation of the center porphyrin, thus there is no fluorescence quenching of the center porphyrin.

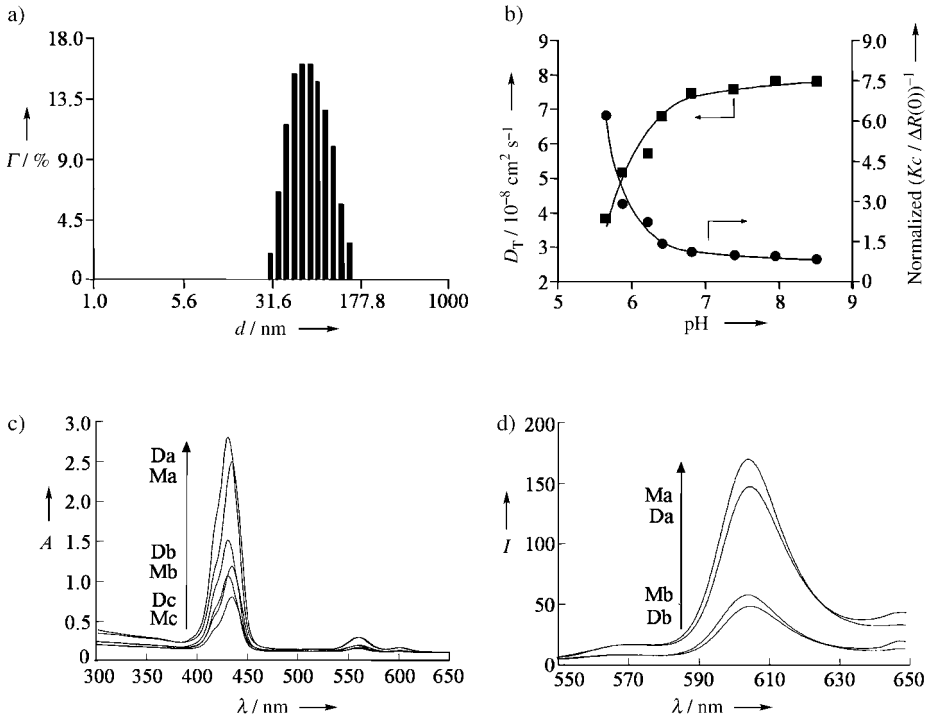

Figure 2. Physical properties of DP and DP/m. a) DLS histogram of DP/m in 150 mM NaCl at 25 °C; d = diameter. b) Dependency of the translational diffusion coefficients D_T (■) and normalized (Kc/$\Delta R(0)$)$^{-1}$ (●) on the pH value for DP/m (1 mg mL^{-1}), measured by DLS and SLS, respectively, at 25 °C. c) Electronic absorption spectra of DP alone and DP/m in PBS (pH 7.4; a = 12 μM, b = 4 μM, c = 2 μM, D: DP, M: DP/m). d) Fluorescence emission spectra of DP alone and DP/m in PBS (pH 7.4; a = 12 μM, b = 4 μM, D: DP, M: DP/m).

increased with an increased apparent molecular weight below pH 6.4 (Figure 2b), and finally precipitated at pH 5.6, which indicates the acid-responsive feature of the micelles. Protonation of DP occurred under acidic pH conditions and resulted in the diminution of the electrostatic interaction between DP and PEG-*b*-PLL. Thus, the well-defined core–shell structure may become more diffuse and a merging of the micelles may take place. This pH-responsive behavior of the micelles allows their effective accumulation in solid tumors in response to the low pH value of the tumor tissue[8] or in an endosomal compartment in the tumor cells while achieving stable circulation in the bloodstream.

The electronic absorption and emission spectra of DP and DP/m are shown in Figure 2c and d, respectively. Unlike low-molecular-weight PSs,[9] DP clearly maintained its absorption and emission intensity in spite of the formation of micelles. The incorporation of DP into the micelles resulted in a 5-nm red-shift for the Soret band of the porphyrin core and a hypochromicity of about 5% (Figure 2c). Both of these effects are likely to be caused by the formation of an electrostatic assembly of charged porphyrins and oppositely charged compounds.[10] The shrinkage of the hydrophobic dendrimer frameworks, which arises from the relaxation of the charge repulsion of the negatively charged DP surface by the formation of an electrostatic assembly, may contribute to the hypochromicity.[11] Interestingly, although the local concentration of DP within each micelle is assumed to be extremely high, DP/m emitted a more intense fluorescence at 610 nm (Figure 2d). Unlike conventional PSs, the dendritic envelope of DP is able to prevent the porphyrin core from undergoing collisional quenching, even at an appreciably high concentration that induces self-quenching of the conventional PSs (Figure 1a).[12] Thus, encapsulation of the porphyrins by the dendritic envelope in the micellar structure is most likely to prevent fluorescence quenching (Figures 1b and 2d). Also, the high microviscosity in the micellar core could restrain the internal molecular motion of DP, which might lead to the inhibition of the nonradiative decay and is related to the increased fluorescence intensity of DP/m (Figure 2d).[13] In connection with this observation, the photoinduced oxygen consumption of DP and DP/m was measured in phosphate-buffered saline (PBS) containing 10% fetal bovine serum (FBS) as a singlet oxygen acceptor (Figure 3). Very interestingly, the result revealed that the oxygen consumption level of

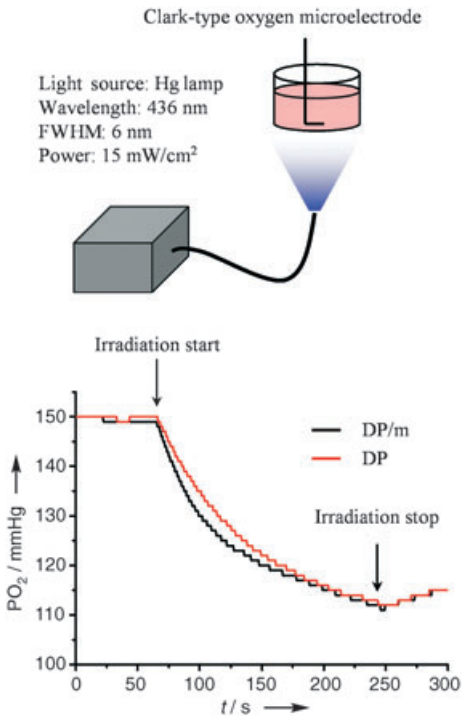

Figure 3. Experimental setup for the measurement of oxygen consumption and the results obtained. FWHM = full width of half maximum height.

DP/m was almost identical to that of the free DP in PBS, which indicates that the singlet oxygen molecules can successfully escape the micellar structure. From the standpoint of the application to PDT, the DP ensures an effective photochemical reaction of the porphyrin core regardless of the local concentrations. It is possible that the DP/m attain an elevated concentration of local singlet oxygen, which cannot be achieved by other formulations containing conventional PSs.

However, the cellular uptake of free DP and DP/m increased with the incubation time, and DP/m showed six- to eightfold higher uptake levels than free DP (Figure 4a). In

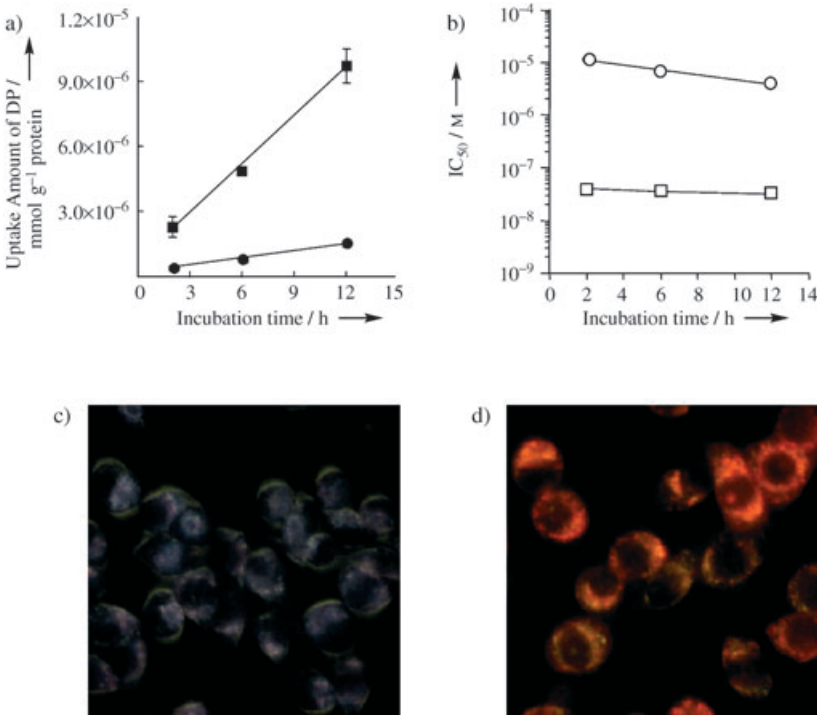

Figure 4. Results of in vitro evaluations of DP and DP/m. a) Cellular uptake level of DP (●) and DP/m (■) as a function of incubation time ($n=3$). LLC cells were incubated with dendrimers and the micelles at 12 μM of DP-equivalent concentration. b) Incubation time dependency of 50% growth inhibitory concentration (IC_{50}) of DP (○) and DP/m (□) after photoirradiation. c, d) Microscopy images of LLC cells incubated with 10 μM of DP (c) and DP/m (d) for 8 h. A Zeiss filter set (excitation: BP 395–440 nm; beam splitter: FT 460 nm; emission: LP 470 nm) was used.

view of the negatively charged surface of mammalian cells,[8] charge neutralization of DP by PEG-*b*-PLL could improve the cellular uptake of DP/m. The improved uptake of DP/m was also confirmed by microscopic observations (Figure 4c and d). No fluorescence quenching was observed for the cells incubated with DP/m, although the sensitizers are assumed to be extremely concentrated in the subcellular organelles such as the endosomes and lysosomes. This observation contrasts with the fact that the conventional mesochlorin e_6 conjugated *N*-(2-hydroxypropyl) methacrylamide (HPMA) copolymers exhibit fluorescence quenching when the cells are incubated with HPMA at an extremely high concentration.[14] The dendritic envelope of DP could prevent aggregation of the porphyrin in the subcellular loci, thus ensuring effective production of singlet oxygen for the photocytotoxicity.

Notably, the photocytotoxicity of DP/m was remarkably improved compared to that of free DP (Figure 4b). Incorporation of DP into the micelles resulted in an approximately 130–280-fold increased photocytotoxicity (Figure 4b). Such a distinctly enhanced photocytotoxicity of DP/m may not be fully explained by the six- to eightfold increase in their cellular uptake shown in Figure 4a; therefore, DP/m may have specific mechanisms to increase their photocytotoxicity. Recently, PEGylated chlorin e_6 and PEG-based polymeric micelles were reported to show an enhanced localization in several cytoplasmic organelles including the mitochondria.[15] Presumably the outer PEG layer of the DP/m and the microenvironment around DP, as mentioned above, may have a role in altering the intracellular mechanism of DP to increase the photocytotoxicity. Recently, several interesting observations concerning the intracellular mechanism have been reported. For example, Berg et al. proposed a photochemical internalization (PCI) in which the photodamage to endosomal membranes can burst the endocytic vesicles, which allows endosomal escape of macromolecules into the cytosol.[16] From this point of view, DP/m may localize in the cytoplasmic organelles susceptible to photodamage following endosomal escape of the micelles during photoirradiation. The DP/m are assumed to produce a significantly high concentration of singlet oxygen as a result of effective separation of the center porphyrin by the dendritic envelope. The mechanism for the efficient generation of singlet oxygen within the micellar structure remains to be explained. However, a dendritic structure that prevents aggregation of the center porphyrin should be essential for the enhanced photocytotoxicity of DP/m. Further investigation to address the detailed mechanisms of the enhanced photocytotoxicity of DP/m, together with dendrimer size and morphological effect, is now in progress.

In summary, the photodynamic efficacy of the DP was dramatically improved by inclusion into micelles. This process resulted in a more than two orders of magnitude increase in the photocytotoxicity compared with that of the free DP, as a result of the accumulated singlet oxygen in the intracellular compartment as well as the modulated intracellular localization related to the micellar structure. Furthermore, the DP/m system has a relevant size range (ca. 100 nm) and high stability for intravenous administration, with resultant EPR effect, and may have a high utility for in vivo PDT of cancer and macular degeneration, the study of which is now in progress.

Experimental Section

PEG-*b*-PLL was synthesized by the polymerization of the *N*-carboxy anhydride of N^{ε}-Z-L-lysine initiated by CH_3O-PEG-NH_2 (12000 g mol^{-1}) in DMF, followed by deprotection of the Z group according to a previously reported method.[17] The M_w/M_n ratio and degree of polymerization of PLL were determined to be 1.11:1 and 41 by gel-permeation chromatography and ^{1}H NMR spectroscopy,

respectively. DP was synthesized as previously described,[6] and its purity was confirmed by a single peak in the MALDI-TOF mass spectrum (8030 g mol^{-1}). The DLS and SLS measurements of DP/m were performed using a Photal dynamic laser-scattering DLS-7000 spectrometer (Otsuka Electronics Co., Ltd., Osaka, Japan). The UV/Vis and fluorescence spectra were measured on a V-550 spectrophotometer and on an FP-777 spectrofluorometer (JASCO, Tokyo, Japan), respectively.

Lewis lung carcinoma (LLC) cells were used in the cell culture studies. In the quantitative analysis of the cellular uptake of DP, the cells incubated with free DP and DP/m were lyzed in 5% SDS solution, followed by measurement of the fluorescence intensity at 609 nm (excitation at 432 nm). In the cytotoxicity assay, the cells were photoirradiated for 10 minutes with broadband visible light using a xenon lamp (150 W) equipped with a filter passing light of 400–700 nm (fluence energy: 180 kJ cm^{-2}). The viability of the cells was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Oxygen consumption was measured by using a Clark-type oxygen microelectrode with a tip diameter of 200 μm (PO_2-100DW, Eikou Kagaku Co., Ltd., Tokyo, Japan). The microelectrode was inserted into the PBS, which contained 3.13 μM of DP or DP/m and 10% FBS as a singlet oxygen acceptor, so that the tip was 100 mm above the bottom of the solution. An Hg lamp (436 nm, FWHM: 6 nm, 15 mW cm^{-2}) was used for light irradiation. The solution was static and exposed to the atmosphere. Before each measurement, the system was calibrated in saline solution bubbled with air, in which the oxygen partial pressure was assumed to be 150 mm Hg.

Received: August 10, 2004

Keywords: block copolymers · dendrimers · drug delivery · micelles · porphyrinoids

[1] R. K. Pandey, G. Zhang in *Porphyrin Handbook*, *Vol. 6* (Eds.: K. M. Kadish, K. M. Smith, R. Guilard), Academic Press, **2000**, pp. 157–230.
[2] N. Nishiyama, H. R. Stapert, G. D. Zhang, D. Takasu, D. L. Jiang, T. Nagano, T. Aida, K. Kataoka, *Bioconjugate Chem.* **2003**, *14*, 58–66.
[3] a) A. W. Bosman, H. M. Jassen, E. W. Meijer, *Chem. Rev.* **1999**, *99*, 1665–1688; b) S. Hecht, J. M. J. Frechet, *Angew. Chem.* **2001**, *113*, 76–94; *Angew. Chem. Int. Ed.* **2001**, *40*, 74–91; c) M. Fischer, F. Vogtle, *Angew. Chem.* **1999**, *111*, 934–955; *Angew. Chem. Int. Ed.* **1999**, *38*, 884–905; d) H. R. Ihre, L. Gagne, J. M. J. Frechet, F. C. Szoka, Jr., *Bioconjugate Chem.* **2002**, *13*, 453–461; e) J. F. Kukowska-Latallo, A. U. Bielinska, J. Johnson, R. Spindler, D. A. Tomalia, J. R. Baker, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 4897–4902.
[4] a) Y. N. Konan, R. Gurny, E. Allemann, *J. Photochem. Photobiol. B* **2002**, *66*, 89–106; b) Y. Matsumura, H. Maeda, *Cancer Res.* **1986**, *46*, 6387–6392; c) M. Yokoyama, T. Okano, Y. Sakurai, S. Fukushima, K. Okamoto, K. Kataoka, *J. Drug Targeting* **1999**, *7*, 171–186.
[5] Y. Kakizawa, K. Kataoka, *Adv. Drug Delivery Rev.* **2002**, *54*, 203–222, and references therein.
[6] a) N. Tomioka, D. Takasu, T. Takahashi, T. Aida, *Angew. Chem.* **1998**, *110*, 1611–1614; *Angew. Chem. Int. Ed.* **1998**, *37*, 1531–1534; b) T. Aida, D.-L. Jiang in *The Porphyrin Handbook*, *Vol. 3* (Eds: K. M. Kadish, K. M. Smith, R. Guilard), Academic Press, **2003**, pp. 369–384.
[7] H. R. Stapert, N. Nishiyama, D. L. Jiang, T. Aida, K. Kataoka, *Langmuir* **2000**, *16*, 8182–8188.
[8] a) E. J. Ambros, D. M. Easty, P. C. T. Jones, *Br. J. Cancer* **1958**, *12*, 439–447; b) S. E. Kornguth, T. Kalinke, H. I. Robins, J. D. Cohen, P. Turski, *Cancer Res.* **1989**, *49*, 6390–6395.
[9] S. A. Gerhardt, J. W. Lewis, D. S. Kliger, J. Z. Zhang, U. Simonis, *J. Phys. Chem. A* **2003**, *107*, 2763–2767.
[10] a) N. C. Maiti, S. Mazumdar, N. Periasamy, *J. Phys. Chem. B* **1998**, *102*, 1528–1538; b) K. M. Kadish, G. B. Maiya, C. Araullo, R. Guilard, *Inorg. Chem.* **1989**, *28*, 2725–2731.
[11] R. Sadamoto, N. Tomioka, T. Aida, *J. Am. Chem. Soc.* **1996**, *118*, 3978–3979.
[12] T. Sato, D.-L. Jiang, T. Aida, *J. Am. Chem. Soc.* **1999**, *121*, 10658–10659.
[13] S. Basu, *J. Photochem. Photobiol. A: Chem.* **1991**, *56*, 339–347.
[14] N. Nishiyama, A. Nori, A. Malugin, Y. Kasuya, P. Kopeckova, J. Kopecek, *Cancer Res.* **2003**, *63*, 7876–7882.
[15] a) M. R. Hamblin, J. L. Miller, T. Hasan, *Cancer Res.* **2001**, *61*, 7155–7162; b) R. Savic, L. Luo, A. Eisenberg, D. Maysinger, *Science* **2003**, *300*, 615–618.
[16] a) K. Berg, P. K. Selbo, L. Prasmickaite, T. E. Tjelle, K. Sandvig, J. Moan, G. Gaudernack, O. Fodstad, S. Kjolsrud, H. Anholt, G. H. Rodal, S. K. Rodal, A. Hogest, *Cancer Res.* **1999**, *59*, 1180–1183; b) L. Prasmickaite, A. Høgset, P. K. Selbo, B. Ø. Engesæter, M. Hellum, K. Berg, *Br. J. Cancer* **2002**, *86*, 652–657.
[17] A. Harada, K. Kataoka, *Macromolecules* **1995**, *28*, 5294–5299.

