

Supramolecular nanocarriers integrated with dendrimers encapsulating photosensitizers for effective photodynamic therapy and photochemical gene delivery†

Nobuhiro Nishiyama,^{ac} Woo-Dong Jang^{bd} and Kazunori Kataoka^{*abd}

Received (in Montpellier, France) 3rd November 2006, Accepted 4th January 2007

First published as an Advance Article on the web 15th February 2007

DOI: 10.1039/b616050f

Recently, biomedical applications of dendrimers, three-dimensional tree-like macromolecules, have received much attention. In this paper, we introduce new biomedical applications of functional dendrimers: dendrimers encapsulating photosensitizers composed of a center dye molecule surrounded by poly(benzyl ether) dendrons with ionic peripheral groups as a new type of photosensitizer (PS) for photodynamic therapy (PDT). Dendrimers encapsulating porphyrin and with ionic peripheral groups (DPs) spontaneously formed polyion complex (PIC) micelles through electrostatic interactions with oppositely-charged block copolymers, and showed no self-quenching of the center dye molecule inside the micellar core due to a unique DP structure, leading to remarkable *in vitro* photocytotoxicity. The DP-incorporated micelles showed successful treatment of choroidal neovascularization (CNV) in rats without any sign of side effects. Thus, the DP-incorporated micelles are expected to be an innovative PS formulation for successful PDT against ophthalmic diseases. In this paper, we also review polymeric micelles incorporating phthalocyanine core dendrimers (DPc) for cancer PDT and novel light-responsive supramolecular gene carriers integrated with DPc for site-directed transfection *in vivo*.

^a Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0022, Japan. E-mail: kataoka@bmw.t.u-tokyo.ac.jp; Fax: +81-3-5841-7139; Tel: +81-3-5841-7138

^b Department of Materials Science and Engineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan

^c Center for NanoBio Integration, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan

^d Department of Chemistry, College of Science, Yonsei University, 134 Sinchondong, Seodaemun-gu, Seoul 120-749, Korea

† This paper was published as part of the special issue on Dendrimers and Dendritic Polymers: Design, Properties and Applications.

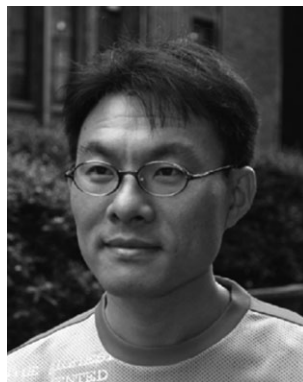
Introduction

Photodynamic therapy (PDT) has been attracting increasing attention as a promising method for the treatment of solid tumors and ophthalmic diseases.^{1–3} PDT involves administration of photosensitizers (PSs) to the body, followed by activation of PSs at the diseased site using light of specific wavelength. Upon photoirradiation, PSs undergo photosensitized reaction processes of type I and type II. The former produces free radicals or superoxide anions resulting from hydrogen- or electron transfer from the photoexcited PSs,



Nobuhiro Nishiyama, PhD, is an Assistant Professor of Clinical Biotechnology at the Graduate School of Medicine of the University of Tokyo, Japan. He received a PhD degree in Materials Engineering from the University of Tokyo in 2001. He was a Postdoctoral Fellow at the University of Utah and an Assistant Professor at the University of Tokyo Hospital. His main research interest

concerns the biomedical applications of intelligent nanodevices for drug and gene delivery.



Woo-Dong Jang, PhD, is an Assistant Professor at the Chemistry Department of Yonsei University, Korea. He received a BSc degree in Polymer Chemistry from Kyungpook National University, Korea, and a PhD degree in Chemistry and Biotechnology from the University of Tokyo, Japan. He was a Postdoctoral Fellow at the Japan Science and Technology Agency and an Assistant

Professor at the University of Tokyo. His main research interest concerns the biomedical application of polymeric materials.

whereas the latter generates cytotoxic singlet oxygen ($^1\text{O}_2$) by the energy transfer. In general, the type II reaction is known to be more efficient in PDT.

A challenge in PDT is to create efficient PSs, which show highly selective photocytotoxicity to the diseased tissues, and an increasing number of PSs are presently being explored in preclinical and clinical studies.^{1,2} PSs generally have large π -conjugation domains, such as a porphyrin structure, to obtain a high quantum yield and effective energy absorption. Therefore, most conventional PSs easily form aggregates in aqueous media through their π - π stacking and hydrophobic interactions, resulting in the self-quenching of the excited state. This may decrease the photodynamic effect of the PS after its accumulation in the diseased tissue. On the other hand, there is also a strong incentive to develop effective drug delivery systems (DDS) for PSs, to enhance the selectivity and effectiveness of PDT as well as prevent side effects caused by non-specific distribution of PSs in the body, such as prolonged skin hypersensitivity to sunlight.² To date, a variety of DDS, including polymer-PS conjugates,⁴ long-circulating liposomes⁵ and polymeric micelles,⁶ have been examined; however, the aforementioned properties of PSs, *i.e.*, a strong tendency to form aggregates and the related self-quenching effect, might interrupt the development of effective PS formulations. In general, it might be difficult to effectively incorporate such hydrophobic substances into drug carriers. Also, increased loading of PSs into drug carriers could change the modality of photochemical reaction from type II to type I due to aggregate formation, leading to diminished photodynamic efficacy.⁷ Thus, both efficiencies of the PS delivery and photochemical reactions of PS itself should be considered in the development of DDS in PDT.

Recently, we have developed a new type of PS based on a dendritic architecture, *i.e.*, dendrimers encapsulating porphyrin with ionic peripheral groups (DPs), in which the centre porphyrin is surrounded by the 3rd generation of poly(benzyl ether) dendrons (Fig. 1).⁸ Unlike conventional PSs, DPs ensure a high efficacy of singlet oxygen production even at a high concentration, since the dendritic envelopes of DPs can prevent aggregation of the center porphyrin (Fig. 2). Thus, it is assumed that the encapsulation of DPs into drug carriers

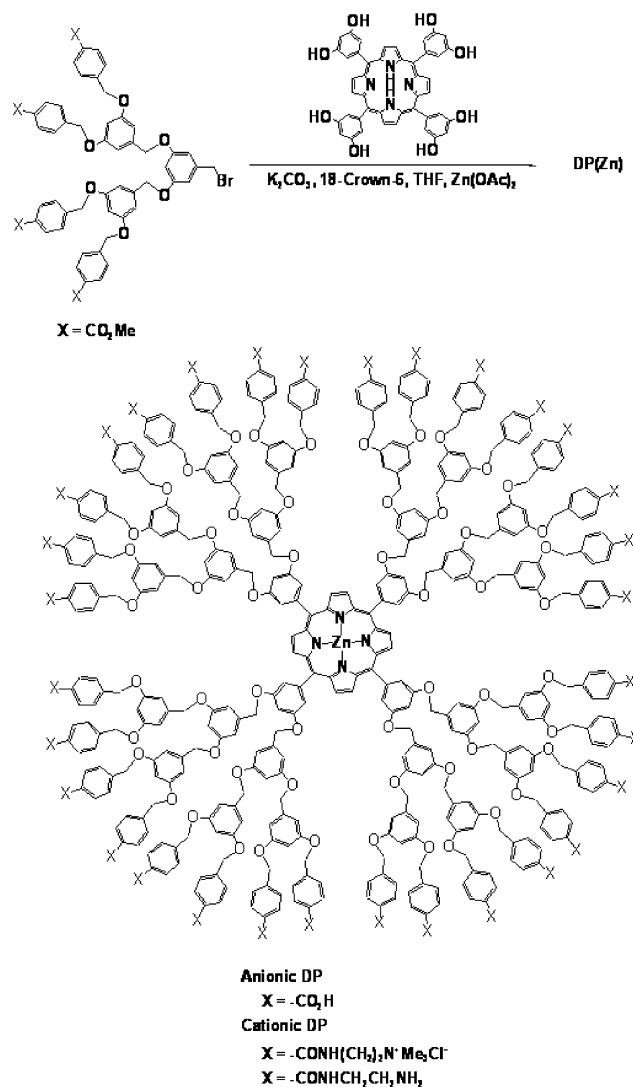


Fig. 1 Chemical structures of dendrimers encapsulating porphyrin with ionic peripheral groups (DPs) and the synthetic scheme.

might not compromise the singlet oxygen production efficiency. In addition, the peripheries of DPs can be modified with a variety of functional groups, providing water-solubility and practical functions such as tissue-targetability. Also, the introduction of charged ionic groups into the periphery of a DP allows its stable incorporation into a supramolecular nanocarrier, a polyion complex (PIC) micelle, through electrostatic interactions with oppositely-charged block copolymers as schematically shown in Fig. 2.

Polymeric micelles, self-assemblies of block copolymers, are characterized by a size of several tens of nanometres, a fairly narrow size distribution, remarkably low critical micelle concentration (c.m.c.) and segregated core-shell structure, and they have recently received considerable attention as a promising modality of drug carriers.^{9–11} It has been demonstrated that polymeric micelles could circulate stably in a bloodstream and, therefore, accumulate effectively in solid tumors¹² due to the enhanced permeability and retention (EPR) effect, characterized by microvascular hyperpermeability and impaired lymphatic drainage in tumor tissues.¹³ Also, we have



Kazunori Kataoka, PhD, is a Professor of Biomaterials at the Graduate School of Engineering of the University of Tokyo, Japan. Since 2004, he has also held a joint position at the Graduate School of Medicine of the University of Tokyo as a Professor of Clinical Biotechnology. He received a PhD from the University of Tokyo in 1979. Dr Kataoka is the author of more than 350 scientific papers in international journals and a recipient of many awards. His current major research interests include the development of new polymeric carrier systems, especially block copolymer micelles, for drug and gene targeting.

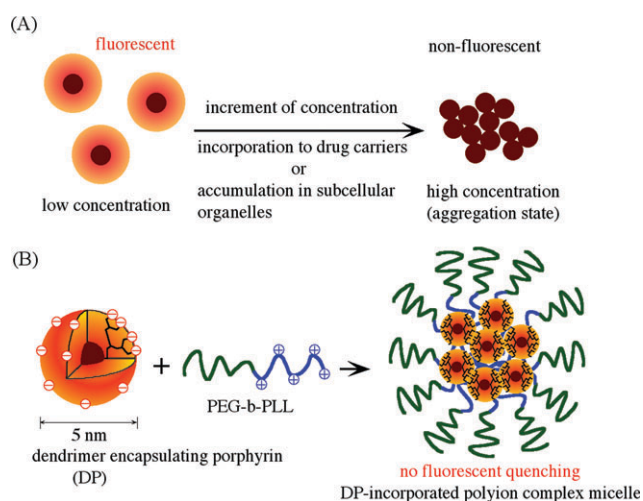


Fig. 2 (A) Conventional PSs form aggregates at a high concentration, resulting in their self-quenching. (B) Formation of DP-incorporated polyion complex (PIC) micelles through electrostatic interactions between anionic DPs and poly(ethylene glycol)-block-poly(L-lysine) (PEG-*b*-PLL) copolymers. The dendritic structure of DP can sterically prevent aggregation of the center porphyrin, thus there is no fluorescent quenching of the center porphyrin. [Ref. 12, copyright permission from Wiley-VCH].

recently reported that polymeric micelles also accumulated in the choroidal neovascularization (CNV) site in rat models,¹⁴ suggesting the targeted therapy of ophthalmic diseases by polymeric micelles. The PIC micelle, a new type of polymeric micelle formed through the electrostatic interaction between a pair of charged block copolymers and oppositely-charged macromolecules,¹⁵ is expected to be a targetable carrier system for biologically active molecules, including proteins¹⁶ and nucleic acids^{17,18} as well as ionic DPs.^{19–22}

In this paper, we review recent advances in the field of PIC micelles incorporating photosensitizer core dendrimers for effective PDT. Also, we introduce our new technology for the control of transgene expression by using gene nanocarriers integrated with dendrimers encapsulating photosensitizers.

Dendrimers encapsulating porphyrins (DPs) as a new photosensitizer

Synthesis of ionic DPs

There are two different synthetic strategies towards ionic DPs; a divergent and a convergent growth approach are generally employed to construct dendrimer frameworks.²³ DPs have poly(benzyl ether) dendritic wedges, which were first synthesized by Fréchet and Hawker using the convergent approach.²⁴ Aida *et al.* reported the first example of a dendrimer encapsulating a metalloporphyrin, in which the zinc porphyrin functionality is covalently encapsulated by poly(benzyl ether) dendritic wedges with methoxycarbonyl groups on the exterior surface, on the basis of Fréchet and Hawker's convergent approach.²⁵ Fig. 1 shows the synthetic scheme towards DPs. Alkaline-mediated coupling of 5,10,15,20-tetrakis(3',5'-dihydroxyphenyl)porphine with methoxycarbonyl-terminated aryl ether dendritic bromides

gave a DP, which was metalated with $\text{Zn}(\text{OAc})_2$, followed by hydrolysis of the exterior MeO_2C -groups with KOH . Dialysis of the reaction mixture for 2 days in fresh water to remove excess KOH , followed by evaporation to dryness, gave an anionic DP bearing 32 carboxylic groups on the periphery. Also, the synthesis of a cationic DP, bearing 32 quaternary ammonium groups on the periphery, was performed by amidation of the anionic DP with 1-amino-2-(trimethylammonium)ethane, followed by purification by column chromatography and preparative HPLC using THF as an eluent. Likewise, a DP bearing 32 primary amino groups was prepared by amidation of the anionic DP with *N*-(trifluoroacetyl)ethylene-1,2-diamine, followed by quantitative deprotection of the trifluoroacetyl group. The perfection of cationic DPs after amidation of anionic DP was confirmed by MALDI-TOF-MS.²¹

Properties of ionic DPs as a photosensitizer for PDT

As described in the Introduction, DPs show a unique photochemical property that the center porphyrin might not be quenched even at a high concentration due to its spatial isolation in the focal core of the dendrimer. Thus, the photochemical reaction of DPs may not be affected by their subcellular localization and local concentrations. Regarding the photochemical properties, ionic DPs absorb light at 415, 434 (Soret bands) and 559 nm (Q-band) and emit fluorescent light at 610 and 660 nm. In our previous study, the singlet oxygen quantum yield of DPs was measured by direct observation of the $\text{O}_2 (^1\Delta_g) \rightarrow \text{O}_2 (^3\Sigma_g^-)$ transition at 1270 nm ((0,0) vibronic band) in MeOD.⁸ It was demonstrated that ionic DPs showed similar singlet oxygen quantum yield to a conventional PS, protoporphyrin IX. Thus, the center porphyrin possesses a high singlet oxygen quantum yield in spite of its dendritic structure. In the photodynamic action of DPs, only the singlet oxygen or other reactive oxygenic species (ROS) is assumed to play an essential role, because the direct interaction of dye radicals with other compounds should be minimal in the dendritic architecture. Also, DPs may be hardly photobleached during photochemical reactions, although this effect remains to be elucidated. It is generally known that singlet oxygen has a quite short lifetime in an aqueous milieu, and the diffusion distance of $^1\text{O}_2$ is estimated to be less than 10 nm.² Nevertheless, the 3-D structure of dendrimers with the size of 5 nm appeared not to be a barrier for the $^1\text{O}_2$ -induced photodynamic effect, because ionic DPs showed comparable or even more efficient photocytotoxicity compared with conventional PSs.⁸ Possibly, the lifetime of $^1\text{O}_2$ may be prolonged inside the hydrophobic dendritic structure of DPs.

DPs are hydrophilic macromolecules (~ 5 nm), so that they are assumed to be taken up by the cells through endocytic pathways. Nevertheless, the ionic DPs showed different cellular associations depending on the peripheral charged groups. In our previous study, cationic DP showed rapid association to the negatively-charged plasma membrane through electrostatic interaction, followed by internalization by adsorptive endocytosis, whereas anionic DPs were slowly internalized by fluid phase endocytosis due to the electrostatic repulsion.⁸ Thus, both cationic and anionic DPs finally localized in

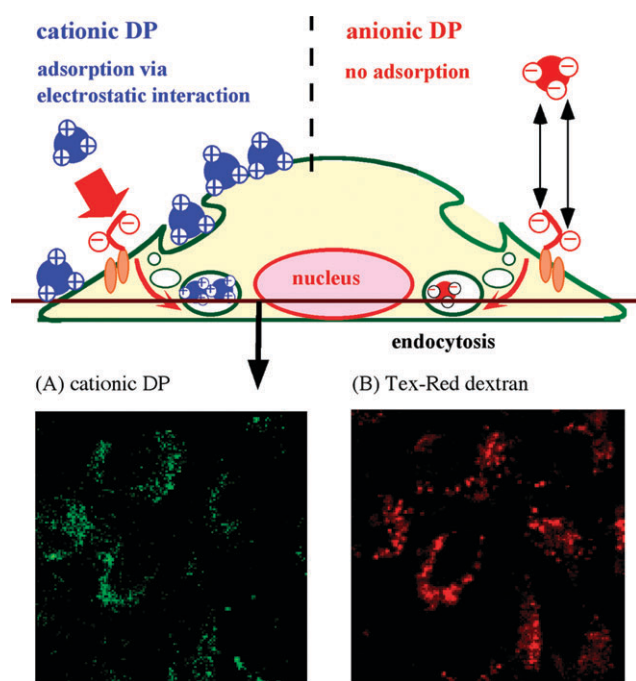


Fig. 3 Schematic illustration of the cellular association manners of cationic and anionic DPs, and confocal images of cationic DP (A) and Tex-Red dextran, an endocytosis marker (B) in LLC cells after incubation for 3 h at 37 °C. [Ref. 8, copyright permission from American Chemical Society].

endosomal compartments, as demonstrated by colocalization with Tex-Red dextran, a fluid phase endocytic marker (Fig. 3). Although cationic DP showed only 22–25 times higher cellular association than anionic DP, the photocytotoxicity ($^1\text{O}_2$ -induced cytotoxicity) of cationic DP was 230 times higher than that of anionic DP.⁸ Such a difference in the photocytotoxicity between cationic and anionic DPs may be due to different interactions of both compounds with cellular components. That is, cationic DP is likely to electrostatically interact with negatively-charged cell membranes, photodamaging membrane components, which may be susceptible to $^1\text{O}_2$ -induced cell death.

Fluorescent microscopy observation using organelle-specific dyes revealed that the photodamage by DPs maintained the characteristic structure of cell membranes and intracellular organelles (the plasma membrane, mitochondrion and lysosome), whereas such organelles were severely photodisrupted by conventional PSs.⁸ Nevertheless, cationic DP induced efficient cell death. In this regard, the fluorescence of Rhodamine 123 (Rh123), a mitochondrion marker, was significantly attenuated by the photodamage by cationic DP. The fluorescence intensity of Rh123 is known to be correlated with the amount of adenosine triphosphate (ATP) in the cell.²⁶ Therefore, cationic DP may have clipped ATP production in the cell, leading to efficient cell death. The eliminated ATP production may be caused by exhaustion of intracellular oxygen or photodamage to the plasma membrane.^{27,28} Also, there is another possibility that the photodamage to the cell membranes could disrupt the endosome/lysosome, localizing DPs in cytoplasmic organelles, which are susceptible to $^1\text{O}_2$ -induced cell death.²⁹ For example, it was reported that the

photodamage to the mitochondrion induced apoptosis through the cytoplasmic release of cytochrome c.³⁰ The exact molecular target of ionic DPs is an issue to be clarified in the future.

Low dark toxicity of PS is one of the important criteria for assessing the usefulness of PS because unwanted toxicity to normal tissues is one of major side effects in clinical PDT.³¹ Ionic DPs showed extremely low dark toxicity even after prolonged incubation of 72 h.⁸ The extremely low dark toxicity of DPs may result from the distinctive intracellular disposition characteristics due to their relative large size (~ 5 nm).

DP-incorporated polyion complex (PIC) micelles for effective PDT

Physicochemical properties of DP-incorporated PIC micelles

The DP-incorporated PIC micelles are formed from a pair of charged block copolymers of poly(ethylene glycol)-*block*-poly(L-lysine) (PEG-*b*-PLL) or poly(ethylene glycol)-*block*-poly(aspartic acid) (PEG-*b*-PAA) and oppositely-charged DPs with 32 charged groups on the periphery (Fig. 2B). Simple mixing of block ionomers and ionic DPs at a stoichiometric charge ratio led to the spontaneous formation of the PIC micelles. The PIC micelles prepared from negatively-charged DP and positively-charged PEG-*b*-PLL had a diameter of approximately 64 nm with an extremely narrow size distribution in physiological saline solution. The spherical shape of the DP-incorporated micelles was confirmed by observation with atomic force microscopy (AFM) and field emission-transmission electron microscopy (FE-TEM). The static light scattering (SLS) measurement revealed that an individual PIC micelle contains an average of 38 DP molecules.¹⁹ From the standpoint of the application as drug carriers, the stability of the PIC micelles against salt concentrations might be primarily important, since the electrostatic shielding of polyelectrolytes with salt ions might destabilize the PIC structure.³² In this regard, the PIC micelles consisting of cationic DP bearing 32 quaternary ammonium groups-PEG-*b*-PAA showed a higher stability against NaCl concentrations than those of PLL₂₇-PEG-*b*-PAA.¹⁹ This may be explained by the assumption that the PIC from a rigid dendrimer may produce a smaller entropy gain upon dissociation than that from a flexible PLL homopolymer. In the case of the PIC micelles consisting of cationic DP bearing 32 primary amino groups-PEG-*b*-PAA or anionic DP-PEG-*b*-PLL, the complexation between DPs and block copolymers might be accomplished by the formation of hydrogen bonds between carboxylic acid and primary amine groups after proton transfer from the acid amine. Therefore, the resulting polyion complex micelles showed a remarkable stability against an increase in NaCl concentrations up to 1500 mM, 10 times higher than the physiological concentration.^{19,21}

The PIC micelles of anionic DP-PEG-*b*-PLL showed pH-dependent structural changes.²² In this study, the translational diffusion coefficient (D_T) and normalized ($K_C/\Delta R(0)$)⁻¹ (normalized to the micelle at pH 7.4) of the PIC micelles, where D_T is related to the hydrodynamic diameter based on

the Stokes–Einstein equation, and the normalized $(Kc/\Delta R(0))^{-1}$ value is related to the changes in the apparent molecular weight of the micelles, were measured under different pH conditions by dynamic light scattering (DLS) and SLS measurements, respectively. Both the hydrodynamic diameter and normalized $(Kc/\Delta R(0))^{-1}$ value remained constant in the pH range from 6.4 to 8.5. However, when the pH was below 6.4, the PIC micelles showed a gradual increase in the diameter and apparent molecular weight, and finally underwent precipitation at pH 5.6, indicating the acid-responsive feature of the micelles. Under acidic pH conditions, the carboxylic acid groups of DP might be considerably protonated, leading to diminution of the electrostatic interaction between anionic DP and PEG-*b*-PLL, thus the well-defined core-shell structure may become more obscure and a merging of the micelles may take place. The similar pH-dependent structural changes of the PIC micelles were also observed for the system of cationic DP bearing 32 primary amino groups–PEG-*b*-PAA.²¹ Such a pH-responsive behavior of the micelles allows their effective accumulation in solid tumors in response to a low pH condition in the tumor tissue (\sim pH 6.5)³³ or an intracellular acidic endosomal compartment (\sim pH 5.0) while their stable circulation in the bloodstream is expected.

In vitro photodynamic effect of DP-incorporated PIC micelles

In the UV-Vis absorption spectra, the PIC micelles of anionic DP–PEG-*b*-PLL showed a red shift of 5 nm for the Soret band of the porphyrin core and a hypochromicity of about 5%,²² which seems to be consistent with the previous reports on the electrostatic assembly of charged porphyrins and oppositely-charged compounds.^{34,35} The cancellation of the charge repulsion of the negatively-charged DP surface by the formation of the electrostatic assembly may lead to shrinkage of the hydrophobic dendrimer frameworks, which may be related to the hypochromicity.²⁵ Importantly, the PIC micelles showed no fluorescent quenching of DP (630 nm), although the local concentration of DP is assumed to be extremely high in the core of the PIC micelles.²² Similarly, no fluorescent quenching of DP in the PIC micelles was observed for the system of cationic DP bearing 32 primary amino groups–PEG-*b*-PAA.²¹ It is worth noting that no fluorescent quenching of DP was microscopically observed inside the cell incubated with the DP-incorporated PIC micelles,²² suggesting the effective photochemical reaction in the living cell directed the enhanced photocytotoxicity. It is hypothesized that the dendritic envelope of DP might prevent the collisional quenching of the center porphyrin even at an extremely high concentration. This unique photochemical property of the DP-incorporated PIC micelles might not be achieved by other conventional PSs and PS formulations.

In regard to the efficiency of the photochemical reactions, the DP-incorporated PIC micelles showed an oxygen consumption rate comparable to free DP in phosphate-buffered saline containing fetal bovine serum (FBS) as a singlet oxygen acceptor (Fig. 4).²² This result suggests that the singlet oxygen molecules produced from DP can escape from the micellar structure and react with proteins of FBS. Thus, the DP-incorporated PIC micelles might have the capacity to effi-

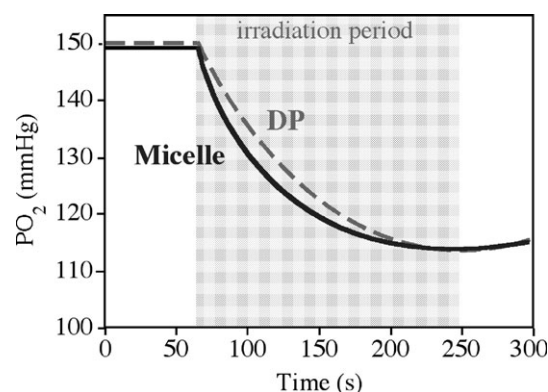


Fig. 4 Profiles of the oxygen consumption by anionic DP (---) and the DP-incorporated micelles (—) in phosphate-buffered saline solution containing 10% fetal bovine serum (FBS). The light irradiation and the oxygen partial pressure measurement were performed using an Hg lamp and a Clark-type oxygen microelectrode, respectively. [Ref. 12, copyright permission from Wiley-VCH].

ciently photo-oxidize biomolecules in the solution without their structural changes. Possibly, the DP-incorporated PIC micelles may achieve the elevated concentration of local singlet oxygen, which may not be obtained by other formulations containing conventional PSs.

Very interestingly, the PIC micelles of anionic DP–PEG-*b*-PLL showed approximately 280 times more efficient photocytotoxicity (against Lewis lung carcinoma (LLC) cells) compared with free anionic DP (Fig. 5), although incorporation of DP into the PIC micelles resulted in an only 6–8 times increase in the cellular uptake.²² From a practical standpoint, such a manner of the photocytotoxicity enhanced by the micelle formulation may avoid long-term phototoxicity after PDT, since the DP-incorporated micelles are assumed to gradually dissociate to constituent DP and block copolymers in the body. It may be hypothesized that the DP-incorporated

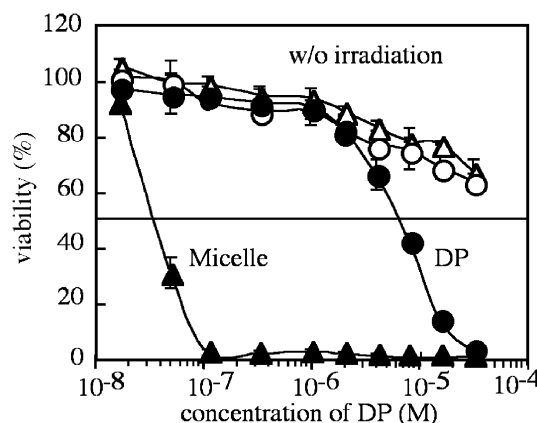


Fig. 5 The photocytotoxicity (closed symbols) and the dark toxicity (open symbols) of anionic DP (circles) and the DP-incorporated micelles (triangles) against LLC cells. In this assay, the cells were photoirradiated for 10 min with broadband visible light using a xenon lamp (150 W) equipped with a filter passing light of 400–700 nm (fluence: 180 kJ cm⁻²). The cell viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

micelles may have a specific mechanism to enhance their photocytotoxicity. Recently, PEGylated chlorin-*e*₆ as well as PEG-based polymeric micelles were reported to show enhanced localization in several cytoplasmic organelles, including the mitochondrion.^{30,36} Thus, the PEG shell of the PIC micelles may have a role in altering the intracellular mechanism of DP to increase the photocytotoxicity. Otherwise, the photodamage-induced disruption of the endosomal/lysosomal membranes may assist the localization of DP in the cytoplasmic organelles, which may be susceptible to ¹O₂-induced cell death. In any case, the remarkably increased concentration of local singlet oxygen by encapsulation into the PIC micelles is likely to contribute significantly to the enhanced photocytotoxicity of DP. Further investigations to address the detailed mechanisms of the enhanced photocytotoxicity of the DP-incorporated micelles are now in progress in our research group.

PDT using DP-incorporated PIC micelles

The exclusive age-related macular degeneration (AMD), a condition caused by choroidal neovascularization (CNV), is a leading cause of visual loss in developed countries.³ Recently, Visudyne[®], a liposomal formulation of verteporfin, has been demonstrated to be effective for the treatment of AMD, and has been approved for clinical use.³⁷ However, PDT with Visudyne requires repeated treatments every three months, and many patients nevertheless suffer from visual loss due to recurrence of CNV. Therefore, there is a strong incentive to develop more effective formulations of PSs for the PDT treatment against AMD.

Recently, we applied the PIC micelles of anionic DP-PEG-*b*-PLL for PDT of experimental CNV in rats, which was created by laser photocoagulation.³⁸ When DP-incorporated micelles were intravenously administered, they showed effective and selective accumulation in the CNV lesions.³⁸ Laser irradiation (5–50 J cm⁻²) at 4 h after iv administration of DP-incorporated micelles resulted in successful occlusion of CNV in 60–72% of the tested animals. Importantly, approximately 80% of the tested animals showed maintained CNV occlusion even 7 days after laser irradiation.³⁸ In contrast, it was reported that PDT with Visudyne under the same experimental conditions showed less effectiveness in CNV occlusion and resulted in recurrence of CNV 7 days after the treatment.³⁹ Thus, DP-incorporated micelles might be an effective PS formulation, and may not require the repeated treatments, which are problems of other PS formulations. In this study, the skin photodamage was also evaluated by photoirradiating the mouse abdominal skin with broadband visible light (Xenon lamp equipped with a filter passing light of 377–700 nm) 4 h after iv injection of DP-incorporated micelles or Photofrin, a clinically used PS formulation for cancer therapy. As a result, mice treated with DP-incorporated micelles did not experience macroscopically observable skin damage, whereas those treated with Photofrin displayed severe skin damage.³⁸ Thus, DP-incorporated micelles might circumvent skin hyperphotosensitivity, a major side effect of current PDT, due to their reduced accumulation in the skin. From these *in vivo* results, we can conclude that DP-incorporated micelles

may have an innovative PS formulation for the treatment of ophthalmologic diseases.

Dendrimer encapsulating phthalocyanine as a photosensitizer for cancer therapy

We have demonstrated that DP-incorporated micelles are a potent PS formulation for PDT against ophthalmologic diseases. However, DPs have relatively short excitation wavelengths (*i.e.*, 430 and 560 nm), which might be a limitation for PDT except for the transparent tissues such as ophthalmologic organs. Skin tissue has melanin dyes, which absorb short wavelength light to prevent photochemical genetic disorder, and also heme proteins of red blood cells account for most of the light absorption in the visible region. Such light absorption by human bodies prevents the excitation of PSs for photochemical reactions. Therefore, PSs should have long absorption wavelength for the PDT of deeper lesions such as solid tumors.

In this regard, several phthalocyanine molecules have been widely studied as potential PSs with an appropriate absorption wavelength for practical PDT application.^{2,40} The phthalocyanines have an optical absorption at approximately 680 nm, where the light can penetrate tissues 2 times deeper than Photofrin (630 nm). Recently, we have synthesized dendrimers encapsulating phthalocyanine with ionic peripheral groups (DPc, Fig. 6) with the maximum absorption at 685 nm according to the synthetic procedure established by Ng *et al.*,⁴¹ and prepared DPc-incorporated micelles, having the size of approximately 50 nm, through the electrostatic interaction between anionic DPc and PEG-*b*-PLL.⁴² DPc-incorporated micelles were stable in a phosphate-buffered solution containing 10% FBS, maintaining the size and polydispersity of the micelles. The micelle formation was accompanied by a shift of maximum absorption wavelength of DPc from 685 nm

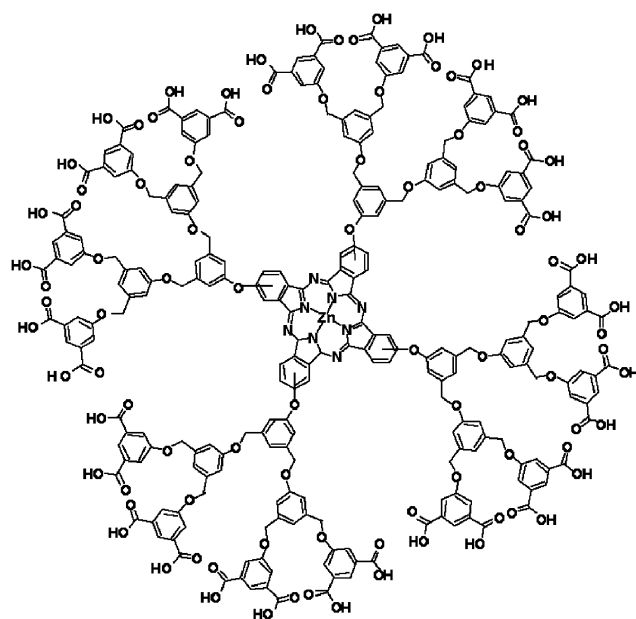


Fig. 6 Chemical structure of dendrimer encapsulating phthalocyanine (DPc).

to 630 nm, indicating some interactions between the phthalocyanine units of DPc (*i.e.*, aggregate formation) in the micellar core. The relatively small dendritic wedges may be insufficient to prevent such interactions. As a result, DPc-incorporated micelles showed reduced oxygen consumption rates compared with DPc alone.⁴² Nevertheless, DPc-incorporated micelles showed significant enhancement of the light-induced cytotoxicity, which greatly relied on the photoirradiation time. In contrast, free DPc did not exhibit such enhancement of the photocytotoxicity regardless of irradiation time. Thus, DPc-incorporated micelles achieved approximately 100-fold photocytotoxicity compared with free DPc after 60 min photoirradiation. The treatment of solid tumors by PDT with DPc-incorporated micelles is ongoing in our laboratory, and the results will be reported elsewhere in the near future.

Photochemical gene delivery using dendrimer encapsulating phthalocyanine (DPc)

The DPc-incorporated micelles are taken up by the cells through the endocytic pathway, preferentially localizing in the endosome or lysosome. Upon photoirradiation, DPc-incorporated micelles may photochemically disrupt the endosomal/lysosomal membrane to relocate in the subcellular organelles susceptible to $^1\text{O}_2$ -induced cell death. This process is available for the light-induced cytoplasmic delivery of macromolecular compounds such as plasmid DNA (pDNA), which are impermeable to cell membranes and easily digested in the endosome or lysosome. This concept was “photochemical internalization (PCI)” by Berg and Høgstet *et al.*^{29,43–46} It should be noted that the light dose necessary for PCI is much lower than that for PDT, ensuring the low photocytotoxicity of PCI. Recently, we also carried out *in vitro* PCI-mediated gene transfection using a combination of the DPc-PEG-*b*-PLL and pDNA-PEG-*b*-PLL micelles, and achieved 100-fold photochemical enhancement of the transgene expression while maintaining 80% cell viability over a wide range of DPc concentrations and light doses.⁴⁷ Moreover, we optimized the chemical structures of PEG-*b*-polycations forming the pDNA-incorporated micelles for the PCI-mediated transfection, and found that the use of PEG-*b*-polycations having the repeated ethylenediamine units in the side chain allowed approximately 1000-fold light-selective gene transfection,⁴⁸ suggesting that the PCI and the so-called proton sponge effect⁴⁹ may work synergistically to enhance the transfection efficiency.

From the standpoint of *in vivo* applications of the PCI-mediated transfection, gene carriers should be equipped with a photosensitizing unit as one component, motivating us to develop the ternary complexes composed of pDNA, quadruplicated cationic peptide (CP₄) containing nuclear localization signal (NLS) sequence and anionic DPc (Fig. 7).⁵⁰ The simple addition of anionic DPc to the cationic pDNA-CP₄ complexes led to the spontaneous formation of the ternary complexes having the pDNA-CP₄ core surrounded by the DPc envelope, and the obtained nanoparticles had the size of 130 nm with a narrow distribution. Interestingly, such nanoparticles were not obtained by the addition of poly(aspartic acid) homopolymer with a polymerization degree of 26 to the pDNA-CP₄ com-

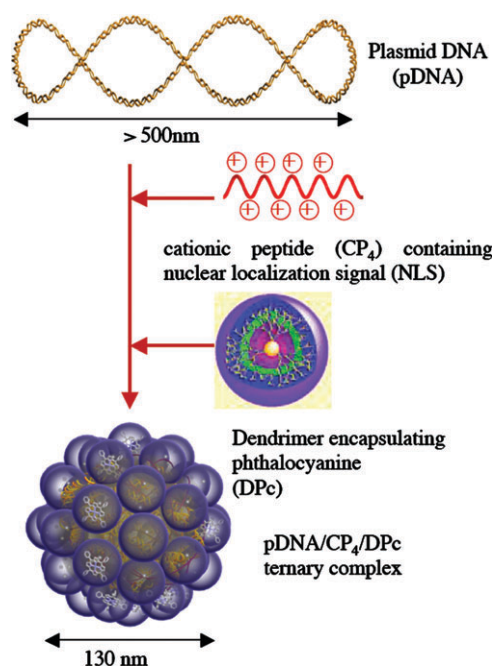


Fig. 7 Formation of the pDNA-CP₄-DPc ternary complexes as light-responsive gene carriers for site-directed gene transfer.

plexes, suggesting that the 3-D structure of DPc might play an essential role in the formation of the ternary complexes. Also, such ternary complexes are definitely discriminated from the layer-by-layer assemblies,^{51,52} because a core of hard materials is not required for the formation of the ternary complexes. The pDNA-CP₄-DPc ternary complexes are assumed to activate the transgene expression in a light-inducible manner according to the following processes: (1) internalization of the ternary complexes through endocytosis, (2) release of DPc from the ternary complexes due to protonation of the peripheral carboxyl groups of DPc under acidic conditions in the endosome, (3) interaction with the endosomal membrane due to the hydrophobic nature of the dendrimer framework, (4) light-induced endosomal escape of the pDNA-CP₄ complexes through the photochemical disruption of the endosomal membrane, and (5) nuclear transport of the pDNA-CP₄ complexes guided by the NLS sequence.⁵³ As a result, the ternary complexes showed a more than 100-fold light-induced enhancement of *in vitro* transgene expression while maintaining the cell viability.⁵⁰ The PCI-mediated transfection with the ternary complexes was not accompanied by long-term toxicity, which was observed by transfection using polyethylenimine (PEI), a well-known highly transfectable polycation. Thus, the ternary complexes might avoid the cytotoxicity induced by buffering polycations, because the cytotoxic events might occur only during the photoirradiation. To demonstrate the potential of the ternary complexes for *in vivo* gene transfer, the ternary complexes were subjected to subconjunctival injection in rats, followed by laser irradiation with a semiconductor laser (689 nm) at 2 h post-injection (Fig. 8). The PCI-mediated gene transfer with the ternary complexes resulted in appreciable gene expression of the fluorescent protein (*Venus*) only at the laser-irradiated site in the conjunctiva.⁵⁰ In contrast, the subconjunctival injection of the pDNA-PEI complexes failed

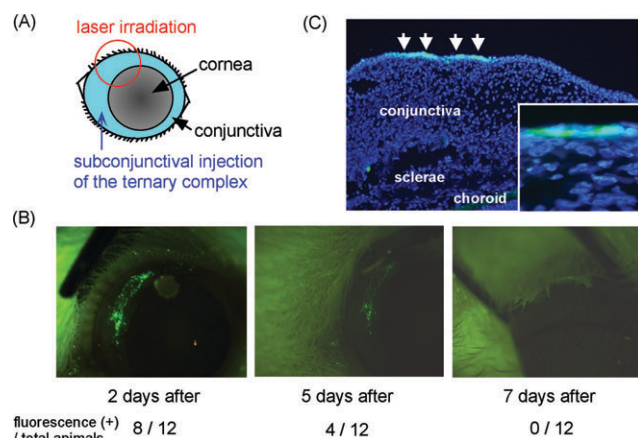


Fig. 8 Light-induced gene transfer to the rat conjunctival tissue. (A) Scheme for *in vivo* transfection. Rats were given a subconjunctival injection (colored in light blue) of the ternary complexes containing pDNA encoding a fluorescent protein, *Venus*. At 2 h post-injection, a part of the conjunctiva (red circle) was irradiated by the laser. (B) Fluorescent images of *Venus* expression in the rat eye at 2, 5 and 7 days after the transfection. The rates of the fluorescent-positive eyes (fluorescent (+)/total eyes) are indicated below the images. (C) Fluorescent image of the *Venus* expression at 2 days after the transfection in the frozen section of the conjunctival tissue. The cell nuclei were stained in blue. [Ref. 50.]

in the gene transfection. Thus, we succeeded in the control of the *in vivo* transgene expression by using light-responsive gene carriers integrated with DPs.

Future prospects

In this paper, we have reviewed the characteristics of the DP-incorporated PIC micelles from the standpoint of the photochemical reactions and *in vitro* and *in vivo* photodynamic effect. Our results revealed that the DP-incorporated micelles possess unique photochemical properties and show remarkable *in vitro* photodynamic effect, which have not been achieved by other PS formulations. The PDT with DP-incorporated micelles succeeded in the treatment of rat AMD models, without any sign of side effects, facilitating the development of the DP-incorporated micelles for cancer photodynamic therapy. Also, the PCI-mediated transfection with gene carriers integrated with DPc allowed the control of transgene expression in the body using light irradiation as an external stimulus. Importantly, the PCI-mediated transfection may not require as high an irradiation energy as PDT, suggesting its potential use for the treatment of deeper tissues, to which PDT is not applicable.

The recent advance in laser technology will allow diverse clinical applications of PDT. For example, the two-photon excitation by using near infrared lasers may have great potential for the PDT of diseases in deeper tissue (e.g., brain tumors). The possibility of two-photon absorption is known to depend linearly on the intrinsic two-photon cross section of the compounds, δ , and quadratically on the incident light intensity.⁵⁴ Although the two-photon cross section of molecules is typically small compared with one-photon absorption, neodymium:YAG (Nd:YAG) laser emitting at 1064 nm or

tunable solid state lasers generating far-red/near infra-red light could significantly increase the possibility of two-photon absorption of the compounds due to high laser output.⁵⁵ In the light of such situations, the development of the delivery systems to improve the biodistribution and enhance the photodynamic efficacy of PSs will be a key technology to effective PDT. Our nanocarriers integrated with dendrimers encapsulating photosensitizers are expected to be a clinically useful PS formulation for PDT and gene therapy.

Acknowledgements

We thank Dr Takuzo Aida, professor in the Department of Chemistry and Biotechnology, Graduate School of Engineering, the University of Tokyo, for valuable discussion on the synthesis of dendrimers encapsulating porphyrins. Also, the authors are grateful to Dr Yasuhiro Tamaki, professor in the Department of Ophthalmology, Graduate School of Medicine, the University of Tokyo, for the accomplishment of the animal experiments. The studies reviewed in this work was supported in part by New Energy and Industrial Technology Development Organization (NEDO) of Japan.

References

- 1 D. E. J. G. J. Dolmans, D. Fukumura and R. K. Jain, *Nat. Rev. Cancer*, 2003, **3**, 380–387.
- 2 I. J. Macdonald and T. J. Dougherty, *J. Porphyrins Phthalocyanines*, 2001, **5**, 105–129.
- 3 R. Z. Renno and J. W. Miller, *Adv. Drug Delivery Rev.*, 2001, **52**, 63–78.
- 4 M. Tijerina, P. Kopeckova and J. Kopecek, *Photochem. Photobiol.*, 2003, **77**, 645–652.
- 5 A. S. L. Derycke and P. A. M. de White, *Adv. Drug Delivery Rev.*, 2004, **56**, 17–30.
- 6 D. Le Garrec, J. Taillefier, J. E. Van Lier, V. Lenaerts and J. C. Leroux, *J. Drug Targeting*, 2002, **10**, 429–437.
- 7 L. I. Grossweiner, A. S. Patel and J. B. Grossweiner, *Photochem. Photobiol.*, 1982, **36**, 159–167.
- 8 N. Nishiyama, H. R. Stapert, G. D. Zhang, D. Takasu, D.-L. Jiang, T. Nagano, T. Aida and K. Kataoka, *Bioconjugate Chem.*, 2003, **14**, 58–66.
- 9 K. Kataoka, A. Harada and Y. Nagasaki, *Adv. Drug Delivery Rev.*, 2001, **47**, 113–131.
- 10 N. Nishiyama and K. Kataoka, *Adv. Polym. Sci.*, 2006, **193**, 67–101.
- 11 N. Nishiyama and K. Kataoka, *Pharmacol. Ther.*, 2006, **113**, 630–648.
- 12 N. Nishiyama, S. Okazaki, H. Cabral, M. Miyamoto, Y. Kato, Y. Sugiyama, K. Nishio, Y. Matsumura and K. Kataoka, *Cancer Res.*, 2003, **63**, 8977–8983.
- 13 Y. Matsumura and H. Maeda, *Cancer Res.*, 1986, **46**, 6387–6392.
- 14 R. Ideta, Y. Yanagi, Y. Tamaki, F. Tasaka, A. Harada and K. Kataoka, *FEBS Lett.*, 2004, **557**, 21–25.
- 15 A. Harada and K. Kataoka, *Science*, 1999, **283**, 65–67.
- 16 A. Harada and K. Kataoka, *J. Am. Chem. Soc.*, 2003, **125**, 15306–15307.
- 17 K. Itaka, N. Kanayama, N. Nishiyama, Y. Yamasaki, K. Nakamura, H. Kawaguchi and K. Kataoka, *J. Am. Chem. Soc.*, 2004, **126**, 13612–13613.
- 18 K. Itaka, K. Yamauchi, A. Harada, K. Nakamura, H. Kawaguchi and K. Kataoka, *Biomaterials*, 2003, **24**, 4495–4506.
- 19 H. R. Stapert, N. Nishiyama, D.-L. Jiang, T. Aida and K. Kataoka, *Langmuir*, 2000, **16**, 8182–8188.
- 20 G.-D. Zhang, A. Harada, N. Nishiyama, D.-L. Jiang, H. Koyama, T. Aida and K. Kataoka, *J. Controlled Release*, 2003, **93**, 141–150.

- 21 G.-D. Zhang, N. Nishiyama, A. Harada, D.-L. Jiang, T. Aida and K. Kataoka, *Macromolecules*, 2003, **36**, 1304–1309.
- 22 W.-D. Jang, N. Nishiyama, G.-D. Zhang, A. Harada, D.-L. Jiang, S. Kawauchi, Y. Morimoto, M. Kikuchi, H. Koyama, T. Aida and K. Kataoka, *Angew. Chem., Int. Ed.*, 2005, **44**, 419–423.
- 23 S. M. Grayson and J. M. J. Fréchet, *Chem. Rev.*, 2001, **101**, 3819–3867.
- 24 C. J. Hawker and J. M. J. Fréchet, *J. Am. Chem. Soc.*, 1990, **112**, 7638–7647.
- 25 R. Sadamoto, N. Tomioka and T. Aida, *J. Am. Chem. Soc.*, 1996, **118**, 3978–3979.
- 26 C. S. Downes, M. J. Ord, A. M. Mullinger, A. R. Collinsm and R. T. Johnson, *Carcinogenesis*, 1985, **6**, 1343–1352.
- 27 D. Kessel, Y. Luo, Y. Deng and C. K. Chang, *Photochem. Photobiol.*, 1997, **65**, 422–426.
- 28 M. Dellinger, *Photochem. Photobiol.*, 1996, **64**, 182–187.
- 29 K. Berg, P. K. Selbo, L. Prasmickaite, T. E. Tejel, K. Sandvig, J. Moan, G. Gaudernak, O. Fodstad, S. Kjolsrud, H. Anholt, G. H. Rodal, S. K. Rodal and A. Høgset, *Cancer Res.*, 1999, **59**, 1180–1183.
- 30 M. R. Hamblin, J. L. Miller, I. Rizvi, B. Ortel, E. V. Maytin and T. Hasan, *Cancer Res.*, 2001, **61**, 7155–7162.
- 31 S. Sandberg and I. Romslo, *Biochem. J.*, 1981, **198**, 67–74.
- 32 Y. Kakizawa, A. Harada and K. Kataoka, *J. Am. Chem. Soc.*, 1999, **121**, 11247–11248.
- 33 I. F. Tannock and D. Rotin, *Cancer Res.*, 1989, **49**, 4373–4384.
- 34 N. C. Maiti, S. Mazumdar and N. Periasamy, *J. Phys. Chem. B*, 1998, **102**, 1528–1538.
- 35 K. M. Kadish, G. B. Maiya, C. Araullo and R. Guillard, *Inorg. Chem.*, 1989, **28**, 2725–2731.
- 36 R. Savic, L. Luo, A. Eisenberg and D. Maysinger, *Science*, 2003, **300**, 615–618.
- 37 TAP and VIP Study Group, *Retina*, 2002, **22**, 6–18.
- 38 R. Ideta, F. Tasaka, W.-D. Jang, N. Nishiyama, G.-D. Zhang, A. Harada, Y. Yanagi, Y. Tamaki, T. Aida and K. Kataoka, *Nano Lett.*, 2005, **5**, 2426–2431.
- 39 D. N. Zacks, E. Ezra, Y. Terada, N. Micband, E. Connolly, E. S. Gragoudas and J. W. Miller, *Invest. Ophthalmol. Visual Sci.*, 2002, **43**, 2384–2391.
- 40 J. Moan and K. Berg, *Int. J. Cancer*, 1994, **58**, 865–870.
- 41 A. C. H. Ng, X. Li and D. K. P. Ng, *Macromolecules*, 1999, **32**, 5292–5298.
- 42 W.-D. Jang, Y. Nakagishi, N. Nishiyama, S. Kawauchi, Y. Morimoto, M. Kikuchi and K. Kataoka, *J. Controlled Release*, 2006, **113**, 73–79.
- 43 A. Høgset, L. Prasmickaite, T. E. Tejel and K. Berg, *Hum. Gene Ther.*, 2000, **11**, 869–880.
- 44 L. Prasmickaite, A. Høgset and K. Berg, *Photochem. Photobiol.*, 2001, **73**, 388–395.
- 45 A. Høgset, L. Prasmickaite, M. Hellum, B. Æ. Engesæter, V. M. Olsen, T. Tejel, C. J. Wheeler and K. Berg, *Somatic Cell Mol. Genet.*, 2002, **27**, 97–113.
- 46 A. Høgset, L. Prasmickaite, P. K. Selbo, M. Hellum, B. Æ. Engesæter, A. Bonded and K. Berg, *Adv. Drug Delivery Rev.*, 2004, **56**, 95–115.
- 47 N. Nishiyama, Arnida, W.-D. Jang, K. Date, K. Miyata and K. Kataoka, *J. Drug Targeting*, 2006, **14**, 413–424.
- 48 Arnida, N. Nishiyama, N. Kanayama, W.-D. Jang, Y. Yamasaki and K. Kataoka, *J. Controlled Release*, 2006, **115**, 208–215.
- 49 O. Boussif, F. Lezoualc'h, M. A. Zanta, M. D. Mergny, D. Scherman, B. Demeneix and J. P. Behr, *Proc. Natl. Acad. Sci. U. S. A.*, 1995, **92**, 7297–7301.
- 50 N. Nishiyama, A. Iriyama, W.-D. Jang, K. Miyata, K. Itaka, Y. Inoue, H. Takahashi, Y. Yanagi, H. Koyama and K. Kataoka, *Nat. Mater.*, 2005, **4**, 934–941.
- 51 F. Caruso, R. A. Caruso and H. Möhwald, *Science*, 1998, **282**, 1111–1114.
- 52 E. Donath, G. B. Sukhorukov, F. Caruso, S. A. Davis and H. Möhwald, *Angew. Chem., Int. Ed.*, 1998, **37**, 2201–2205.
- 53 C. Rudolph, C. Plank, J. Lausier, U. Schillinger, R. H. Muller and J. Rosenecker, *J. Biol. Chem.*, 2003, **278**, 11411–11418.
- 54 D. H. Oh, R. J. Stanley, M. Lin, W. K. Hoeffler, S. G. Boxer, M. W. Berns and E. Bauer, *Photochem. Photobiol.*, 1997, **65**, 91–95.
- 55 A. M. R. Fisher, A. L. Murphree and C. J. Gomer, *Lasers Surg. Med.*, 1995, **17**, 2–31.